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Studies on Ester Sulphates

16. Use of ^{35}S-labelled Inorganic Sulphate for Quantitative Studies of Sulphate Conjugation in Liver Extracts *

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A simple method is described for estimation of small amounts of ^{35}S-labelled ester sulphates synthesized *in vitro*. The method is based on the use of $Ba(OH)_2$ for separation of ester sulphates from ^{35}S-labelled nucleotides and inorganic sulphate.

Assay systems are described for determination on the micro-scale of over-all sulphurylating activities and sulphokinases present in liver extracts. An account is also given of an assay system for estimating the PAPS-accumulating activity of liver extracts.

Although several methods have been described for quantitative studies of sulphate conjugation, most of them are based on the use of sulphate acceptors (*e.g.* *m*-aminophenol and *p*-nitrophenol) which, either as such or as sulphates, are determinable by colorimetric procedures.[1–3] Thus, when using such methods, sulphurylation studies are limited to one or a few acceptors.

In 1956, Roy [4] introduced Vlitos' colorimetric method [5] for determination of enzymically synthesized ester sulphates. This method is based on the solubility in chloroform of coloured complexes of methylene blue and certain ester sulphates (*e.g.* *p*-nitrophenyl sulphate, steroid sulphates). The method is useful for determination of a wide range of ester sulphates but, like those previously mentioned, is impractical for use on the micro-scale.

The availability of ^{35}S-labelled inorganic sulphate has enabled more sensitive and specific methods to be devised. Enzymically synthesized radioactive ester sulphates can be isolated by paper chromatography or electrophoresis, eluted, and measured on the basis of their radioactivity.[6] A major disadvantage of

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such determination procedures is, however, that the isolation and elution steps are somewhat time-consuming.

The scope of the present investigation was to develop a rapid method for the estimation of enzymically synthesized ^{35}S-labelled ester sulphates.

EXPERIMENTAL

Liver preparations. Rats (Sprague-Dawley strain)* were stunned by a blow on the head, decapitated and exsanguinated. The liver was rapidly removed, chilled on ice and washed in the ice-cold buffer used for homogenization. The tissue was quickly weighed and homogenized in 4 vols of ice-cold 0.15 M KCl, containing 0.001 M EDTA ** (pH 7.0) in an all-glass Potter-Elvehjem homogenizer.[7] The homogenate was centrifuged in a Spinco model L ultracentrifuge at an average 105 000 *g* for 60 min. The clear supernatant fluid was frozen, and kept at −20°C until used. Tests showed that these preparations did not lose any appreciable amount of over-all sulphurylating activity when stored at this temperature for 1 month. Before use, the enzyme solutions were diluted with 1 vol. of the aforementioned buffer. Thus, 50 µl of such a dilute preparation corresponded to approximately 5 mg of wet weight liver. For further dilution of extracts, the buffer applied for homogenization was used.

The human liver extract was prepared from a biopsy specimen of the liver, taken at cholecystectomy in a 42-year-old woman. The specimen was transported to the laboratory on ice, and was homogenized 20−30 min after it was taken.

Preparation of sulphate-activating enzymes from yeast. A yeast-extract fraction, containing concentrated sulphate-activating enzymes, was prepared by ammonium sulphate fractionation according to Nose and Lipman.[8] Before use, this extract (representing 1 kg of fresh yeast) was dialyzed against 4 litres of 0.01 M Tris/HCl buffer containing 0.001 M EDTA, pH 7.2, for 22 h at + 2−4°C, to remove most of the added inorganic sulphate. One change of buffer was made after the first 5 h. The dialyzed solution was frozen in small fractions and kept at −20°C. Its content of inorganic sulphate was determined.[9]

Chemicals. Carrier-free ^{35}S-labelled inorganic sulphate was obtained as the sodium salt from the Radiochemical Centre, Amersham, England. The disodium salt of ATP was obtained from Sigma Chemical Company, Saint Louis, Mo., USA. Sulphate acceptors *** were mostly commercial products used without further purification. 3β-Hydroxy-5β-androstane-17-one (etiocholane-3β-ol-17-one) was synthesized by Dr. Emiliozzi, and kindly placed at my disposal by Dr. E.-E. Baulieu.†

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* Obtained from Anticimex, Norrviken, Sweden.

** The following abbreviations are used: EDTA, ethylenediamine tetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane; APS, adenosine-5′-phosphosulphate; PAPS, 3′-phosphoadenosine-5′-phosphosulphate; PAP^{35}S, ^{35}S-labelled PAPS; ATP, adenosine triphosphate; DHA, dehydroepiandrosterone; DHAS, dehydroepiandrosterone sulphate; PhS, phenyl sulphate.

*** The following compounds were kindly supplied by Pharmacia, Uppsala, Sweden: 3α-hydroxy-5α-androstane-17-one (androsterone); 3β-hydroxy-5α-androstane-17-one (epiandrosterone); 3-hydroxy-$\Delta^{1,3,5(10)}$-oestratriene-17-one (oestrone); $\Delta^{1,3,5(10)}$-oestratriene-3,16α,17β-triol (oestriol).

3α-Hydroxy-5β-androstane-17-one (ethiocholane-3α-ol-17-one) and 3β-hydroxy-Δ^{5}-androstene-17-one (dehydroepiandrosterone, DHA) were kindly supplied by Schering AG, Berlin, Germany.

The following compounds were obtained from L. Light & Co. Ltd., Colnbrook, England: 20β-hydroxy-5α-pregnane-3-one (*allo*pregnane-20β-ol-3-one); 3α-hydroxy-5β-pregnane-20-one (pregnane-3α-ol-20-one), and 3β-hydroxy-5β-pregnane-20-one (pregnane-3β-ol-20-one).

21-Hydroxy-Δ^{4}-pregnene-3,20-dione (11-desoxycorticosterone) was obtained from Sigma Chem. Co., St.Louis, Mo., U.S.A.

Phenol, *pro analysi*, was obtained from E. Merck AG, Darmstadt, Germany.

Ethanol, spectrographically pure (99 vol.%) was obtained from AB Vin & Spritcentralen, Stockholm, Sweden.

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Deionized water was used for preparation of buffers and other aqueous solutions.

Pipettes. The micropipettes used were manufactured by H. J. Elliott Ltd., E-mil Works, Treforest, Glam., England. Pipettes size 50 and 100 μl had a tolerance of $\pm$ 1 %. The tolerance of pipettes size 20 and 25 μl was $\pm$ 2 %.

Assay of over-all sulphurylating activity. Enzymic over-all sulphurylation was studied by incubation of mixtures containing the following constituents in 120 μl: (*a*) 10—50 mμmoles of sulphate acceptor. Steroids were added in ethanolic solution, the ethanol being evaporated *in vacuo* before addition of other components; (*b*) 50—100 μC of carrier-free ^{35}S-labelled sodium sulphate; (*c*) 50 μl of a buffer solution containing equal parts of 0.3 M KH_2PO_4 (pH 6.8), 0.03 M K_2SO_4 and 0.005 M $MgCl_2$; (*d*) 50 μl of liver supernatant fluid corresponding to 0—10 mg of wet weight liver; (*e*) 400 mμmoles of ATP, disodium salt.

Blanks contained all constituents except acceptor, but otherwise underwent the entire procedure.

Incubation was carried out for 0—60 min at 37.5°C in open test tubes. The sulphurylation process was stopped by heating the tubes in a boiling-water bath for 90 sec followed by cooling in ice-water. All incubations were run at least in duplicate.

Assay of PAPS-accumulating activity. Accumulation of PAPS was studied by incubation of mixtures containing the same constituents as for the assay of over-all sulphurylating activity, except that the acceptor was omitted. The enzyme source used was a female rat liver extract stored for 5 months at —20°C. Incubation was carried out for 0—15 min at 37.5°C in open test tubes. The sulphate-activating process was stopped by cooling the tubes in ice-water, and the addition of 10 μl of 0.039 M EDTA,[10] pH 6.4. 20 mμmoles of phenol were added, as well as 10 μl of rabbit or rat liver supernatant fluid prepared as described above. Incubation was then continued for 60 min. The reaction was stopped by heating the tubes in a boiling-water bath for 90 sec, followed by cooling in ice-water. The active sulphate accumulated during the first incubation period was thus transformed into phenyl sulphate during the second incubation period. Phenyl sulphate was determined as stated below.

Blanks contained all constituents for the assay of PAPS-accumulating activity, but were kept in ice-water during the first incubation period. In all other respects, blanks were handled the same as assay tubes. All incubations were run at least in duplicate.

Assay of sulphokinase activity. The yeast extract prepared as described was used as a PAP^{35}S-synthesizing agent. The preparation was allowed to accumulate PAP^{35}S in an incubation mixture composed as follows: (*a*) 1—2 mC of carrier-free ^{35}S-labelled sodium sulphate; (*b*) 1.0 ml of equal parts of 0.3 M KH_2PO_4 (pH 6.8), 0.03 M K_2SO_4 and 0.005 M $MgCl_2$; (*c*) 1.0 ml of yeast extract (dialyzed); (*d*) 8 μmoles of ATP, disodium salt. Total volume: 2.4 ml.

Incubation was carried out in air for 30 min at 37.5°C. Sulphate activation was stopped by cooling the incubation mixture in ice-water, and the addition of 0.2 ml of 0.039 M EDTA, pH 7.0.

100 μl of this incubation mixture, corresponding to 7.0 mμmoles of ^{35}S-labelled PAPS (with the yeast extract in question), was then transferred to tubes containing 50 μl of a liver supernatant fluid (representing 0—2.0 mg of wet weight liver) together with 10 mμmoles of the acceptor. Steroids were added in ethanolic solution, the ethanol being evaporated *in vacuo* before addition of other components. Incubation was then carried out at 37.5°C for 0—60 min. The sulphate-transferring reaction was stopped by heating the tubes in a boiling-water bath for 90 sec, followed by cooling in ice-water. Synthesized ester sulphates were determined as described in the next section.

Blanks contained all constituents except the acceptor, but otherwise underwent the entire procedure. All incubations were run at least in duplicate.

Determination of ester sulphates formed. Inorganic sulphate and nucleotides were precipitated with $Ba(OH)_2$. The ester sulphates remaining in solution could be estimated from their content of ^{35}S-labelled sulphate. The following *reagents* were used: (1) 0.05 M H_2SO_4; (2) 0.1 % phenolphthalein in ethanolic solution; (3) 0.1 M $Ba(OH)_2$ containing 5 ml of aqueous ammonia per litre; immediately before use, a few drops of reagent 2 were added to this solution.

The *procedure* was as follows: 1 ml of 0.05 M H_2SO_4 was added to one incubation tube and the heat-denatured protein was homogenized with a glass rod. 1 ml of $Ba(OH)_2$ reagent was added, the content of the tube was mixed, and the heavy precipitate centri-

fuged down at 1000 *g* for 10 min. An aliquot of the supernatant fluid was transferred to another tube, and CO_2 was quickly bubbled through the solution, care being taken not to change the pH of the solution to the acid side.

Precipitated $BaCO_3$ was filtered off (Munktell filter paper No. 00H). Aliquots of the filtrate were plated in duplicate on frosted aluminium plates (infinite thinness), and counted in a Geiger-Müller counter (mica end-window tube, 1.9 mg/cm^2).

The aliquots of plated solutions presumed to contain ester sulphates as the only radioactive compounds were checked for purity by means of two-dimensional ascending paper chromatography and autoradiography.[11] Solvent I: phenol-water (4/1 by weight); solvent II: butanol-acetic acid-water (12/3/5 by vol.).

Sulphate determinations [9] on liver preparations from two adult male and two adult female rats showed, in each case, that the inorganic sulphate content of 50 µl of the undiluted preparations was less than 1 % of the sulphate added as K_2SO_4 to the incubation tubes. If the amount of inorganic sulphate in liver preparations was neglected, the specific activity of the inorganic sulphate present in the tubes could be estimated, which permitted the absolute amounts of radioactive ester sulphates formed to be calculated.

The *recovery* of certain ester sulphates with the determination procedure described was estimated as follows. ^{35}S-labelled ester sulphates of androsterone, *epi*androsterone, dehydro*epi*androsterone (DHA), etiocholane-3α-ol-17-one, etiocholane-3β-ol-17-one, *allo*pregnane-20β-ol-3-one, pregnane-3α-ol-20-one, pregnane-3β-ol-20-one, 11-desoxycorticosterone, oestrone, oestriol, ethanol and phenol were prepared biosynthetically, by use of rat liver extract in the assay system for over-all sulphurylation.[4,11,12] The esters were isolated by the determination method described above.

Aliquots of these esters were added to tubes containing 50 µl of undiluted rat liver extract and other constituents present in the incubation mixtures used for assay of over-all sulphurylation. Final protein concentration:[20] 8.33 mg/ml (reference: crystallised bovine plasma albumin, Armour Pharm. Co. Ltd., Eastbourne, England). The tubes were heated in a boiling-water bath for 90 sec, cooled in ice-water, and then submitted to the procedure described for ester sulphate determination.

Statistical methods. The standard deviation (*s*) of a single determination of ester sulphate synthesis was calculated from differences (*d*) between duplicate assays by the following formula:

$$s = \sqrt{\Sigma d^2/2n}$$

where n = number of pairs. Means and standard errors of the means were calculated by conventional methods.[13]

RESULTS

Isolation and recovery of synthesized ester sulphates. ^{35}S-labelled ester sulphates of androsterone, *epi*androsterone, dehydro*epi*androsterone (DHA), etiocholane-3α-ol-17-one, etiocholane-3β-ol-17-one, *allo*pregnane-20β-ol-3-one, pregnane-3α-ol-20-one, pregnane-3β-ol-20-one, 11-desoxycorticosterone, oestrone, oestriol, ethanol and phenol were synthesized by the rat liver extracts. The position of the ester sulphate of oestriol on the chromatograms suggested that only a monosulphate of the steroid was synthesized.[6]

By using $Ba(OH)_2$ in the method applied for ester sulphate determination, it was possible to precipitate and remove ^{35}S-labelled inorganic sulphate and 3′-phosphoadenosine-5′-phosphosulphate (PAPS) from the incubation mixtures.

In 5 experiments, 95.1, 97.4, 96.2, 95.0, and 99.0 %, an average 96.5 % of 9.2 mµmoles of PAPS synthesized by the yeast extract, and added to incubation mixtures, was removed by heating the mixture and precipitation with $Ba(OH)_2$.

Aliquots of the final solutions presumed to contain ester sulphates as the only ^{35}S-labelled compounds were checked for purity by means of paper chro-

matography, as described above. The results of such control experiments also showed that ^{35}S-labelled nucleotides (APS, PAPS), as well as inorganic sulphate, had been effectively removed from the incubation mixtures, whereas synthesized ester sulphates remained in solution. Fig. 1 illustrates the radioactive homogeneity of dehydro*epi*androsterone sulphate (DHAS), phenyl sulphate (PhS) and oestrone sulphate (Oestrone-S), synthesized by over-all sulphurylation, and isolated by the method described. The radioactive impurities (X) of the ^{35}S-labelled sodium sulphate are also visualized in this way. However, the radioactivity of these unknown compounds never exceeded 0.05 % of the carrier-free ^{35}S-labelled sulphate used.

During incubation, endogenous compounds (E) present in the extracts were also sulphurylated to a certain extent (Fig. 1). The formation of ester

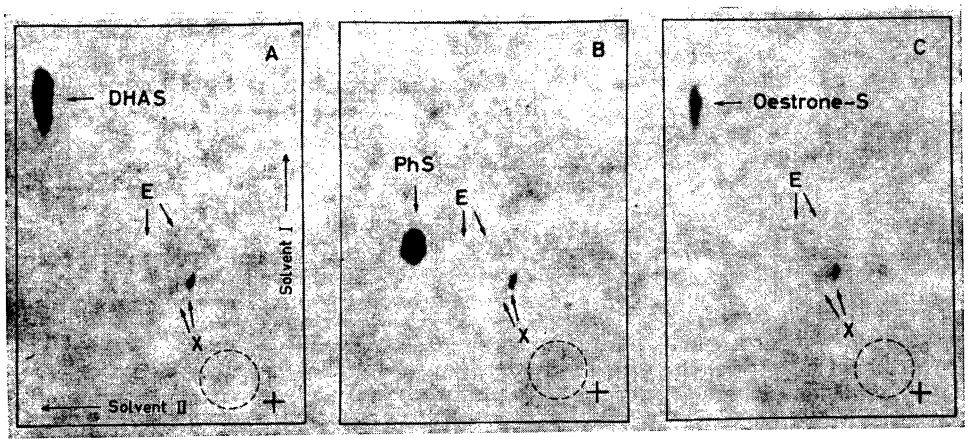

Fig. 1. Autoradiograms of two-dimensional paper chromatograms, illustrating the radioactive compounds present in incubation mixtures after synthesis of dehydro*epi*androsterone sulphate (DHAS), phenyl sulphate (PhS), and oestrone sulphate (Oestrone-S) by over-all sulphurylation. Chromatography was performed on aliquots of incubation mixtures depleted of ^{35}S-labelled nucleotides and inorganic sulphate by means of $Ba(OH)_2$. The following substrates were used: A. Dehydro*epi*androsterone (DHA); B. Phenol; C. Oestrone.

The position of ester sulphates synthesized from endogenous compounds (E), as well as radioactive impurities (X) already present in the solution of carrier-free $Na_2{}^{35}SO_4$, are indicated in the figures. The area within dotted lines should contain adenosine-5′-phosphosulphate (APS), 3′-phosphoadenosine-5′-phosphosulphate (PAPS) and inorganic sulphate if present. Solvent I: Phenol-water (4/1 by weight); solvent II: Butanol-acetic acid-water (12/3/5 by vol.).

sulphates from endogenous compounds was, however, minute in comparison to the formation from exogenous sulphate acceptors assayed.

The recovery of small amounts of some biosynthetically prepared ester sulphates was also estimated in the determination procedure described, including the heating step together with liver extract (Table 1). The recovery of the C_{19} steroid sulphates studied was in the range of 80.1—91.4 %, and that of the C_{21} steroid sulphates in the range of 65.5—76.9 %. The corresponding

Table 1. Recovery of ester sulphates by the determination method described in the text. The conjugates were added to the assay systems for studies of over-all sulphurylation. The amounts of ester sulphate added and recovered are expressed in terms of the sulphate content of the esters. S.E. = standard error of the mean. For further details, see text.

Ester sulphate of	Amount of ester sulphate (mμmoles of sulphate)						Recovery Mean ± S.E. %
	Added	Recovered		Added	Recovered		
		I	II		I	II	
Androsterone	0.386	0.333	0.314	0.195	0.164	0.167	84.4 ± 1.1
*Epi*androsterone	0.346	0.295	0.310	0.295	0.248	0.243	85.2 ± 1.6
Dehydro*epi*androsterone	0.408	0.367	0.336	0.261	0.253	0.252	91.4 ± 3.4
Etiocholane-3α-ol-17-one	0.244	0.186	0.195	0.227	0.187	0.185	80.1 ± 1.4
Etiocholane-3β-ol-17-one	0.318	0.285	0.277	0.280	0.232	0.252	87.6 ± 1.7
*Allo*pregnane-20β-ol-3-one	0.271	0.180	0.200	0.161	0.096	0.100	65.5 ± 3.1
Pregnane-3α-ol-20-one	0.208	0.161	0.171	0.166	0.104	0.107	71.5 ± 4.8
Pregnane-3β-ol-20-one	0.272	0.189	0.205	0.182	0.112	0.119	68.0 ± 3.0
11-Desoxycorticosterone	0.192	0.150	0.167	0.180	0.124	0.131	76.9 ± 3.8
Oestrone	0.170	0.141	0.139	0.093	0.073	0.075	81.2 ± 0.9
Oestriol	0.111	0.101	0.098	0.108	0.109	0.105	94.5 ± 3.0
Ethanol	0.142	0.128	0.127	0.116	0.106	0.111	91.5 ± 1.3
Phenol	0.278	0.259	0.254	0.261	0.229	0.213	88.6 ± 2.5

figures for oestrone sulphate and oestriol sulphate were 81.2 and 94.5 %. Around 90 % of the ester sulphates of ethanol and phenol was recovered.

Over-all sulphurylation as a function of incubation time and enzyme concentration. The determination method described was applied to studies of the over-all sulphurylation of phenol and DHA by rat liver extracts. The formation of PhS by one male and two female extracts was found to be directly proportional to the amount of enzyme solution present, unless extracts corresponding to more than 7.5 mg wet weight liver were added (Fig. 2). However, the amount of two other female liver extracts, corresponding to 2.5 mg wet weight liver, sulphurylated less phenol than would have been proportional to the amount of extract used.

The formation of DHAS by three different female liver extracts, used in amounts less than corresponding to 7.5 mg wet weight liver, was directly proportional to the extract concentration (Fig. 2).

The synthesis of PhS by one male and one female rat liver extract (representing 5 mg wet weight liver) was proportional to the incubation time, at least up to 60 min (Fig. 3). A short lag period in the beginning of the time course was, however, observed with three female liver extracts. The rate of synthesis of DHAS by three female extracts (5 mg wet weight liver) showed a somewhat declining course during the incubation periods studied (Fig. 3).

Accumulation of PAPS as a function of incubation time and enzyme concentration. The ^{35}S-labelled sulphate group of PAPS accumulated by a female rat liver extract (stored at −20°C for 5 months) was transferred to phenol and determined as PhS, as described above. Within certain limits of enzyme con-

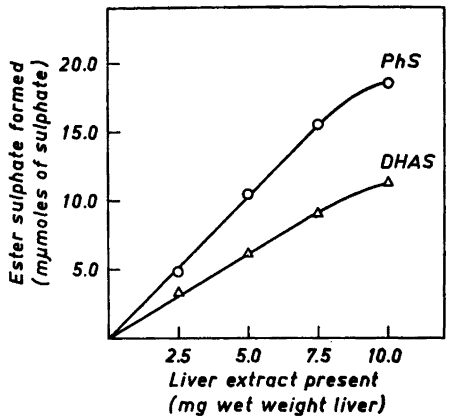

Fig. 2. Effect of varying amounts of a female rat liver extract on the over-all sulphurylation of 40 mμmoles of phenol and 50 mμmoles of DHA. Incubation period: 60 min. The amounts of ester sulphates synthesized, PhS and DHAS, respectively, are expressed in terms of the sulphate content of the esters. The amounts of extract used are expressed in terms of wet weight liver. For experimental conditions, see text.

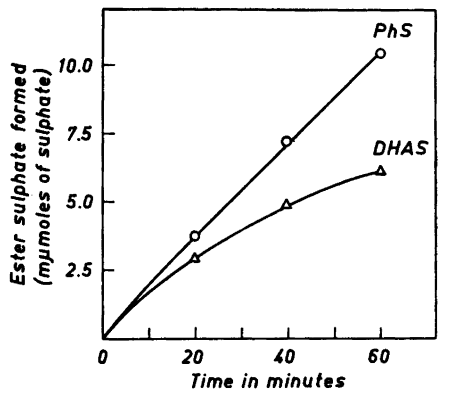

Fig. 3. Rate of over-all sulphurylation of 40 mμmoles of phenol and 50 mμmoles of DHA by a female rat liver extract representing 5 mg wet weight liver. The amounts of ester sulphates synthesized, PhS and DHAS, respectively, are expressed in terms of the sulphate content of the esters. For experimental conditions, see text.

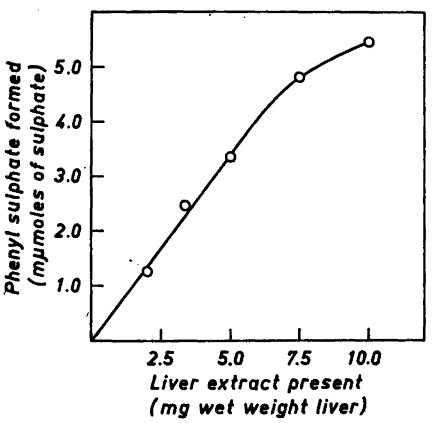

Fig. 4. Effect of varying amounts of a female rat liver extract (stored for 5 months at −20°C) on the accumulation of PAPS. Incubation period: 15 min. Further sulphate activation was stopped by addition of EDTA. The ^{35}S-labelled sulphate group of PAPS was transferred to phenol, and determined as PhS. The amounts of extract used are expressed in terms of wet weight liver. For experimental conditions, see text.

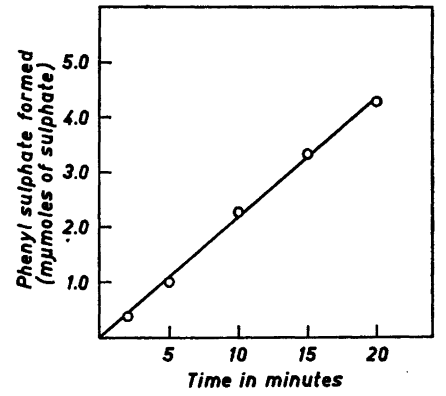

Fig. 5. Rate of accumulation of PAPS by a female rat liver extract (stored for 5 months at −20°C) representing 5 mg wet weight liver. Sulphate activation was stopped by addition of EDTA. The ^{35}S-labelled sulphate group of PAPS was transferred to phenol, and determined as PhS. For experimental conditions, see text.

centration (representing up to 5 mg wet weight liver), the accumulation of PAPS was found to be directly proportional to the amount of enzyme solution present if accumulation was allowed to proceed for 15 min (Fig. 4). At high enzyme concentrations, however, a decline in PAPS accumulation was noted. The rate of accumulation of PAPS by an amount of extract corresponding to 5 mg wet weight liver was constant for 20 min (Fig. 5).

Sulphokinase activity as a function of incubation time and enzyme concentration. The phenol and DHA sulphokinase activity of a dilute human liver extract was assayed in the presence of a given amount of ^{35}S-labelled PAPS and EDTA, as described above. If incubation was run for 60 min, the amount of PhS and DHAS synthesized was directly proportional to the amount of liver extract present (representing up to 2 mg wet weight liver; Fig. 6). When the amount of extract corresponded to 1 mg wet weight liver, the time course of sulphate esterification, exemplified by synthesis of PhS, showed a slow levelling off during incubation (Fig. 7).

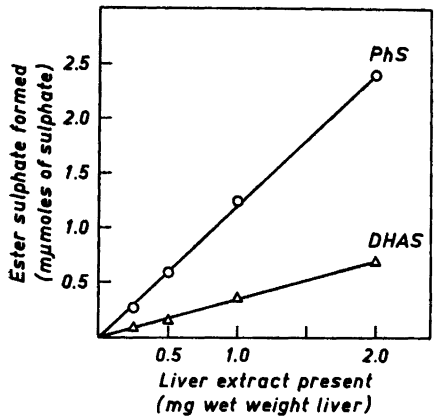

Fig. 6. Effect of varying amounts of a human liver extract on the synthesis of PhS and DHAS from 10 mμmoles of phenol and DHA, respectively, in the presence of EDTA and ^{35}S-labelled PAPS. Incubation period: 60 min. The amounts of ester sulphates synthesized are expressed in terms of their sulphate content. The amounts of extract used are expressed in terms of wet weight liver. For experimental conditions, see text.

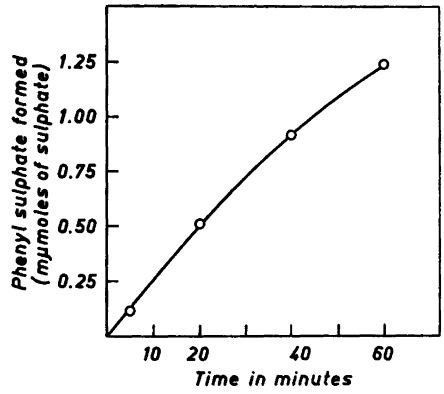

Fig. 7. Rate of formation of PhS from 10 mμmoles of phenol, in the presence of EDTA and ^{35}S-labelled PAPS, by a human liver extract representing 1 mg wet weight liver. The amounts of PhS synthesized are expressed in terms of its sulphate content. For experimental conditions, see text.

Statistical calculations. From 23 duplicate assays on the over-all sulphurylation of both phenol and DHA by liver extracts yielding ester sulphates in the range of 1.30—3.93 and 1.98—3.50 mμmoles/assay, respectively, the standard deviation of a single determination was calculated to be ± 0.09 mμmoles of PhS and ± 0.10 mμmoles of DHAS. The coefficient of variation was 3.4 and 3.8 %, respectively.

A standard deviation of ± 0.05 mμmoles was calculated from 8 duplicate assays on PAPS-accumulating activity of liver extracts (determined as PhS) in the range of 0.71—1.18 mμmoles/assay. Coefficient of variation: 5.4 %.

From 16 duplicate assays on the phenol sulphokinase activity of liver extracts, yielding PhS in the range of 0.01—2.39 mμmoles/assay, the standard deviation was calculated to be ± 0.09 mμmoles. Coefficient of variation: 11.6 %.

From 12 duplicate assays on the DHA sulphokinase activity of liver extracts yielding DHAS in the range of 0.04—1.08 mμmoles/assay, the standard deviation was calculated to be ± 0.03 mμmoles. Coefficient of variation: 7.8 %.

DISCUSSION

The present results showed that by use of $Ba(OH)_2$, it was possible to separate *in vitro* synthesized ^{35}S-labelled ester sulphates from ^{35}S-labelled inorganic sulphate and nucleotides (APS, PAPS). Since paper chromatographic control experiments also showed that plated solutions contained ester sulphates only, the radioactivity measured was taken as an expression of the amount of these compounds synthesized. The amount of sulphate present in extracts was either known or negligible. The specific activity of the sulphate content of the incubation mixtures could therefore be calculated, which implied that the absolute quantities of ester sulphates synthesized could be computed. The radioactive impurities present in the carrier-free $Na_2{}^{35}SO_4$ solution obtained did not disturb the assays, since they always constituted an extremely small part of the total radioactivity used for each assay, and occurred to the same extent in blanks as in assay tubes.

The nature of the ester sulphates synthesized by rat liver extracts from endogenous compounds has previously been studied by Spencer.[14] The minute amounts of these ester sulphates synthesized from endogenous compounds were, however, taken into account by running blanks for each kind of incubation period and enzyme concentration used.

The recovery of steroid sulphates in the determination procedure was probably dependent on the structure of the conjugates, since five C_{19} steroid sulphates were recovered to a greater extent than the ester sulphates of four C_{21} steroids. Of the two oestrogens studied, the more polar one (oestriol monosulphate) was recovered to the greatest extent. This observation, as well as the aforementioned effect of steroid structure on recovery of the esters, suggested that the loss of esters noted in the determination method was probably due to adsorption of the conjugates to heat-denatured proteins removed during the procedure. This assumption was further substantiated by the fact that many steroid sulphates are known not to be split by heating for a short time (90 sec) at neutral pH.[15] It must, however, be pointed out that minute quantities of esters were used in these experiments. Other ^{35}S-labelled ester sulphates synthesized *in vitro* should also be possible to estimate by the procedure described if data on recovery experiments were known.

In order to determine suitable conditions for the assay of over-all sulphurylation by liver extracts, the influence of incubation time and enzyme concentration on the synthesis of PhS and DHAS was studied.

A direct proportionality was observed between the amount of extract used and the amount of PhS synthesized, unless too highly concentrated extracts were used (Fig. 2). However, the amount of a few extracts, corresponding to 2.5 mg wet weight liver, synthesized PhS to a lesser extent than could be expected from such a direct proportionality. Since the process leading to the formation of ester sulphates consists of a series of reactions, involving both sulphate activation and transfer to the acceptor, it could not be determined which enzyme activity limited the over-all sulphurylation of phenol.

In the case of DHA sulphurylation it might, however, be assumed that a limited DHA sulphokinase activity determined the synthesis of DHAS, since the extracts used were able to form PAPS in amounts permitting synthesis of PhS to exceed that of DHAS. In the latter case, a direct proportionality existed between the amount of extract used and the amount of DHAS synthesized, provided that the extracts were not too highly concentrated (Fig. 2).

The rate of conjugation of phenol by some extracts was proportional to time for at least 60 min (Fig. 3). The same observation was made by Bernstein and McGilvery [16] using *m*-aminophenol for assay of over-all sulphurylating activity of unfractionated rat liver supernatant fluid. However, a few other extracts used in the present study showed a short initial lag period. Such a time course of over-all sulphurylation of phenols was reported [17–19] by several workers when liver extracts fractionated by ammonium sulphate were used.

For comparative studies of over-all sulphurylation by liver extracts, the amount of extract chosen for the assays should, if possible, be such that the amount of ester sulphates synthesized is directly proportional to the concentration of the extract. An amount of extract representing 5 mg wet weight liver and an incubation period of 60 min might, therefore, be regarded as appropriate for the standard assay of over-all sulphurylation. This was despite the fact that the rate of formation of ester sulphates (PhS and DHAS) was seldom constant during this long incubation time. However, even during shorter incubation periods, these rates of reaction were not constant.

Data obtained by studying over-all sulphurylating activities of tissue extracts do not permit any definite conclusions about the single enzymes involved. They might, nevertheless, be regarded as of greater physiological interest than separate data on, for instance, sulphokinases or sulphate-activating enzymes.

The present method of ester sulphate determination did, however, also allow the latter activities to be studied separately.

The accumulation of PAPS by the stored extract was found to be directly proportional to the enzyme concentration, unless too highly concentrated solutions were used (Fig. 4). The relatively low capacity of concentrated enzyme solutions to accumulate PAPS was previously described by Brunngraber.[10] He, as well as Spencer,[14] found that the amount of PAPS possible to accumulate by an unfractionated rat liver extract was limited. Brunngraber suggested that this limitation could be achieved by a PAPS-degrading enzyme (3′-nucleotidase) present in the extracts, or by utilization of PAPS to esterify endogenous compounds present in the enzyme preparations.

Spencer [14] showed, however, that the decrease in PAPS concentration due to the last-mentioned esterification of endogenous compound was minute.

Spencer also demonstrated that the activity of the 3′-nucleotidase was very high in fresh extracts, but that it diminished greatly after storage for 3 months at −20°C.

In the present study, the liver extract stored for 5 months was found to accumulate PAPS at a constant rate (Fig. 5). This finding deviated from the observations of both Brunngraber [10] and Spencer,[14] who stated that the rate of PAPS accumulation levelled off during the incubation period observed. This discrepancy might, however, be explained if the extract used in the present study contained nucleotidase of lower activity than, for instance, the stored extract used by Spencer.

Thus, for the reasons given above, it is not possible to estimate the PAPS-synthesizing activity of unfractionated liver extracts, but only the ability of extracts to accumulate PAPS, the latter activity being a function of both synthesizing and degrading enzymic processes.

If comparisons between different liver extracts with respect to PAPS-accumulating activity are to be made on the basis of the procedure outlined above, the extracts compared should be kept frozen for the same period of time before this activity is assayed.

An incubation period of 15 min for PAPS accumulation, and an amount of liver extract corresponding to 5 mg wet weight liver, are suggested for such assays. Since, however, the properties of the 3′-nucleotidase have not yet been fully investigated, data obtained from estimations of PAPS-accumulating activity should be interpreted cautiously, and only great differences between extracts be taken into account.

For the assay of sulphokinases, a standardized amount of PAPS was used in every estimation. The amount chosen in these experiments was that accumulated by the yeast extract in 100 μl of incubation mixture during 30 min incubation. Further sulphate activation by the yeast or liver extracts was effectively stopped by the addition of EDTA.

Nose and Lipmann,[8] in their study of sulphokinases, used a yeast extract as PAPS generator, although they did not use a given amount of PAPS in their assays. An assay procedure more like the present one was described by Brunngraber,[10] who used isolated PAPS and EDTA.

To ensure that added PAPS would be present in excess, liver extracts were diluted prior to assay of sulphokinase activity. As might be expected, synthesis of PhS and DHAS was directly proportional to the amount of liver extract present (Fig. 6).

The rate of formation of ester sulphates in this kind of enzyme assay was exemplified by studies on the synthesis of PhS (Fig. 7). The time course was approximately the same as that described by Brunngraber [10] for synthesis of *m*-aminophenyl sulphate in the presence of isolated PAPS and EDTA, at least during the first 30 min of incubation.

Since the amount of esters synthesized was directly proportional to the extract concentrations used, any amount of extract representing up to 2 mg wet weight liver could be used in a standard assay system. Liver extract corresponding to 0.5—1 mg wet weight liver was, however, considered to be a suitable amount for standard assay of sulphokinase activity, if incubation was carried out for 60 min.

Statistical calculations were performed in order to test the reliability of the data obtained when applying the determination procedure to the different assay systems. The methodological errors computed in this way were considered to be satisfactorily low in every case.

The liver extract used for studies on sulphokinases was prepared from a human liver biopsy specimen taken at abdominal operation. Since only minute quantities of tissue were necessary to carry out assays on sulphokinases, it should be possible, however, to study such enzyme activities even in small percutaneous specimens of organs. Further data on sulphate conjugation by human liver extracts will be reported elsewhere.

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