somes of 30s and 45s); (4) ribonucleic acid of low molecular weight, together with solubilized protein that includes all the amino acid-activation enzymes of the complex.

3. When the whole membrane complex or protoplasts are incubated with labelled amino acids, labelled protein appears first in the ribosomal and phospholipoprotein fractions. The greater part of this labelled protein is found in the phospholipoprotein fraction, and there is no evidence that this is derived from the ribosomes.

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Enzymic Desulphation of L-Serine *O*[^{35}S]-sulphate: the Intracellular Localization of the Enzyme

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Dodgson, Lloyd & Tudball (1961*a*, *b*) reported that an enzyme system, capable of liberating inorganic [^{35}S]sulphate from L-serine *O*[^{35}S]-sulphate, is present in rat liver. The enzyme system could be extracted from fresh rat-liver suspensions with water. Studies on the localization of the enzyme system in the rat-liver cell and its distribution in other rat tissues and in other species have now been made.

MATERIALS AND METHODS

L-Serine O[^{35}S]*-sulphate.* This was prepared and diluted with carrier L-serine *O*-sulphate as described by Dodgson *et al.* (1961*b*).

Enzyme sources. Pig, sheep, chicken and ox livers and ox pancreas were slaughterhouse specimens. Human liver was a post-mortem sample provided by the Department of Pathology. Periwinkles (*Littorina littorea*) and limpets (*Patella vulgata*) were collected at low tide from the beach at Sully, Glamorgan. Snails (*Helix pomatia*) were purchased from L. Haig, Beambrook, Newdigate, Surrey. Brewer's yeast was kindly provided by Brain's Brewery, Cardiff. Salmon (*Salmo salar*) liver was obtained from the fish-market. Frogs (*Rana temporaria*) were kindly supplied by the Department of Zoology. Taka-diastase (lactose-free) was a gift from Parke, Davis and Co. Ltd., and acetone-dried cells of *Alcaligenes metalcaligenes* (see Dodgson, Melville, Spencer & Williams, 1954) were prepared at the M.R.C. Antibiotic Research Station, Clevedon.

Fractionation of cellular components of rat liver. M.R.C. hooded rats were used throughout. Fractionations of cellular components in sucrose media were carried out according to the procedures of Hogeboom, Schneider & Pallade (1948) (hyperosmotic sucrose, 0·88M) and Schneider (1948) (isosmotic sucrose, 0·25M). The separated fractions were resuspended in the appropriate sucrose medium for enzyme assay. Preliminary experiments showed that sucrose did not interfere with the assay method.

Uncontaminated cell nuclei. These were obtained by method 1 of Roodyn (1956). For enzyme assay the nuclei were resuspended in the fractionation medium (0·25M-sucrose containing 1·8mM-$CaCl_2$), which did not interfere with the assay method.

Determination of enzyme activity

A procedure based on that described by Dodgson *et al.* (1961*b*) was used. For the assay of whole tissue suspensions 50μl. of 0·02M-L-serine *O*[^{35}S]-sulphate in 0·5M-tris–acetic acid buffer, pH 7·0, was incubated with an equal volume of the enzyme preparation. These volumes were doubled for the assay of the crude cell-nuclei fractions and halved for all other enzyme assays. Except where otherwise stated, incubation was for 3 hr. at 38°. Enzyme action was stopped by placing the reaction tubes in boiling water for 30 sec. Control determinations consisted of substrate and enzyme incubated separately and mixed immediately before the boiling-water treatment. Precipitated protein was removed by centrifuging and 20μl. of the clear supernatant was applied as a spot (7 mm. diameter, prepared by alternate spotting and drying) to Whatman no. 1 paper and subjected to electrophoresis for 2 hr. at a potential gradient of 12v/cm. in the presence of 0·1M-ammonium acetate. Tests, controls and markers of L-serine *O*[^{35}S]-sulphate and inorganic $^{35}SO_4^{2-}$ ions ($Na_2{}^{35}SO_4$) were run on the same paper. After drying and cutting into suitable strips, radioactive zones were detected with the C. 100 Actigraph automatic strip-scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) as described by Dodgson *et al.* (1961*b*). The areas of the peaks on the recording chart (corresponding to enzymically liberated inorganic $^{35}SO_4^{2-}$ ions and residual L-serine *O*[^{35}S]-sulphate) were cut out and weighed on a microbalance, and the weights used to calculate enzyme activity.

The following checks on the validity and limitations of the method were made.

Variation in weights of standard areas of recording chart paper. Standard areas (varying between 4 and 17·5 cm.^{2}) were cut from different regions of the recording chart paper and weighed. The variation in the weights of identical areas was less than $\pm$2·5%.

Reproducibility of the electrophoresis procedure. Variations in the count rate (and hence of areas of peaks on the recording chart) of identical amounts of radioactively labelled material could arise as a result of the electrophoresis procedure (e.g. from uneven spreading). This possibility was checked by subjecting several 20μl. samples of the substrate solution to paper electrophoresis. Within the limits imposed by variations in the density of the chart paper, the weights of the areas of the peaks of radioactivity obtained were reproducible.

In assaying the activity of different enzyme preparations, variations in the amounts of product liberated and of substrate remaining are to be expected. It was therefore necessary to show that the amounts of product (or substrate remaining) and the areas of the peaks on the recording chart were linearly related. Inorganic $^{35}SO_4^{2-}$ ions or L-serine *O*[^{35}S]-sulphate, in various concentrations and representing 0–100% desulphation of substrate, were subjected to electrophoresis and the strips analysed. In each case a linear relationship between concentration and peak area was obtained.

As a final check on the electrophoresis procedure, standard amounts of inorganic $^{35}SO_4^{2-}$ ions and L-serine *O*[^{35}S]-sulphate were subjected to electrophoresis, separately and as mixtures. The areas of the peaks obtained with the mixtures were identical with those obtained when the components were run separately.

Recovery of radioactivity from enzyme preparations. The recovery of radioactivity from incubation mixtures of enzyme and substrate was always checked by means of an additional control experiment in which water was substituted for the enzyme preparation. The total radioactivity recovered from the test and normal control determinations could then be compared with that recovered in the absence of enzyme preparation. Table 2 shows that recoveries from enzyme preparations were somewhat variable but were sufficiently good for the purpose of the present study.

EXPERIMENTAL AND RESULTS

Fractionation of intracellular components of rat liver

Table 1 shows the activities of the various cellular fractions obtained by simple differential centrifuging of rat-liver suspensions in hyper- and iso-osmotic sucrose solutions. Enzyme activity was concentrated mainly in the high-speed supernatant fraction although appreciable activity was also present in the so-called 'nuclear' fraction. However, microscopic examination of this fraction showed the presence of unruptured cells and relatively little enzyme activity could be detected in uncontaminated nuclei isolated by the procedure of Roodyn (1956). The low enzyme activity associated with the 'mitochondrial' and 'microsomal' fractions is probably not significant and no attempt was made to resolve these fractions further by density gradient centrifuging procedures.

Comparative activities of male and female rat livers

During studies on the microsomal arylsulphatase of rat liver, Dodgson, Spencer & Thomas (1953) noted that the livers of male rats were almost twice as active as those of females. The relative activities of male and female rat livers towards L-serine *O*[^{35}S]-sulphate were checked as follows. The livers of five male and five female rats were weighed and squeezed through fine (1 mm.^{2}) brass-wire meshes in order to remove connective tissue. The pulps were suspended with a glass homogenizer in sufficient ice-cold water to give a final concentration of 10% (w/v), and the activity of each suspension was

determined as described earlier. The liberation of inorganic $^{35}SO_4^{2-}$ ions, expressed as a percentage of the available ^{35}S, averaged 33·3 % (range 32·1–35·9 %) for male rats, whereas the corresponding figures for females was 26·8 % (range 22·5–30·6 %). It is doubtful whether these differences are significant in view of the relatively small numbers of animals tested.

Distribution of enzyme activity in other rat tissues and in other species

Except where otherwise stated, a 20 % (w/v) suspension of the tissue in ice-cold water was frozen and thawed, resuspended, and centrifuged for 20 min. at 0° and 2000*g*. The activity of the supernatant was then determined as described pre-

Table 1. *Enzymic liberation of inorganic $^{35}SO_4^{2-}$ ions from* L-*serine* O[^{35}S]-*sulphate by fractions obtained by the differential centrifuging of rat-liver suspensions in sucrose solutions*

See text for experimental details. The enzyme activities of the fractions are expressed as percentages of that obtained with the whole-liver suspension. Ranges are given in parentheses.

Fractionation medium	No. of experiments	Fraction	Activity (%)
Hyperosmotic sucrose	7	Nuclei	21·8 (14·5–32·0)
		Mitochondria	5·1 (0–13·0)
		Microsomes	8·3 (0–15·9)
		Final supernatant	58·0 (43·6–71·6)
Iso-osmotic sucrose	1	Nuclei	37·0
		Mitochondria	5·8
		Microsomes	4·5
		Final supernatant	68·0
Iso-osmotic sucrose –$CaCl_2$	3	Uncontaminated nuclei*	2·5 (0–7·6)

* Prepared by the method of Roodyn (1956).

Table 2. *Liberation of inorganic $^{35}SO_4^{2-}$ ions from* L-*serine* O[^{35}S]-*sulphate by aqueous extracts of rat tissues and of other organisms*

See text for experimental details.

Species	Tissue	Initial concn. of tissue suspension (%, w/v)	Incubation period (hr.)	Percentage of available ^{35}S liberated as inorganic sulphate	Recovery of ^{35}S from incubation mixture (%)
Rat	Kidney	20	22	31·3	93·7
	Heart	20	22	0	96·2
	Spleen	20	22	< 5·0	96·5
	Lung	20	18	< 5·0	96·0
	Pancreas	20*	18·5	25·3	85·5
	Small intestine	20	3·5	0	90·3
Ox	Liver	20	3	63·1	—
	Pancreas	20	3	31·0	106·2
Human	Liver	20	4	22·0	86·7
Mouse	Liver	10	3	15·0	105·0
Pig	Liver	15	3	46·6	103·4
Sheep	Liver	20	3	54·1	—
Chicken	Liver	10	4	39·9	—
Frog (*Rana temporaria*)	Liver	20*	3	6·5	94·7
Salmon (*Salmo salar*)	Liver	20*	3	40·3	93·8
Snail (*Helix pomatia*)	Digestive juice	†	20·5	0	101·1
Limpet (*Patella vulgata*)	Digestive gland	20	2·5	0	88·9
Periwinkle (*Littorina littorea*)	Digestive gland	20	2·5	0	96·9
Taka-diastase (*Aspergillus oryzae*)	—	5*	4	0	95·0
Alcaligenes metalcaligenes	—	5*	4	0	88·5
Baker's yeast	—	20	3	0	105·0
Brewer's yeast	—	20*	18	< 5·0	97·3

* Whole tissue suspensions used. † Undiluted juice dialysed for 16 hr.

viously, although in some cases a long incubation period was used (see Table 2).

The enzyme was present in the livers of all the mammalian species examined and was also found in rat kidney and pancreas and in ox pancreas. Appreciable activity was also obtained with chicken and fish (salmon) livers but not with frog liver or with any of the lower organisms that were examined.

DISCUSSION

Desulphation of L-serine O[^{35}S]-sulphate can be achieved by mammalian, avian and fish liver extracts, and, although the responsible enzyme system does not appear to be particularly active, this view must be accepted with reserve until the optimum conditions for activity have been completely defined. Dodgson *et al.* (1961*b*) have pointed out that the desulphation may not necessarily be due to a simple hydrolase (a sulphatase), but preliminary efforts to establish the exact mechanism of the process have been hindered by the crude nature of the rat-liver preparations. Certainly no known sulphatase can be responsible for the activity. Chondro-, glyco- and myro-sulphatase do not occur in rat livers (Dohlman, 1956; Dodgson & Lloyd, 1961; Baum & Dodgson, 1957) and cellulose polysulphatase (found in at least one mollusc) is apparently specific for β-linked polyglucans containing sulphate in the 2- or 3-position of the glucose residues (Takahashi & Egami, 1961). Cortisone 21-sulphatase occurs in the digestive juice of the snail (*Helix pomatia*) but L-serine O-sulphate is not a substrate for the enzyme (Dodgson, 1961). 3β-Steroid sulphatase and arylsulphatases A, B and C do occur in mammalian livers but are localized in the particulate material of the liver cell (Roy, 1957, 1960; Dodgson, Spencer & Thomas, 1953, 1955).

Neuhaus & Byrne (1959) and Borkenhagen & Kennedy (1959) have shown that rat and chicken livers possess a phosphatase that is active towards L-serine O-phosphate (O-phosphoserine). A similar enzyme is also present in baker's yeast (Schramm, 1958). No enzyme activity towards L-serine O[^{35}S]-sulphate could be detected in baker's yeast in the present study, nevertheless the possibility that the same enzyme is attacking both substrates cannot yet be excluded.

The occurrence, in proteins, of L-serine O-phosphate is well known but the presence of the corresponding sulphate ester has never been demonstrated. Free L-serine cannot be sulphated enzymically by particle-free rat-liver preparations which are able to sulphate steroids, phenols, amines and simple alcohols (Spencer, 1960; Dodgson *et al.* 1961*b*), but this does not exclude the possibility that protein-bound serine residues can be sulphated. Free L-tyrosine cannot be sulphated by rat-liver preparations (see, for example, Grimes, 1959) but occurs in fibrinogen as the corresponding O-sulphate ester (Bettelheim, 1954). It may be of significance that the enzyme systems responsible for the desulphation of L-serine O[^{35}S]-sulphate and for the sulphation of hydroxylated compounds co-exist in the cell sap. Attempts to achieve the enzymic sulphation of free L-serine have therefore always been made with enzyme preparations that contain the desulphating system.

SUMMARY

1. Further observations have been made on a method of determining the activity of the rat-liver enzyme system which is able to desulphate L-serine O[^{35}S]-sulphate.

2. The desulphating enzyme is mainly localized in the cell sap of the liver cell. Its distribution in the cell differs from that of any known mammalian sulphatase enzyme.

3. The enzyme is present in human, ox, sheep, pig, mouse, chicken and salmon livers, but significant amounts could not be detected in a number of lower organisms that were examined.

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