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The Metabolism of Dipotassium 2-Hydroxy-5-Nitrophenyl [^{35}S]Sulphate, a Substrate for Lysosomal Arylsulphatases A and B

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The metabolic fate of dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulphate ([^{35}S]NCS), a chromogenic substrate for lysosomal arylsulphatases A and B, has been studied in rats. Intraperitoneal injection of [^{35}S]NCS into free-ranging animals is followed by excretion of the bulk of the radioactivity in the urine within 24hr., less than 13% being eliminated as inorganic [^{35}S]sulphate. Most of the urinary radioactivity can be accounted for as [^{35}S]NCS, but small amounts of a labelled metabolite are also present. Experiments in which [^{35}S]NCS was injected intravenously into anaesthetized rats with bile-duct and bladder cannulae confirm that the ester is rapidly excreted in the urine. However, small amounts of radioactivity appear in bile, mainly in the form of the metabolite detected in urine. When [^{35}S]NCS is perfused through the isolated rat liver, about 35% of the dose is hydrolysed within 3hr. Similar results are obtained if [^{35}S]NCS is injected into anaesthetized rats in which kidney function has been eliminated by ligature of the renal pedicles. The labelled metabolite has been isolated from bile obtained by perfusing several rat livers with blood containing a total of 100mg. of [^{35}S]NCS. It has been identified as 2-β-glucuronosido-5-nitrophenyl [^{35}S]sulphate. The implications of the various findings are discussed. The Appendix describes the preparation of [^{35}S]NCS.

Mammalian tissues contain three arylsulphatase enzymes (aryl sulphate sulphohydrolases, EC 3.1.6.1), designated A, B and C, that can hydrolyse aryl sulphate esters (Dodgson, Spencer & Thomas, 1955*a*). Though the specificities of the enzymes are not yet absolutely defined it is clear that arylsulphatases A and B, which are lysosomal in origin (Viala & Gianetto, 1955), show high activity and affinity for such substrates as dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate), and comparatively low activity towards simpler aryl sulphates such as potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate. In contrast, arylsulphatase C, a microsomal enzyme, is particularly active towards these simpler aryl sulphates (see Dodgson, Spencer & Thomas, 1954).

In attempts to elucidate the physiological role of the arylsulphatases, work in these, and other, Laboratories has been directed towards investigating the metabolic fate of aryl sulphate esters after their administration to experimental animals. Early studies with simple esters, such as potassium phenyl sulphate (Rhode, 1923; Sperber, 1948; Garton & Williams, 1949) and ^{35}S-labelled potassium phenyl and naphthyl sulphates (Hawkins & Young, 1954), revealed that such compounds undergo little hydrolysis *in vivo*. In contrast, Hanahan & Everett (1950) have shown that the ^{35}S-labelled sulphate ester of the phenolic hormone oestrone is almost completely desulphated when administered to female rats, and there is also some evidence to show that ^{131}I-labelled 3,3′,5-tri-iodo-L-thyronine *O*-sulphate is partially desulphated *in vivo* (Roche, Michel, Closon & Michel, 1958). These results might suggest that only the sulphate esters of 'physiologically important' phenols are extensively hydrolysed in the living animal. However, Dodgson & Tudball (1960) found that potassium *p*-nitrophenyl [^{35}S]sulphate (the chromogenic substrate generally used for measuring arylsulphatase C) underwent appreciable hydrolysis in rats, although the parent phenol can hardly be considered as 'physiologically important'. On the basis of specificity studies *in vitro* (see Dodgson, Rose & Tudball, 1959) it was tentatively assumed that arylsulphatase C was the enzyme responsible for the hydrolysis of the ester *in vivo*. Dodgson &

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Tudball (1960) also pointed out that the failure of potassium phenyl [^{35}S]sulphate to undergo appreciable hydrolysis *in vivo* was also in accord with the known specificity of the mammalian arylsulphatase enzymes.

The present paper is concerned with the metabolism of ^{35}S-labelled nitrocatechol sulphate, the chromogenic substrate generally used to measure the activity of the mammalian lysosomal arylsulphatases A and B (Roy, 1953, 1954; Dodgson *et al.* 1955*a*).

MATERIALS AND METHODS

Dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulphate. [^{35}S]-NCS* was prepared by monosulphation of 4-nitrocatechol with chloro[^{35}S]sulphonic acid as described in the Appendix (Flynn, Rose & Tudball, 1967). The specific radioactivity of the preparation was about 31 μc/mg.

Potassium p-nitrophenyl [^{35}S]sulphate. This was prepared by the method of Dodgson & Tudball (1960), but with carrier-free chloro[^{35}S]sulphonic acid. The specific radioactivity of the product was about 38 μc/mg. Descending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25, by vol.) showed the compound to be homogeneous and free from inorganic [^{35}S]sulphate.

Experimental animals. Male and female M.R.C. hooded rats were used throughout. Intraperitoneal injections of [^{35}S]NCS were carried out while the animals were under general ether anaesthesia. The animals were placed in metabolism cages that permitted separate collection of urine and faeces, and were subsequently allowed water and food without restriction. Urine samples were collected after 2, 24 and 48 hr., funnel washings being added to the appropriate sample. Samples were filtered through a glass-wool plug and, whenever possible, were assayed immediately for radioactivity; otherwise they were stored at 2° until required.

In experiments on rats with bile-duct and bladder cannulae, animals were first lightly anaesthetized with ether and the trachea was exposed and cannulated (Portex tubing, PP270, external diameter 2·8 mm.) to facilitate breathing. The jugular vein was then cannulated (Portex tubing, PP30, external diameter 1 mm.) and Nembutal (10–15 mg./kg. body wt.) was injected via the cannula, followed by 0·5 ml. of iso-osmotic aq. 0·9% NaCl. The bladder and bile duct were cannulated (Portex tubing, PP00, external diameter 0·628 mm.) through a mid-line abdominal incision, and [^{35}S]NCS was then injected via the cannula in the jugular vein. Aq. 0·9% NaCl was injected every 30 min. and Nembutal every 2·5 hr. Bile and urine samples were collected at suitable time intervals. At the end of the experiment the animal was killed with an overdose of Nembutal.

Measurement of radioactivity in urine and bile. Bile and urine samples were made up to a known volume with water and portions were removed for the estimation of inorganic [^{35}S]sulphate (as $Ba^{35}SO_4$) and total [^{35}S]sulphate (as $Ba^{35}SO_4$ after acid hydrolysis) according to the method of Lloyd (1961).

The precipitated $Ba^{35}SO_4$ was plated at infinite thickness in plastic planchets, giving a surface area of 1 cm.^{2}. Measurement of radioactivity was made with a thin-end-window Geiger–Müller tube (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). For samples having low radioactivity, a windowless gas-flow counter was used. Corrections for background, self-absorption, decay and coincidence were made and sufficient counts were recorded to give a standard error of less than 2%.

Measurement of ^{35}S content of faeces and carcasses. The total ^{35}S contents of faeces and of the whole rat carcass were precipitated and counted as $Ba^{35}SO_4$ after oxidation with fuming HNO_3, according to the method of Young, Edson & McCarter (1949).

Detection of radioactivity on paper chromatograms, silica-gel chromatograms and paper electrophoresis strips. After drying in a current of warm air, paper chromatograms and paper electrophoresis strips were kept in contact with Ilford Industrial B X-ray film for periods of 14 days. Silica-gel chromatograms were dried in an oven at 40° and kept in contact with X-ray film for periods of up to 21 days.

Liver perfusion. Isolated rat livers were perfused with blood containing either [^{35}S]NCS or potassium *p*-nitrophenyl [^{35}S]sulphate in an apparatus that was essentially that of Miller, Bly, Watson & Bale (1951). The perfusion medium consisted of homologous blood collected from 8–10 lightly anaesthetized rats by means of a cannula inserted into the abdominal aorta towards the heart. Blood (approx. 75 ml.) was collected into a vessel containing 25 ml. of heparinized iso-osmotic aq. 0·9% NaCl (20 mg. of heparin/25 ml. of NaCl soln.), and the whole was filtered through 15-denier micro-mesh nylon (to remove small clots), before being introduced into the perfusion apparatus.

The liver was removed from the donor animal by the procedure described by Miller *et al.* (1951). Approximately 20 min. elapsed during the whole of the operational procedure; the time taken for the actual removal and transference of the liver was usually less than 3 min.

Blood containing the ^{35}S-labelled sulphate ester under investigation was circulated in the apparatus for 2–3 hr. before insertion of the liver into the system and samples (0·5 ml.) were removed every 30 min. during this period. These control samples were assayed for possible hydrolysis of the ester, in the absence of the liver, under the conditions prevailing in the perfusion apparatus. After the insertion of the liver into the system, samples of blood (0·5 ml.) and bile (approx. 0·2 ml.) were collected every 30 min.

The blood samples were centrifuged to remove erythrocytes, the supernatant was diluted to a known volume with water and portions were taken for the assay of inorganic [^{35}S]sulphate and total [^{35}S]sulphate. Bile samples were made up to a known volume and treated in the same way. Preliminary experiments showed that neither [^{35}S]NCS nor potassium *p*-nitrophenyl [^{35}S]sulphate sedimented with the erythrocytes, nor did either ester significantly interfere with the subsequent assay procedure.

EXPERIMENTAL AND RESULTS

Free-ranging male and female rats were injected intraperitoneally and intravenously with [^{35}S]NCS in doses in the range 0·1–2·4 mg. in 0·4 ml. of water.

* Abbreviation: [^{35}S]NCS, dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulphate.

Table 1. *Distribution of* ^{35}S *in the urine and faeces of rats injected intraperitoneally with* [^{35}S]*NCS*

The results are mean values with the ranges in parantheses.

Sex	No. of animals	Dose (mg.)	Distribution of ^{35}S in urine (% of ^{35}S injected)						Total ^{35}S content of faeces (% of ^{35}S injected)	^{35}S recovered (% of ^{35}S injected)
			Total sulphate fraction			Inorganic sulphate fraction				
			2 hr.	24 hr.	48 hr.	2 hr.	24 hr.	48 hr.		
Male	3	2·4	82·8 (79·0–85·0)	12·0 (6·8–16·0)	1·7 (1·4–2·4)	3·9 (2·4–4·8)	4·5 (4·0–5·5)	0·8 (0·8–0·9)	—	96·5
Female	4	2·4	74·8 (57·5–84·8)	19·8 (13·6–34·0)	2·2 (1·7–2·8)	3·2 (2·9–3·5)	3·7 (2·1–7·2)	1·9 (1·1–2·8)	—	96·8
Male	3	0·8	66·0 (53·2–68·3)	30·7 (20·6–40·0)	1·9 (0·9–2·8)	4·4 (3·7–5·1)	7·2 (6·1–8·3)	0·7 (0·6–0·8)	1·5 (0·9–1·8)	100·1
Male	3	0·1	53·4 (48·5–58·4)	46·3 (44·2–48·5)	1·3 (1·0–1·5)	4·5 (4·2–4·8)	7·4 (7·3–7·5)	1·5 (1·0–2·3)	1·3 (0·9–1·9)	102·3

Table 2. *Excretion of* ^{35}S *in the urine after the injection of* 2·4 *mg. of* [^{35}S]*NCS into anaesthetized rats with bile-duct and bladder cannulae*

Time (min.)... ...	Distribution of ^{35}S in urine (% of ^{35}S injected)													
	20	35	50	65	80	95	110	125	140	155	170	230	290	350
Animal 1														
Total sulphate	8·9	15·5	19·9	11·9	10·0	6·1	3·8	2·9	4·2	1·8	1·4	2·7	0·9	0·57
Inorganic sulphate	0·5	1·3	1·3	1·1	0·9	0·6	0·5	0·5	0·7	0·3	0·7	0·7	0·3	0·1
Animal 2														
Total sulphate	22·1	23·0	14·6	10·9	6·2	4·8	2·4	1·2	2·1	3·7	1·4	0·8	0·7	0·2
Inorganic sulphate	0·9	1·2	1·7	0·9	0·4	0·4	0·5	0·5	0·5	0·7	0·1	0·1	0·2	0·1
Animal 3														
Total sulphate	30·3	10·7	17·1	9·8	7·4	4·2	2·1	4·1	1·1	2·7	1·4	0·8	0·3	0·5
Inorganic sulphate	2·1	0·9	1·7	0·9	0·5	0·6	0·3	0·5	0·4	0·7	0·3	0·3	0·1	0·0

	Animal 1	Animal 2	Animal 3
Total [^{35}S]sulphate excreted in 5 hr. 50 min. (% of ^{35}S injected)	100·0	94·1	92·5
Inorganic [^{35}S]sulphate excreted in 5 hr. 50 min. (% of ^{35}S injected)	9·5	8·0	9·3

Urine was collected after 2, 24 and 48hr. and faeces were collected over the 48hr. period. At the end of this period the animals were killed and the whole carcasses taken for determination of residual ^{35}S. Table 1 records the results but does not include the carcass ^{35}S values, since these were always less than 0·2% of the injected dose. Regardless of sex, dose and mode of administration, about 90–95% of the dose appeared in the urine within 24hr., and the rapid excretion of the ester from the animal is shown by the fact that about 60–80% of the administered radioactivity was excreted within 2hr. Less than 13% (and in most experiments, less than 10%) of the injected radioactivity was present as inorganic [^{35}S]sulphate, indicating that little hydrolysis of the ester had taken place. A small amount of radioactivity was detected in the faeces.

[^{35}S]NCS was also injected intravenously into rats possessing bile-duct and bladder cannulae. Bile and urine samples were collected at suitable time intervals over a period of about 6hr. In initial experiments bile samples were assayed individually. However, the amount of radioactivity appearing in samples collected after 1hr. was very small, and in subsequent experiments the bile samples were pooled before assay. The distribution of radioactivity in the urine is shown in Table 2. The greater part of the dose was excreted within the first 65min. and virtually the whole of the dose (90–100%) within 6hr., 8·0–9·5% of this being present as inorganic [^{35}S]sulphate. The total biliary radioactivity was only a small percentage of the injected radioactivity (1·75%); 0·49% was present as inorganic [^{35}S]sulphate.

Qualitative examination of ^{35}S in bile and urine. The urine and bile of a bladder- and bile-duct-cannulated rat that had received 2·4mg. of [^{35}S]-NCS, as described above, were subjected to paper chromatography for 16hr. on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25, by vol.); radioautograms were then prepared. Control experiments in which [^{35}S]NCS was added to freshly voided urine and bile (1mg./ml.) and kept at room temperature for 6hr. before chromatography established that the ester was stable under the conditions of the experiment.

Radioautograms of the test samples of both bile and urine revealed that the bulk of the radioactivity was present as unchanged [^{35}S]NCS. Small amounts of inorganic [^{35}S]sulphate and an unknown ^{35}S-labelled component were also present. The unknown component had a mobility intermediate between those of inorganic [^{35}S]sulphate and [^{35}S]NCS; its identification is described below.

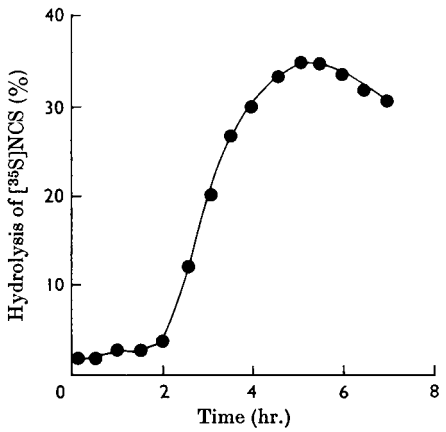

Fig. 1. Time-course of the hydrolysis of 24mg. of [^{35}S]NCS by the isolated perfused rat liver.

Liver perfusion experiments

The experiments described above indicate that [^{35}S]NCS undergoes relatively little hydrolysis in the intact animal in spite of the widespread distribution of arylsulphatases A and B in rat tissues. Several explanations may be offered, one being that the extremely rapid excretion of the ester results in minimal exposure of substrate to enzymes. In attempts to eliminate the problem of rapid excretion, isolated rat livers were perfused with rat blood containing 24–26mg. of [^{35}S]NCS.

Quantitative examination of blood. Relatively little hydrolysis of the ester occurred (approx. 2–3% in 2hr.) in the absence of the liver, but after the introduction of the latter there was an initial rapid hydrolysis of the ester over a period of 3hr. (see Fig. 1). This hydrolysis may be presumed to occur within the liver, since separate experiments in which livers were perfused with blood alone showed that there was no release of arylsulphatase activity into the circulation during perfusion. After 3hr. the amount of inorganic [^{35}S]sulphate in each blood sample gradually decreased, suggesting utilization of the liberated sulphate.

Dodgson & Tudball (1960) have shown that potassium *p*-nitrophenyl [^{35}S]sulphate undergoes desulphation after its injection into the living animal. For the purpose of comparison, isolated rat livers were therefore perfused with blood containing 26·1mg. of this ester. Considerable hydrolysis (approx. 40%) of the ester occurred during the first 3hr. after the introduction of the liver. Subsequently, as with [^{35}S]NCS, the amount of inorganic [^{35}S]sulphate appearing in each successive sample declined, suggesting utilization by the liver.

Quantitative examination of bile. The total volume of bile produced during the perfusion of livers with blood containing [^{35}S]NCS was used for the estimation of total and inorganic [^{35}S]sulphate.

In a typical experiment the amount of ^{35}S excreted in the bile (expressed as a percentage of ^{35}S initially added to the blood) was 2·2%; the greater part of this biliary ^{35}S (80%) was present in the form of ester sulphate.

Experiments on rats with renal vessel ligation

The results of the liver perfusion experiments demonstrated that appreciable desulphation of [^{35}S]NCS occurred under these 'closed system' conditions. In order to simulate these conditions in the living animal, [^{35}S]NCS (2·4mg.) was injected into a rat in which kidney function had been eliminated by ligation of the renal pedicles. Blood samples were withdrawn from the animal at 30min. intervals after injection and assayed for radioactivity. Considerable hydrolysis of the ester occurred over a period of approx. 3hr. (Fig. 2) and this phase was followed by a period of apparent sulphate utilization similar to that observed with perfused livers.

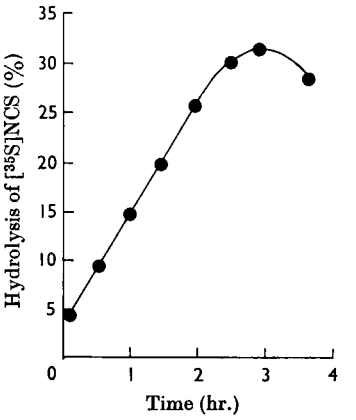

Fig. 2. Time-course of the hydrolysis of 2·4mg. of [^{35}S]NCS intravenously injected into an anaesthetized rat in which kidney function had been eliminated by ligation of the renal pedicles.

Isolation and identification of the ^{35}S-labelled metabolite of [^{35}S]NCS

Paper chromatography showed that the unknown ^{35}S-labelled component present in the urine and bile after injection of [^{35}S]NCS was also present in bile collected from isolated rat livers perfused with blood containing [^{35}S]NCS. Several liver perfusions were performed (total of 100mg. of [^{35}S]NCS), which allowed the collection of considerable quantities of bile containing the metabolite; this was separated and purified by chromatography on thick-layer silica-gel plates. The plates were prepared by applying a slurry of silica gel (Whatman silica gel, S.G.41, 60g. in 117ml. of water) to 20cm. × 20cm. glass plates, with a Unoplan leveller (Shandon and Co.) set at 1mm. The plates were dried first at room temperature for 4–6hr. and then at 105° for 4hr. Bile samples were applied as streaks (50–150μl.) to a line 1·5cm. from the bottom edge of each glass plate. After drying, the samples were subjected to ascending chromatography in benzene–ethyl methyl ketone–ethanol–water (3:3:3:1, by vol.) (Wusteman, Dodgson, Lloyd, Rose & Tudball, 1964). By superimposing a radioautogram of the silica-gel chromatogram over the silica-gel plate, the unknown ^{35}S-labelled component was located and the appropriate area was then scraped off and eluted with ice-cold water. The eluate was reduced to a small volume, reapplied to a silica-gel plate and rechromatographed, detected and eluted as before. Thin-layer chromatography of the concentrated eluate (hereafter referred to as eluate B) showed the presence of a single radioactive component that also gave a strong positive reaction for glucuronic acid when sprayed with ethanolic naphtharesorcinol (0·2% solution)–phosphoric acid reagent (22:1, v/v) according to the directions of Randerath (1963). Eluate B was stored in the frozen state until required.

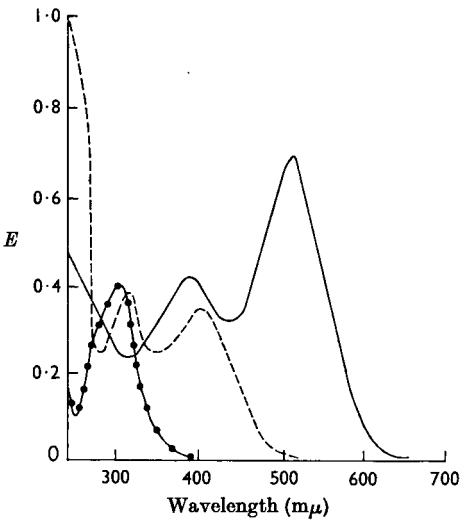

Fig. 3. Absorption spectra of eluate B (see text for details) after various treatments. ●——●, In the presence of 0·1N-NaOH (final concn.); ----, in the presence of 0·1N-NaOH (final concn.) but after an initial treatment for 5min. at 100° with an equal volume of 4N-HCl; ——, in the presence of 0·1N-NaOH (final concn.) but after an initial treatment for 1hr. at 100° with an equal volume of 4N-HCl. The curves are not quantitatively related to each other.

Absorption spectrum. A portion of eluate B was made 0·1N with respect to NaOH and examined spectrophotometrically. Strong u.v. absorption was noted, with $\lambda_{max.}$ 307mμ (Fig. 3).

Mild acid hydrolysis. A portion of eluate B was treated at 100° for 5min. with an equal volume of 4N-HCl. Aryl sulphate esters are normally hydrolysed by such treatment, whereas glucuronides are largely unaffected (see, e.g., Garton, Robinson & Williams, 1949). Tests on a portion of the cooled solution revealed the presence of inorganic [^{35}S]-sulphate (detected as $Ba^{35}SO_4$), whereas similar tests on the original eluate were negative. The remainder of the solution was made 0·1N with respect to NaOH and examined spectrophotometrically. The spectrum (Fig. 3) was very different from that obtained before treatment with acid.

Prolonged acid hydrolysis. A further portion of eluate B was hydrolysed with an equal volume of 4N-HCl for 1hr. at 100°; 4-nitrocatechol was liberated and detected spectrophotometrically (Fig. 3) after the hydrolysate had been made 0·1N with respect to NaOH.

Incubation with β-glucuronidase. From the results outlined above it seemed clear that nitrocatechol [^{35}S]sulphate had become associated with a glucuronic acid residue. Accordingly, portions of eluate B were incubated with equal volumes of a solution of β-glucuronidase (Bacterial Preparation, type I; Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0·1M-phosphate buffer, pH6·8 (400 Fishman units/ml.), for 30min. at 37°. After incubation, portions (usually 0·2ml.) of the mixture (applied as streaks) were subjected to thin-layer chromatography in the usual solvent system. Control experiments were performed in which β-glucuronidase was omitted from the incubation mixture, and suitable markers of [^{35}S]NCS, inorganic [^{35}S]-sulphate and untreated eluate B were also included on the plates. Dried plates were sprayed for glucuronic acid and radioautograms were also prepared.

Incubation with β-glucuronidase resulted in the disappearance of the radioactive glucuronic acid-containing band attributable to the metabolite and the appearance of a radioactive band with a mobility identical with that of authentic [^{35}S]NCS. In separate experiments in which spraying for glucuronic acid was omitted, the area of the plate corresponding to the new band was eluted with 0·1N-NaOH and its identity confirmed as [^{35}S]NCS by its u.v.-absorption spectrum ($\lambda_{max.}$ 405mμ). Further experiments showed that [^{35}S]NCS was not produced if saccharo-(1→4)-lactone (a powerful inhibitor of β-glucuronidase) was incorporated in the initial incubation mixture at a concentration of 1mM.

Incubation with arylsulphatase. Eluate B was incubated at 37° for 1hr. with an equal volume of a solution of a β-glucuronidase-free preparation of the arylsulphatase of *Alcaligenes metalcaligenes* (20mg./ml.) in 0·1M-tris–acetate buffer, pH8·7 (Dodgson, Spencer & Williams, 1955*b*). Control experiments were performed in which the enzyme preparation was omitted. After incubation, portions (usually 150μl.) of the incubation mixtures were subjected to thin-layer chromatography; radioautograms were then prepared. Incubation with arylsulphatase resulted in the disappearance of the labelled metabolite and the appearance of inorganic [^{35}S]sulphate. A faintly yellow-coloured, non-radioactive band was also apparent when the plates were exposed to ammonia vapour and this band reacted positively to the naphtharesorcinol spray for glucuronic acid. In separate experiments the area corresponding to this band was eluted with water and a portion was made 0·1N with respect to NaOH and examined spectrophotometrically. The absorption spectrum was virtually identical with that obtained for the product of mild acid hydrolysis (cf. Fig. 3). A further portion was treated at 100° for 1hr. with an equal volume of 4N-HCl, when 4-nitrocatechol (identified spectrophotometrically after being made 0·1N with respect to NaOH) was liberated. Similarly, 4-nitrocatechol was liberated if treatment with β-glucuronidase, as described earlier, was substituted for acid hydrolysis.

The experiments described above collectively establish the identity of the metabolite as a glucuronic acid conjugate of [^{35}S]NCS and, since complete acid hydrolysis yields 4-nitrocatechol, the double conjugate can be identified as 2-β-glucuronosido-5-nitrophenyl [^{35}S]sulphate.

Biochemical synthesis of 2-β-glucuronosido-5-nitrophenyl [^{35}S]sulphate. Mammalian livers possess microsomal glucuronyltransferase systems [UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17], which transfer glucuronic acid from UDP-glucuronate to a wide variety of acceptors. Attempts to use [^{35}S]NCS as an acceptor in the rat-liver system were unsuccessful, possibly owing to the presence of endogenous acceptors (G. J. Dutton, personal communication). However, some transfer of glucuronic acid to [^{35}S]-NCS was achieved with rabbit-liver microsomal preparations obtained by the procedure of Isselbacher, Chrabas & Quinn (1962). Such preparations, when assayed under the conditions described by Isselbacher *et al.* (1962), were able to conjugate 150–160mμmoles of *p*-nitrophenol/hr. Under identical conditions, but with [^{35}S]NCS (in amounts of 0·5–5·0μmoles) instead of *p*-nitrophenol, the amount of transfer achieved was 0–8·4mμmoles of [^{35}S]NCS conjugated/hr. Subsequently, electrophoresis of the incubation mixtures was carried out on Whatman no. 3 paper in the presence of 0·1M-

sodium acetate–acetic acid buffer, pH 4·2, for 4hr. at a potential gradient of 8 v/cm. Radioautograms revealed the presence of a new radioactive component having the same electrophoretic mobility as the 2-β-glucuronosido-5-nitrophenyl [35S]sulphate isolated from rat bile.

DISCUSSION

A variety of physiological roles are presently ascribed to lysosomal enzyme systems, including those of scavenging and digestion of intracellular materials and of materials arriving in the cell from outside sources (see de Duve & Wattiaux, 1966). There is, as yet, no clear indication of the part played by arylsulphatases A and B in the normal functioning of lysosomes although the former enzyme may be involved in the degradation of cerebroside sulphate esters (Mehl & Jatzkewitz, 1965; Austin *et al.* 1964). Roy (1960) suggests that arylsulphatases A and B cannot act as such *in vivo*, since they would become functional only when the lysosomes were ruptured. Moreover, even if they were liberated they would be effectively inhibited by ions such as P_i and Cl^- present in the cell. Roy (1960) concludes that either arylsulphatases A and B are inactive *in vivo* or that the properties of the enzymes in the intact cell are very different from those exhibited *in vitro*. Some of these views would not be in accord with present concepts of lysosomal action, which, for the most part, imply that lysosomal enzymes are not normally released into the main body of the cell.

The present studies on the metabolic fate of [35S]NCS in free-ranging rats and in anaesthetized rats with bladder cannulae appear to support the view that arylsulphatases A and B are relatively inactive *in vivo*, since only slight hydrolysis of the injected ester can be observed under these circumstances. However, it seems more probable that the observation is a reflection of the extremely rapid rate at which the ester is excreted via the kidneys (cf. Table 2). Under these circumstances any contact between substrate and enzymes would be minimal. This reasoning receives support from the finding that considerable hydrolysis of [35S]NCS can occur in the isolated perfused rat liver. Moreover, the extent of this hydrolysis may indeed be greater than is apparent from the experimental results, since there is evidence that the liberated sulphate (the quantitation of which is the basis of the assessment of the degree of hydrolysis) is being utilized by the liver. The continuing production of bile by the perfused liver, coupled with the fact that leakage of lysosomal arylsulphatase activity into the blood does not occur during perfusion, suggests that the liver cells remain viable and intact. Electron microscopy reveals an apparently normal lysosome population in livers perfused under similar conditions (G. Boyd, personal communication). The concept that lysosomal leakage of arylsulphatases A and B could be responsible for [35S]NCS hydrolysis in the perfused liver is also shown to be unlikely by the fact that extensive hydrolysis of the ester also occurs in whole rats with ligated renal pedicles. The microsomal arylsulphatase C may be responsible for the observed hydrolysis; however, recent studies in these Laboratories (F. A. Rose & D. W. Milsom, unpublished work) have confirmed that nitrocatechol sulphate is an extremely poor substrate for this enzyme.

It seems unlikely therefore that injected [35S]-NCS is able, under favourable circumstances, to penetrate lysosomal particles and to undergo hydrolysis followed by release of products. Certainly these preliminary studies indicate that further work in this direction would be worth while. The results also illustrate the possible value of the isolated perfused liver, together with radioactively labelled lysosomal enzyme substrates, in the study of lysosomal function and the mechanisms whereby small highly polar molecules are enabled to penetrate lysosomal membranes.

The conversion of a small part of injected [35S]-NCS into 2-β-glucuronosido-5-nitrophenyl [35S]-sulphate is noteworthy. First, it again confirms that [35S]NCS is able to penetrate cells, where it may be presumed to encounter the glucuronyltransferase systems in the endoplasmic reticulum. Secondly, although double conjugates are not unknown in Nature, this particular one is probably a unique example of two highly polar conjugate groups existing in juxtaposition. Molecular models of the structure show that the uronic acid residue can attach itself to the benzene ring through the β-linkage with a considerable and surprising degree of freedom. Other analogous double acid conjugates are known to feature to some extent in the metabolism of steroids (see, e.g., Troen, Nilsson Wiqvist & Diczfalusy, 1961; Wengle & Böstrom, 1963), but generally the two acidic groups are remote from each other on the conjugated molecule.

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APPENDIX

Preparation of Dipotassium 2-Hydroxy-5-Nitrophenyl [^{35}S]Sulphate

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Elbs persulphate oxidation of *p*-nitrophenol (Smith, 1951) is employed for the preparation of dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS†), the chromogenic substrate for arylsulphatases A and B (see also Roy, 1953; Dodgson & Spencer, 1956). The method may be adapted for the preparation of [^{35}S]NCS by using ^{35}S-labelled potassium persulphate. The latter may conveniently be obtained by an exchange reaction between potassium persulphate and chloro[^{35}S]sulphonic acid. However, dilution of label is inevitable and the yield of [^{35}S]NCS is low, particularly when the work is on a small scale (yields of less than 5% are obtained; N. Tudball, unpublished work). It has therefore been necessary to devise a method that produces [^{35}S]NCS in relatively high yield and of high specific radioactivity. The method developed involves the direct sulphation of 4-nitrocatechol with sufficient chloro[^{35}S]sulphonic acid to sulphate only one of the two available phenolic hydroxyl groups. The substitution-directing properties of the nitro group ensure that the correct hydroxyl group is sulphated.

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† Abbreviations: [^{35}S]NCS, dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulphate; NCS, unlabelled dipotassium 2-hydroxy-5-nitrophenyl sulphate.

METHOD

Preparation of 4-nitrocatechol. 4-Nitrocatechol may be prepared by the method of Weselsky & Benedikt (1882), but the procedure is laborious and yields are low. It is more conveniently obtained from NCS prepared by large-scale Elbs persulphate oxidation of *p*-nitrophenol according to the directions of Roy (1953) as modified by Dodgson & Spencer (1956).

NCS (4g.) was boiled under reflux with 75ml. of 2N-HCl for 1hr. and the liberated 4-nitrocatechol was extracted from the cooled yellow solution with ether. The ether extract was shaken with 20ml. of water containing sufficient

KOH to give a red-coloured aqueous phase (pH 10–11) after shaking. This phase was separated and extracted several times with ether, the extracts being discarded. It was then carefully acidified (pH 2–3) with 2N-HCl and extracted with several portions of ether. A small quantity of anhydrous Na_2SO_4 was added to the combined ethereal extracts to remove traces of water, the mixture was stirred, and the Na_2SO_4 was removed by centrifuging. The ethereal extract was evaporated to dryness in a shallow dish, whereupon pale-yellow needles of 4-nitrocatechol were deposited. The yield was 89% of the theoretical value. Paper chromatography of the compound in butan-1-ol–acetic acid–water (50:12:25, by vol.) showed it to be homogeneous. Spectrophotometric examination in 0·1N-NaOH gave results identical with authentic 4-nitrocatechol ($\lambda_{max.}$ 510; $\epsilon_{max.}$ 12300; Smith, 1951).

Monosulphation of 4-nitrocatechol. Diethylaniline (1·82ml.) and carbon disulphide (1·6ml.) were mixed in a short thick-walled glass tube (internal diameter 2·5cm.) and cooled in an ice–salt mixture at −5°. Chloro[^{35}S]-sulphonic acid (0·35ml., specific radioactivity 9·7mc/mmole of S; The Radiochemical Centre, Amersham, Bucks.) was slowly added, with stirring, care being taken to keep the temperature below 5°. A greenish-brown syrup was formed. The mixture was allowed to attain room temperature, and then finely powdered 4-nitrocatechol (0·75g.) was added and the whole was stirred until it was homogeneous. After the mixture had been kept at room temperature overnight, the syrup that separated out was removed and poured, with stirring, into a mixture of KOH (2·74g.) and $Ba(OH)_2,8H_2O$ (0·7g.) in 25ml. of water, the temperature being kept below 35°. A blood-red mixture formed, which was extracted five times with ether to remove excess of diethylaniline. The aqueous phase was adjusted to pH 3–4 with N-H_2SO_4 and unchanged 4-nitrocatechol was removed by extraction with five 30ml. portions of ether. $BaCl_2$ was added to precipitate inorganic $^{35}SO_4^{2-}$ ions, and $Ba^{35}SO_4$ was removed by centrifugation. The supernatant was passed through a column (14cm. × 1·5cm. diam.) of cation-exchange resin (Dowex 50; H^+ form; 200–400 mesh; Dow Chemical Co., Midland, Mich., U.S.A.) to eliminate Ba^{2+} and K^+ ions, and the acid eluate was collected in a beaker surrounded by ice. Ag_2CO_3 was added to the eluate until the colour of the latter became distinctly yellow and the precipitated AgCl was removed by centrifugation. The supernatant was passed through a Dowex 50 column (H^+ form, 200–400 mesh) to eliminate Ag^+ ions, and the acid eluate was again collected in the cold. The eluate was adjusted to pH 11 with aq. 5% (w/v) KOH and reduced to dryness *in vacuo* at 37°. The product was recrystallized from a small volume of water. To ensure complete purity, the product was converted into the monopotassium salt according to the method of Dodgson & Spencer (1956), and recrystallized from water. The faintly beige-coloured monopotassium nitrocatechol [^{35}S]sulphate was suspended in a small volume of water and conc. KOH added until complete solution was attained. The dipotassium salt was then crystallized by dropwise addition of ethanol until the needle-like crystals no longer continued to form. Continued addition of ethanol beyond this point resulted in the appearance of a fluffy orange–red material that appeared to contain other sulphate esters of 4-nitrocatechol. The crystalline product [yield 0·4g. (approx. 27% of the theoretical yield), specific radioactivity about 31μc/mg.] was separated by filtration and dried over P_2O_5 *in vacuo* at 60° (cf. Roy, 1958) (Found: C, 23·4; H, 0·93; N, 4·2; SO_4, 31·2; K, 25·6; $C_6H_3K_2NO_7S$ requires C, 23·1; H, 0·96; N, 4·5; SO_4, 30·8; K, 25·0%).

Characterization of the sulphate ester formed

Since in 4-nitrocatechol two hydroxyl groups are available for sulphation it was obviously necessary to compare the product obtained with authentic NCS prepared by Elbs persulphate oxidation of *p*-nitrophenol.

Electrophoresis. Paper electrophoresis in the system described previously showed that the compound had the same mobility as authentic NCS. Radioautography of the dried electrophoretogram showed that the ^{35}S-labelled ester moved as a discrete spot free of inorganic [^{35}S]-sulphate ions.

Spectrophotometric analysis. The u.v. and visible spectra of the prepared compound were identical with those of authentic NCS ($\lambda_{max.}$ 405mμ; $\epsilon_{max.}$ 18300). The infrared spectra of [^{35}S]NCS and NCS prepared by monosulphation of 4-nitrocatechol and of NCS prepared by Elbs persulphate oxidation were also examined and were found to be identical.

Comparison of relative activity of arylsulphatase towards the preparations. The relative ability of [^{35}S]NCS prepared by monosulphation of 4-nitrocatechol and authentic NCS to serve as substrates for the same enzyme preparation was determined. An acetone-dried preparation of the viscera of the common limpet (*Patella vulgata*) was used for this purpose, conditions described by Powell (1959) being used. The activity of the enzyme preparation, with increasing time, towards the two substrates was identical.

Isotope dilution. The common identity of the ^{35}S-labelled sulphate ester prepared by monosulphation of 4-nitrocatechol and authentic NCS was confirmed by isotope-dilution analysis.

DISCUSSION

The monosulphation of 4-nitrocatechol provides a novel route for the synthesis of [^{35}S]NCS. The main advantage of the method lies in the direct use of chloro[^{35}S]sulphonic acid as a sulphating agent, so that the resultant compound is formed in relatively high yield and with high specific radioactivity.

The monosulphation method described is a modification of the procedure normally used for the sulphation of monohydric phenols (Burkhardt & Lapworth, 1926). A previous observation of Dodgson, Rose & Spencer (1955) showed that it was possible to sulphate only one hydroxyl group of 4-chlorocatechol provided that the amount of sulphating reagent used was limited to that theoretically necessary to sulphate one hydroxyl group only. The hydroxyl group *para* to the substituent chlorine atom was preferentially sulphated and the explanation of this was thought to be related to the *ortho–para*-directing nature of the chloro substituent. A similar situation occurs in

the sulphation of 4-nitrocatechol, although in this case the *meta*-directing influence of the nitro group ensures that sulphation occurs preferentially on the hydroxyl group *meta* to this substituent. The preparation probably contained other sulphate esters of nitrocatechol, but these were removed in the recrystallization step. The extent of the formation of these other esters was not determined, but it may be concluded that they represented only a minor fraction of the total yield of ester sulphate.

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