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Synthesis of ^{35}S -Labelled Arylsulphates by Intact Animals and by Tissue Preparations, with Particular Reference to L-Tyrosine O-Sulphate

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The combination of sulphuric acid with phenolic compounds to form arylsulphates is well known to occur in the animal body and certain arylsulphates are present normally in the urine of higher animals. Recently a new arylsulphate, namely L-tyrosine O-sulphate, has been reported in the urine of normal and abnormal human subjects (Tallan, Bella, Stein & Moore, 1955). The possible occurrence of this compound in the urine of species other than man has been investigated in the present work.

The synthesis of arylsulphates by tissue preparations has been studied in some detail (e.g. Bernstein & McGilvery, 1952; DeMeio, Wizerkaniuk & Fabiani, 1953; Robbins & Lipmann, 1957; Segal, 1957). Attempts to synthesize L-tyrosine O-sulphate, however, from L-tyrosine by various tissue preparations have so far been unsuccessful (Segal, 1957; Dodgson, Rose & Tudball, 1959). Synthesis of this particular arylsulphate has been investigated in the present work with preparations of the liver and kidney tissue from several species. The technique employed was capable of detecting the synthesis of small amounts of arylsulphate and depended on the use of ^{35}S -labelled sulphate as a source of sulphate. The tissue preparations used were active in synthesizing several arylsulphates from phenolic substrates under differing experimental conditions. It was not possible, however, to demonstrate synthesis of L-tyrosine O-sulphate from L-tyrosine.

MATERIALS

The potassium salts of phenylsulphuric acid and of *p*-cresylsulphuric acid were prepared by the method of Feigenbaum & Neuberg (1941). The *p*-toluidine salts of the products were prepared (Barton & Young, 1943), and had satisfactory melting points.

L-Tyrosine O-sulphate. The monopotassium salt of this compound was prepared as described by Tallan *et al.* (1955) and was isolated as the dihydrate (Found: N, 4.1; S, 9.4; K, 11.8. Calc. for $\text{C}_9\text{H}_{10}\text{O}_6\text{NSK}\cdot 2\text{H}_2\text{O}$: N, 4.2; S, 9.6; K, 11.7%).

Potassium *p*-ethylphenylsulphate. Freshly distilled chlorosulphonic acid (1.6 ml., 2.9 g.) was added dropwise, and with shaking, to a solution containing 10 ml. of carbon disulphide and 10 ml. of dimethylaniline cooled in a bath containing chipped ice. Freshly distilled *p*-ethylphenol (3 g.) was added to this mixture, which was warmed to room temperature and shaken mechanically for 1 hr. This reaction mixture was added slowly, and with continuous stirring, to an ice-cold solution containing 7 g. of KOH in 10 ml. of water. The resulting mixture was centrifuged at 500 *g* for several minutes and the supernatant liquor was discarded. The precipitate was stirred with 10 ml. of ethanol and the mixture was centrifuged as before. The supernatant was discarded, the precipitate was transferred to a Buchner funnel and was dried by suction. The resulting pale-yellow solid was washed several times with ether and yielded 2.8 g. of material, which was dissolved in 10 ml. of hot water and was rapidly filtered. Large plates were obtained from the pale-yellow filtrate (Found: C, 40.1; H, 3.4; S, 13.3; K, 16.3. Calc. for $\text{C}_8\text{H}_9\text{O}_4\text{SK}$: C, 40.0; H, 3.8; S, 13.3; K, 16.3%).

Potassium *p*-acetylphenylsulphate. This was prepared as described by Dodgson & Spencer (1953) (Found: C, 37.9; H, 2.7; S, 12.4. Calc. for $\text{C}_8\text{H}_7\text{O}_5\text{SK}$: C, 37.8; H, 2.8; S, 12.6%).

L-Tyrosine methyl ester. This compound was prepared by the method of Fischer & Schrauth (1907). The product had m.p. 134–136°, $[\alpha]_D^{25} + 30^\circ$ in methanol (*c.* 1). Fischer & Schrauth give m.p. 135–136°, $[\alpha]_D^{20} + 26^\circ$ in methanol (Found: C, 61.1; H, 6.3; N, 7.2. Calc. for $\text{C}_{10}\text{H}_{13}\text{O}_3\text{N}$: C, 61.5; H, 6.7; N, 7.2%).

L-Tyrosine amide. This was prepared from L-tyrosine methyl ester by the method of Blau & Waley (1954). Colourless crystals were obtained, m.p. 152–153°: L-tyrosine amide has m.p. 153–154° (Found: C, 59.8; H, 6.9; N, 15.6. Calc. for $\text{C}_9\text{H}_{12}\text{O}_3\text{N}_2$: C, 59.9; H, 6.7; N, 15.5%).

Tyramine O-sulphate. This was prepared as described by Dodgson *et al.* (1959). The addition of $\text{Ba}(\text{NO}_3)_2$ solution to an aqueous solution of the compound produced no precipitate. After the compound had been heated in approx. 2N-HCl solution in a boiling-water bath, the addition of $\text{Ba}(\text{NO}_3)_2$ solution yielded a precipitate of BaSO_4 . The compound in aqueous solution gave no colour when diazotized sulphanilic acid solution was added followed by NaOH. After heating the compound in 2N-HCl in a boiling-water bath for several minutes, the addition of a solution of diazotized sulphanilic acid to the cooled solution, followed by NaOH, yielded a red colour. This red was obtained when tyramine solution was treated with diazotized sulphanilic acid followed by NaOH. An aqueous solution of the compound gave a positive ninhydrin test. Paper-chromatographic examination of the compound in three different solvent systems gave evidence of a single spot only. R_F values are shown in Table 2. The compound was identified on chromatograms both by ninhydrin and by diazotized sulphanilic acid. The ultraviolet-absorption spectrum of the compound corresponded to that obtained by Dodgson *et al.* (1959) with tyramine O-sulphate.

METHODS

Column chromatography

A column (14.2 cm. $\times$ 0.6 cm.) of Dowex 1-X8 (200–400 mesh) in the acetate form was prepared according to Tallan *et al.* (1955); the temperature was held constant by circulating cold tap water through a jacket. Pure L-tyrosine O-sulphate placed upon the column was eluted after the passage of 80 ml. of 1.1N-sodium acetate buffer (pH 5.0). The eluate was collected in 2 ml. fractions and the L-tyrosine O-sulphate was determined by the photometric ninhydrin method of Stein & Moore (1954). In order to examine urine for the presence of L-tyrosine O-sulphate the urine sample was concentrated to half its original volume, the pH was adjusted to 5.0 and the sample was centrifuged. A measured portion (3–5 ml.) of this clear supernatant liquid was applied to the column by pipette.

Paper chromatography

Urine was collected for 24 hr. after the administration of $\text{Na}_2^{35}\text{SO}_4$. The urine specimens were extracted by shaking with an equal volume of butan-1-ol; these extracts were evaporated to dryness *in vacuo* and the residues were dissolved in up to 1 ml. of water. These aqueous solutions, or samples of the original urine, were examined by paper chromatography on Whatman no. 1 paper. The chromatograms were developed overnight by the ascending technique without prior equilibration. The average time of running was 16 hr.

Detection of arylsulphates on chromatograms. (a) Colour reagents. To display ninhydrin-sensitive compounds, air-dried chromatograms were dipped in 0.1% (w/v) ninhydrin solution in acetone and again were air-dried. Arylsulphates were detected by suspending air-dried chromatograms in HCl vapour for 10 min. This treatment hydrolyses arylsulphates to H_2SO_4 and the corresponding phenols. The phenols then were displayed by spraying the paper with 0.16% (w/v) diazotized sulphanilic acid solution followed by exposure to NH_3 vapour (Corner & Young, 1955). In the detection of indican on chromatograms, the initial HCl

treatment yielded a blue-grey spot which became red on treatment with diazotized sulphanilic acid solution, and darker red after treatment with NH_3 . With other arylsulphates no coloured spot was apparent until chromatograms were treated with NH_3 vapour.

(b) Radioactivity measurement. Radioactive compounds were detected by passing the strip chromatograms through the automatic recording scanner described by Morrison & Young (1959). Radioactive compounds on the chromatograms gave rise to peaks on an automatic pen-recorded trace.

Where quantitative results were required for experiments with tissue extracts, the areas under the curves obtained on the trace were measured by planimetry and also by cutting out and weighing. It was known by experiment that the radioactivity of compounds on chromatograms was proportional to the areas under the curves obtained on the trace. In order to characterize the arylsulphates synthesized by tissue preparations, R_F values were determined in three different solvent systems. The values so obtained were compared with the R_F values of reference compounds developed at the same time. Results are shown in Table 2.

Housing and dosing of animals

Male rabbits, black-hooded rats, guinea pigs and mice were housed in metabolism cages which permitted the collection of urine separately from faeces. With the exception of rabbits, the animals were maintained on a diet described by Maw (1953). Rabbits were fed on rat cake [J. Murray and Sons (London) Ltd.] and cabbage leaves. The animals had access to water at all times. Each of two rats received 1 mg. of $\text{Na}_2^{35}\text{SO}_4$ (0.5 mc) in 1 ml. of solution, and each of 24 mice received 0.19 mg. of $\text{Na}_2^{35}\text{SO}_4$ (30 μC) in 0.2 ml. of solution by intraperitoneal injection whilst under light ether anaesthesia. A guinea pig received, also by intraperitoneal injection, 1 mg. of $\text{Na}_2^{35}\text{SO}_4$ (0.5 mc) in 1 ml. of solution, and a single rabbit received 10.7 mg. of $\text{Na}_2^{35}\text{SO}_4$ (2.1 mc) in 5 ml. of solution through a stomach tube.

Tissue preparations and incubation

Animals were killed by a blow on the head and the liver or kidney was removed, blotted to remove surface moisture, and was weighed immediately in a chilled container. The tissue was shredded with scissors into an all-glass homogenizer (containing a loose-fitting pestle) cooled to 0°. One of two buffer solutions was used to homogenize the tissue, either 2-amino-2-hydroxymethylpropane-1:3-diol (tris) containing 7.75 ml. of acetic acid/l. of 0.15M-tris, or 0.02M- KH_2PO_4 –0.08M- Na_2HPO_4 . The pH of these buffer solutions was always in the range 7.27–7.40. The buffer at 0° was added to the tissue so as to yield a 30% (w/v) mixture, which was homogenized. The mixture so obtained was centrifuged at 550 g for 7 min. at 0°; the upper layer was removed and is referred to as the 'homogenate'. A sample of this 'homogenate' was centrifuged further at 30 000 g for 30 min. at 0°. Three layers were obtained after this centrifuging; the clear layer in the centre was removed and is referred to as the 'supernatant'.

Samples of the 'homogenate' or 'supernatant' fractions (2 ml. unless stated otherwise) were added to stoppered 25 ml. conical flasks containing either 1 or 2 ml. of substrate and supplements dissolved in the buffer (except MgCl_2). The composition of a typical reaction mixture, in

which the concentrations of added substances are expressed as molarities in the final mixture, was: adenosine triphosphate (ATP; 7.8 mM) prepared freshly; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (7.5 mM) added in water; substrate (0.89 mM); $\text{Na}_2^{35}\text{SO}_4$ (0.68 mM; 60 μC). The final volume was 4 ml. The reaction mixture was shaken while it was incubated in air at 37° for the times indicated. The reaction was stopped by immersing the incubated mixture in a boiling-water bath for 1–2 min. Heating in this way caused no breakdown of the arylsulphates investigated. Coagulated protein was separated by centrifuging, and samples of the clear-yellow supernatant were examined by paper chromatography. Where the incubation resulted in the synthesis of a radioactive arylsulphate, the developed chromatograms contained two radioactive spots. One spot near the origin was due to unchanged free sulphate and the other was due to the arylsulphate synthesized by the tissue preparation. In expressing results quantitatively, the arylsulphate radioactivity has been calculated as a percentage of the total radioactivity. The reproducibility of the method was tested by taking duplicate samples from each of five incubated mixtures in which different amounts of phenylsulphate had been synthesized. Each of these ten samples was examined by paper chromatography on different occasions. Eight of the samples, comprising three duplicate pairs and a sample of each of the other two duplicate pairs, were developed in butan-1-ol-acetic acid-water (4:1:5, by vol.), the remaining two samples were developed in butan-2-ol-aq. 3N- NH_3 soln.-3N- $(\text{NH}_4)_2\text{CO}_3$ (4:3:3, by vol.). From the developed chromatograms, the amount of phenylsulphate synthesized was calculated. The values obtained indicated agreement between the two samples of each of the five duplicate pairs; they were: 10.7, 10.7; 15.7, 17.1; 24.2, 25.2; 31.3, 32.5 and 36.2, 36.8%. Results quoted in this paper are representative single determinations.

RESULTS

Samples of freshly voided normal human urine were found by column chromatography to contain L-tyrosine *O*-sulphate. When samples of normal male rat-urine concentrates were examined by this

technique, no evidence was obtained for the presence of this arylsulphate. L-Tyrosine *O*-sulphate was readily detected when added to normal male rat urine in amount equal to the concentration in normal human urine. The sensitivity of the method used was such that if this arylsulphate had been present in normal male rat urine it was in amount less than 80 μg ./24 hr. urine excretion. In view of the inability to detect L-tyrosine *O*-sulphate in normal male rat urine by column chromatography, it was decided to administer ^{35}S -labelled sulphate to animals and to examine their urine by paper chromatography.

The results are shown in Table 1. After the administration of $\text{Na}_2^{35}\text{SO}_4$ to male rabbits, rats, guinea pigs and mice, it was possible to detect radioactive indican, *p*-cresylsulphate and phenylsulphate in urine concentrates. These three arylsulphates were detected both by their radioactivity and by their colour reactions. No evidence was obtained for the excretion of L-tyrosine *O*-sulphate by any of these species. Butan-1-ol extracts of freshly voided normal human urine were examined also by paper chromatography. It was possible to detect indican, *p*-cresylsulphate and phenylsulphate on the chromatograms.

Studies with tissue preparations

Phenylsulphate-synthesizing activity of various tissues. When attempts to synthesize L-tyrosine *O*-sulphate from L-tyrosine by tissue preparations were unsuccessful, the tissue preparations were tested for arylsulphate-synthesizing activity with phenol as a substrate. The results are shown in Tables 2 and 3. Both 'supernatant' and 'homogenate' fractions of rat-liver preparations synthesized phenylsulphate from phenol in the presence

Table 1. R_F values of arylsulphates present in butan-1-ol extracts of urine of animals of different species

With all species, except man, the animals had received $\text{Na}_2^{35}\text{SO}_4$ and the metabolites listed below were radioactive. Those R_F values which are quoted in parentheses are of reference compounds developed simultaneously with the urine samples. Solvent systems (volumes mixed): *A*, butan-1-ol-acetic acid-water (4:1:5); *B*, butan-2-ol-aq. 3N- NH_3 soln.-3N- $(\text{NH}_4)_2\text{CO}_3$ (4:3:3); *C*, pentan-2-ol-aq. 3N- NH_3 soln.-3N- $(\text{NH}_4)_2\text{CO}_3$ (4:3:3); *D*, butan-1-ol-pyridine-aq. 3N- NH_3 soln. (4:3:3).

Solvent system	R_F in solvent system					Chromatograms		
	Rat	Guinea pig	Mouse	Rabbit	Man	HCl vapour	Diazotized sulphanilic acid followed by NH_3 vapour	Metabolite
<i>A</i>	0.45	0.42	—	—	0.45	Blue	Red	Indican
<i>B</i>	0.29	0.28	0.30	0.29	0.26	Blue	Red	Indican
	0.39 (0.41)	0.39 (0.37)	0.42 (0.38)	0.41 (0.37)	0.34 (0.34)	—	Yellow	Phenylsulphate
	0.50 (0.50)	0.44 (0.46)	0.53 (0.47)	0.48 (0.45)	0.40 (0.43)	—	Orange	<i>p</i> -Cresylsulphate
<i>C</i>	0.09	0.06	0.07	0.07	0.06	Blue	Red	Indican
	0.16 (0.15)	0.12 (0.11)	0.14 (0.13)	—	0.10 (0.09)	—	Yellow	Phenylsulphate
	0.24 (0.23)	0.20 (0.18)	0.24 (0.21)	0.16 (0.15)	0.18 (0.15)	—	Orange	<i>p</i> -Cresylsulphate
<i>D</i>	—	—	0.80	0.75	—	Blue	Red	Indican

Table 2. R_F values of reference arylsulphates compared with those synthesized by tissue preparations

Solvent systems were as described in Table 1. The sulphate giving the R_F values in parentheses was not identified.

Substrate	Arylsulphate	R_F in solvent system					
		A		B		D	
		Reference compound	Metabolite	Reference compound	Metabolite	Reference compound	Metabolite
Phenol	Phenylsulphate	0.49	0.49	0.35	0.34	0.76	0.77
<i>p</i> -Cresol	<i>p</i> -Cresylsulphate	0.61	0.61	0.34	0.31	0.77	0.78
<i>p</i> -Ethylphenol	<i>p</i> -Ethylphenylsulphate	0.70	0.71	0.62	0.61	0.78	0.79
<i>p</i> -Acetylphenol	<i>p</i> -Acetylphenylsulphate	0.54	0.56	0.25	0.25	0.77	0.77
Tyramine	Tyramine <i>O</i> -sulphate	0.34	(0.44)	0.10	(0.33)	0.66	(0.35)

Table 3. Synthesis of phenylsulphate from phenol by 'homogenate' and 'supernatant' fractions prepared from various tissues

Incubation mixtures were prepared as described in the Methods section. 'Homogenate' and 'supernatant' fractions are called H and S respectively. Incubations were carried out at 37° in air for 1 hr. except where otherwise stated.

Expt. no.	Tissue preparation	Conversion of sulphate into phenylsulphate (%)	
		Phosphate buffer	Tris buffer
1	Rat liver H	2*	—
	S	13*	—
2	H	—	12
	S	—	36
3	S	16	36
4	Rabbit liver H	—	37
	S	—	60
5	H	—	23
	S	—	38
6	Guinea-pig liver H	—	24
	S	—	31
7	Human liver† S	—	34
8	Rat kidney S	—	5
9	Rabbit kidney S	—	5

* Incubated for 2 hr.

† The human liver used was a biopsy sample prepared and incubated within 1.5 hr. of excision.

of added Mg^{2+} ions, ATP and $Na_2^{35}SO_4$. The 'supernatant' fractions invariably were more active than the 'homogenate' fractions, irrespective of whether phosphate or tris buffer was used (Table 3, Expts. 1 and 2). When a sample of rat-liver 'supernatant' fraction was prepared and incubated in phosphate buffer, less phenylsulphate synthesis from phenol was observed than when a sample of the same liver was prepared and incubated in tris buffer (Expt. 3). 'Homogenate' or 'supernatant' fractions of the livers of rabbits, guinea pigs and man synthesized phenylsulphate from phenol (Expts. 4, 5, 6 and 7). Rat- and rabbit-kidney 'homogenate' fractions

gave no detectable phenylsulphate synthesis from phenol. The 'supernatant' fractions, however, of the kidney tissue of both these species gave small but significant synthesis of phenylsulphate (Expts. 8 and 9).

It is apparent from the results recorded in Table 3 that the tissue preparations examined had arylsulphate-synthesizing activity, as judged by their ability to synthesize phenylsulphate from phenol. Nevertheless, when they were tested for their ability to synthesize L-tyrosine *O*-sulphate from L-tyrosine, negative results were invariably obtained. ATP is required in arylsulphate biosynthesis because the sulphate radical is transferred to the substrate through the intermediary adenosine 3'-phosphate 5'-phosphosulphate (cf. Robbins & Lipmann, 1956). In the absence of ATP in the present experiments, no synthesis of phenylsulphate or L-tyrosine *O*-sulphate occurred when phenol or L-tyrosine was used as substrate with either rat-liver or guinea-pig-liver preparations.

Requirements for metal ions by tissue preparations. The effects of metal ions on arylsulphate biosynthesis has been little investigated. Magnesium, calcium and manganese were examined for their effect on the synthesis of arylsulphates from phenol and from L-tyrosine. The concentrations of the added ions are not necessarily optimum; they are expressed as molarities in the final incubation mixture. Added magnesium (7.5 mM) was not essential for phenylsulphate synthesis from phenol by rat- or guinea-pig-liver preparations in tris buffer. In one experiment with a rat-liver 'supernatant' preparation, omission of Mg^{2+} ions had no effect upon the extent (11%) of phenylsulphate synthesis. In a second experiment, with a similar preparation, 34% conversion of sulphate into phenylsulphate occurred, compared with 67% conversion when Mg^{2+} ion (7.5 mM) was present. When a guinea-pig 'supernatant' fraction was incubated without added Mg^{2+} ion conversion of sulphate into phenylsulphate was 9%, compared with 11% when Mg^{2+} ion (7.5 mM) was present in a control experiment.

When Ca^{2+} ion (5 mM) was added instead of Mg^{2+} ion in a rat-liver preparation, no phenylsulphate synthesis from phenol was observed. In the presence of equimolar amounts of added Ca^{2+} (5 mM) and Mg^{2+} ion (5 mM), phenylsulphate synthesis from phenol was less (4 %) than in a rat-liver preparation in which Mg^{2+} ion (5 mM) alone was added (11 %). Manganese ions appeared to exert an inhibitory effect since phenylsulphate synthesis by a rat-liver 'supernatant' fraction was less (23 %) with added Mn^{2+} ion (7.5 mM) than in the absence of either added Mn^{2+} or Mg^{2+} ion (34 %). None of these three ions (Mg^{2+} , Ca^{2+} and Mn^{2+}), whether omitted or added to the incubation media, resulted in synthesis of L-tyrosine O-sulphate from L-tyrosine in these tissue preparations.

Biosynthesis of other arylsulphates. In view of this difference in behaviour between phenol and L-tyrosine, the ability of tissue preparations to synthesize arylsulphates from phenolic compounds intermediate in structure between phenol and L-tyrosine was investigated. Both the 'homogenate' and 'supernatant' fraction of rat-liver preparations containing added Mg^{2+} ions, ATP and $\text{Na}_2^{35}\text{SO}_4$ were capable of synthesizing the arylsulphates of *p*-cresol, *p*-ethylphenol and *p*-acetylphenol. The extent of synthesis of the arylsulphates of these three phenols, compared in each case with a control phenylsulphate synthesis, is shown in Table 4 (Expts. 13–15). L-Tyrosine had no effect upon the synthesis of phenylsulphate from phenol by rat-liver preparations (Expt. 16).

When tyramine was used as a substrate, incubation with a rat-liver 'supernatant' fraction resulted in the formation of a small but significant amount of sulphate conjugate. This conjugate had a different R_f from that of synthetic tyramine O-sulphate in three different solvent systems (Table 2). When tyramine and phenol were used together as substrates in a rat-liver 'supernatant' fraction containing added Mg^{2+} ions, ATP and $\text{Na}_2^{35}\text{SO}_4$, phenylsulphate synthesis only was observed, to the same extent as when phenol alone

was used as substrate (Table 4, Expt. 17). No sulphate conjugation was observed from L-tyrosine amide or L-tyrosine methyl ester in a rat-liver 'supernatant' fraction.

Hydrolysis of L-tyrosine O-sulphate. The occurrence in the tissue preparations used of an enzyme which hydrolyses L-tyrosine O-sulphate would provide an explanation for failure to demonstrate synthesis of this compound by these preparations. The possibility that hydrolysis of this arylsulphate occurred in the rat-liver preparations used, was investigated and the results are shown in Table 5. The preliminary incubation of the preparation with phenol, Mg^{2+} ions and ATP, followed by incubation with $\text{Na}_2^{35}\text{SO}_4$ (Expt. 18), resulted in conversion of $^{35}\text{SO}_4^{2-}$ ion into phenylsulphate to the extent of 18 %. A similar value (19 %) was obtained when the preliminary incubation medium contained phenol, Mg^{2+} ion, ATP and L-tyrosine O-sulphate, and the mixture was incubated subsequently with $\text{Na}_2^{35}\text{SO}_4$ (Expt. 20). These two

Table 4. *Synthesis of arylsulphates by the 'supernatant' fraction of rat liver*

An incubation mixture, containing phenol as substrate, was used as a control with each of the substrates investigated. Tris was used in all experiments. Unless stated otherwise, the substrate concentration (expressed as molarity in the final incubation mixture) was 0.89 mM.

Expt. no.	Substrate	Conversion of sulphate into arylsulphate (%)
13	Phenol	31
	<i>p</i> -Cresol	25
14	Phenol	46
	<i>p</i> -Ethylphenol	56
15	Phenol	45
	<i>p</i> -Acetylphenol	38
16	Phenol (0.3 mM)	11
	Phenol (0.3 mM) and L-tyrosine (0.3 mM)	12
17	Phenol (0.45 mM)	49
	Phenol (0.45 mM) and tyramine (0.45 mM)	50

Table 5. *Effect of L-tyrosine O-sulphate on the synthesis of phenylsulphate from phenol by the 'supernatant' fraction of rat liver*

Three reaction mixtures in tris buffer were prepared, containing enzyme (1 ml.), Mg^{2+} ions (6.8 mM), phenol (0.82 mM) and ATP (7.6 mM). Other additions were as shown in column 2. The mixtures were incubated for 30 min. in air at 37°. $\text{Na}_2^{35}\text{SO}_4$ (0.38 mM; 20 μC) was then added to each flask and incubation was continued for a further 45 min. Each sample was then assayed for phenylsulphate. Concentrations are expressed as molarities in the final mixture, whose volume was 2.2 ml.

Expt. no.	Addition to reaction mixture	Total radioactivity on chromatograms (counts/min.)	Radioactive phenylsulphate on chromatograms (counts/min.)	Conversion of sulphate into phenylsulphate (%)
18	None	4324	766	18
19	$\text{Na}_2^{35}\text{SO}_4$ (0.38 mM)	4262	284	7
20	L-Tyrosine O-sulphate (0.36 mM)	4192	795	19

experiments showed that L-tyrosine *O*-sulphate had little effect upon phenylsulphate synthesis. Hydrolysis of L-tyrosine *O*-sulphate during the incubation would have liberated SO_4^{2-} ions, which, by diluting the $\text{Na}_2^{35}\text{SO}_4$, would have reduced the value obtained for apparent conversion of the $^{35}\text{SO}_4^{2-}$ ions into phenylsulphate. The extent of this dilution effect is shown in Expt. 19, in which Na_2SO_4 was present during the preliminary incubation. Incubation of this mixture subsequently with $\text{Na}_2^{35}\text{SO}_4$ gave a value for conversion of $^{35}\text{SO}_4^{2-}$ ions into phenylsulphate of only 7%.

DISCUSSION

The presence of L-tyrosine *O*-sulphate in normal male human urine and its apparent absence from the urine of normal male rabbits, rats, guinea pigs and mice, indicates a species difference in the urinary occurrence of this compound.

Tissue preparations were made from the livers of rabbits, rats, guinea pigs and man, and from the kidney tissue of rabbits and guinea pigs, which were capable of synthesizing phenylsulphate from phenol. With rat-liver preparations this synthesis occurred under a variety of experimental conditions. In addition to phenylsulphate synthesis, rat-liver preparations were capable of synthesizing the arylsulphates of phenol, *p*-cresol, *p*-ethylphenol and *p*-acetylphenol.

Under none of the experimental conditions described in this work, nor with any of the tissues investigated, was it possible to demonstrate synthesis of L-tyrosine *O*-sulphate from L-tyrosine. It is significant that other workers using tissue preparations have failed to demonstrate this synthesis (Segal, 1957; Dodgson *et al.* 1959). The possibility that L-tyrosine *O*-sulphate is formed and hydrolysed subsequently by arylsulphatases present in the enzyme system seems unlikely in view of the results shown in Table 5. Preparations of the type used in the present work contain Type II arylsulphatases (Baum & Dodgson, 1958). These arylsulphatases have been shown to exhibit relatively low affinity towards L-tyrosine *O*-sulphate (Dodgson, Rose & Tudball, 1957; Dodgson *et al.* 1959).

It seems therefore that, with the conditions used, the enzyme system contained in rat-liver tissue can synthesize the corresponding arylsulphates of simple phenols related in structure to L-tyrosine, but not of L-tyrosine itself. When tyramine (i.e. *p*-hydroxyphenylethylamine) was used as a substrate with rat-liver tissue, synthesis of a sulphate conjugate was demonstrated. Evidence at present available suggests that this conjugate is not tyramine *O*-sulphate and there is the possibility that the conjugate is an *N*-substituted

sulphate ester. When L-tyrosine amide and L-tyrosine methyl ester were used as substrates in a rat-liver preparation, no evidence was obtained for synthesis of a sulphate conjugate.

Previous workers have commented upon the unusual behaviour of L-tyrosine *O*-sulphate. Dodgson *et al.* (1959) have pointed out that its presence in bovine fibrinogen is atypical of arylsulphates, which are considered generally to be end products of metabolism. These workers have shown also that the behaviour of this arylsulphate with mammalian arylsulphatase C preparations is unexpected, for it does not undergo hydrolysis. Previous data therefore indicate that the behaviour of L-tyrosine *O*-sulphate is unusual in comparison with other arylsulphates. The present work supports this view.

SUMMARY

1. Examination of normal human urine by column and by paper chromatography confirmed the presence of L-tyrosine *O*-sulphate and revealed the presence of phenylsulphate, *p*-cresylsulphate and indican. Examination of the urine of rabbits, guinea pigs, rats and mice dosed with $\text{Na}_2^{35}\text{SO}_4$ revealed the presence of ^{35}S -labelled phenylsulphate, *p*-cresylsulphate and indican, but not of L-tyrosine *O*-sulphate.

2. By the use of a particle-free system prepared from rat liver and incubated in tris buffer, a brief study has been made of the requirements of the sulphate-esterifying system which forms phenylsulphate from phenol. The arylsulphates of *p*-cresol, *p*-ethylphenol and *p*-acetylphenol were synthesized by this system. Liver- and kidney-tissue fractions of rabbits and guinea pigs synthesized phenylsulphate from phenol, as did a sample of fresh human liver.

3. Tyramine underwent conjugation with sulphate, during incubation with rat-liver-tissue preparation. This biosynthetic sulphate conjugate did not appear to be tyramine *O*-sulphate.

4. No evidence could be obtained for formation of L-tyrosine *O*-sulphate from L-tyrosine with any of the preparations used. This finding supports the results of previous workers.

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The Effect of 'Glucose Cycloacetoacetate' on the Regeneration of Erythrocytes and Restoration of Haemoglobin Level in Experimental Anaemia in Rats

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That phenylhydrazine brings about experimental anaemia accompanied by liver damage has been shown by several workers (Damodaran & Vijayaraghavan, 1943; Chanutin, Lentz & Ludewig, 1953; Helbig, 1955; Imanishi, 1956). It was reported by Vijayaraghavan & Dunn (1950) that an experimental anaemia induced in mice by the injection of phenylhydrazine (6–8 mg./100 g. body weight) responded to the parenteral administration of vitamin B₁₂ by a regular increase in the erythrocytes. It has also been shown by Popper, Koch-Weser & Szento (1949) and Hove & Hardin (1951) that liver damage caused by phenylhydrazine can be prevented by vitamin B₁₂.

One of the important physiological properties of vitamin B₁₂ is its effect on the maintenance of reduced glutathione in liver as reported by Dubnoff (1950), and it has been shown by Nath and associates (Nath & Behki, 1958; Behki, Motlag & Nath, 1958) that 'glucose cycloacetoacetate', a crystalline compound formed by condensing glucose with acetoacetate, when hydrolysed with 2N-hydrochloric acid for 10–15 min. has a similar effect in sparing reduced glutathione in acetoacetate-induced diabetes as well as in scorbutic guinea pigs. Glucose cycloacetoacetate, on such hydrolysis, has been shown by Nath & Bhattathiry (1956*a, b*) to give rise to 1:2-dienol glucose. It has also been reported by Nath & Saikia (1958, 1959) that hydrolysed glucose cycloacetoacetate, like vitamin B₁₂, can prevent the rise of the ratio total cholesterol/lipid phosphorus in the blood and

tissues in experimental atherosclerosis, induced by diets either with a high content of saturated fat or of cholesterol. Therefore hydrolysed glucose cycloacetoacetate was studied to see if it could also behave like vitamin B₁₂ in the regeneration of erythrocytes and restoration of haemoglobin in experimental anaemia induced by phenylhydrazine.

MATERIALS AND METHODS

Male albino rats weighing 90–120 g. were selected and divided into three groups of six or seven each. All were fed on a normal stock diet consisting of wheat flour (75%), casein (10%), yeast powder (5%), Hawk & Oser (1947) salt mixture (5%), ground-nut oil (5%) and cod-liver oil (0.1%). Drinking water was given *ad lib*.

Before making the animals anaemic, the initial haemoglobin level and the erythrocyte count in all the animals were determined from the tail blood. Erythrocytes were counted by the usual method with the help of a Neubauer ruling counting chamber. Haemoglobin was estimated by the method of Wong (1928) with 0.1 ml. of blood, by converting it into Fe³⁺ ions by means of conc. H₂SO₄ and potassium persulphate and by using 3N-KSCN for colour development. The red colour formed was compared against that of the standard iron solution with a green filter in a Lange Universal Colorimeter. The haemoglobin value (in g./100 ml.) was obtained by multiplying the total blood iron (mg.) by the factor 1/3.4.

The experiment was performed for 3 weeks. All the animals were first injected with a single dose of 80 mg. of phenylhydrazine hydrochloride/kg. body wt. The solution of phenylhydrazine for injection was prepared as described by Chanutin *et al.* (1953): a weighed amount of recrystallized phenylhydrazine hydrochloride was dissolved in aq.