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Biochemical Studies of Toxic Agents

4. A STUDY OF ETHEREAL SULPHATE FORMATION *IN VIVO* USING RADIOACTIVE SULPHUR

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The present work was done to throw light on the question of whether administered inorganic sulphate can participate in the formation in the animal body of the so-called 'etheral sulphates'. This matter has long been the subject of controversy. The problem is one which lends itself to investigation by means of tracer isotope techniques, and the present paper is an account of experiments in which etheral sulphate formation *in vivo* was studied with the aid of radioactive sulphur (^{35}S).

In earlier papers of the present series descriptions have been given of the isolation of 2-amino-1-naphthylsulphuric acid from the urine of rats dosed with 2-naphthylamine (Manson & Young, 1950), and the isolation of 1-naphthylsulphuric acid and 2-naphthylsulphuric acid from the urine of rats dosed with 1- and 2-naphthol, respectively (Berenbom & Young, 1951). The formation of 2-amino-1-naphthylsulphuric acid from 2-naphthylamine is an example of etheral sulphate production from a compound which, at least at the time of its administration, does not contain a phenolic group. On the other hand, the conversion of a naphthol to the corresponding naphthylsulphuric acid is typical of a reaction which is known to occur when many phenolic compounds are administered to animals. It appeared, therefore, that 2-naphthylamine and 1- or 2-naphthol would serve as suitable compounds in a study of the participation of administered inorganic sulphate in the biosynthesis of etheral sulphates.

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Three groups of experiments were carried out. The first group consisted of determinations of the distribution of ^{35}S in the inorganic and total sulphate fractions of rat urine (and hence the etheral sulphate fraction) following the administration of radioactive sodium sulphate alone, or simultaneously with the administration of 2-naphthylamine by another route. The results obtained indicated that the administered inorganic sulphate had participated in etheral sulphate formation. This was confirmed in the second group of experiments. In one of these experiments radioactive 2-amino-1-naphthylsulphuric acid was isolated from the urine of rats dosed with 2-naphthylamine and radioactive sodium sulphate by different routes. In a similar experiment radioactive 2-naphthylsulphuric acid was isolated from the urine after the administration of 2-naphthol and radioactive sodium sulphate. In the third group of experiments it was shown that radioactive inorganic sulphate does not exchange in urine with the sulphate of the etheral sulphate fraction.

EXPERIMENTAL AND RESULTS

Animals and dosing. The animals used were male white rats weighing between 170 and 210 g. During the experimental period they were housed in cages which permitted the collection of urine separate from the faeces. The rats were fed on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Ltd.) supplemented with fresh milk and whole wheat bread. In quantitative experiments the animals were allowed to feed twice daily for periods of 30 min. each from a metal cup designed to prevent scattering of food. In qualitative experiments the rats were fed in a separate cage twice daily for periods of 30 min. each and any

urine excreted during these periods was not collected. The animals had access to water at all times.

All dosing was carried out under ether anaesthesia. Solutions of sodium sulphate containing $\text{Na}_2^{35}\text{SO}_4$ were prepared using distilled water and were always administered by intraperitoneal injection. In one experiment, 2-naphthylamine suspended in 1% starch solution was administered by stomach tube. In other experiments 2-naphthylamine dissolved in olive oil or 2-naphthol dissolved in corn oil was injected subcutaneously in the lumbar region just lateral to the midline.

Determination of inorganic sulphate and total sulphate in urine. The method of Polin (1905) was used.

Measurement of ^{35}S in the inorganic sulphate and total sulphate fractions of urine. In order to measure the ^{35}S content of the inorganic sulphate fraction of urine, a small measured sample of urine in a 15 ml. centrifuge tube was made up to 6.0 ml. by the addition of distilled water. Two drops of a 0.04% solution of bromophenol blue in ethanol were then added, followed by N-HCl , drop by drop, until the colour of the solution was yellow without a trace of blue. 4.0 ml. of 95% ethanol were added and the sulphate was precipitated by the addition of 2.0 ml. of benzidine hydrochloride reagent (prepared by dissolving 5.0 g. of purified benzidine hydrochloride in 40 ml. of N-HCl and diluting the solution to 250 ml. with 50% (v/v) aqueous ethanol). The precipitate was stirred and after 30 min. it was separated by centrifuging. The supernatant liquid was discarded. The precipitate was washed in the centrifuge tube with four 5 ml. portions of 95% ethanol. After each washing the precipitate was separated by centrifuging and the supernatant liquid was discarded. The ^{35}S content of the precipitate was then measured in an ionization chamber with a direct current amplification system using an FP-54 tetrode valve (Young, Edson & McCarter, 1949).

In order to measure the ^{35}S content of the total sulphate fraction of the urine, 6.0 ml. of 4N-HCl were added to a measured sample of urine (1–2 ml.) in a 50 ml. beaker, and the solution was evaporated to dryness on a water bath. The beaker was allowed to cool and its contents transferred to a 15 ml. centrifuge tube with addition of small portions of distilled water (total vol. 6.0 ml.) and 95% ethanol (total vol. 4.0 ml.). Benzidine sulphate was then precipitated, washed by the method already described, and its ^{35}S content measured by the method of Young *et al.* (1949).

The ^{35}S content of the ethereal sulphate fraction of the urine was calculated from the difference between the ^{35}S content of the inorganic sulphate fraction and that of the total sulphate fraction.

Measurement of ^{35}S in organic compounds. The method of Young *et al.* (1949) was used. The compound was oxidized by the Carius method and the H_2SO_4 formed in the process was separated as benzidine sulphate, the ^{35}S content of which was then measured.

Distribution of ^{35}S in the inorganic, ethereal and total sulphate fractions of urine following the administration of radioactive $\text{Na}_2^{35}\text{SO}_4$ alone or with 2-naphthylamine

Two rats (weight 182 and 185 g.) each received by intraperitoneal injection 0.5 ml. of a solution of radioactive $\text{Na}_2^{35}\text{SO}_4$ containing 0.1 mg. of sulphate S. The animals were placed in a metabolism cage and the urine was collected for

the periods 0–12, 12–24, 24–28 and 48–72 hr. after dosing. At the end of 72 hr. each rat was again given the same amount of radioactive $\text{Na}_2^{35}\text{SO}_4$ by intraperitoneal injection, and in addition it received by stomach tube 0.050 g. of 2-naphthylamine in 1.0 ml. of 1% starch solution. The rats were returned to the metabolism cage and the urine was collected for the following 72 hr. at the same intervals as before. The urines were analysed for their contents of inorganic sulphate S (IS) and total sulphate S (TS), and from the data obtained the ethereal sulphate S (ES) content was calculated. The ^{35}S contents of the inorganic sulphate fraction (I^{35}S) and the total sulphate fraction (T^{35}S) were measured and from the results obtained the ^{35}S content of the ethereal sulphate fraction (E^{35}S) was calculated.

It should be noted that the amounts of Na_2SO_4 injected (0.1 mg. of sulphate S per rat) were less than 2% of the average daily excretion of TS in the urine of each rat, and the administration of such amounts of inorganic sulphate would not be expected to produce any appreciable disturbance of normal metabolism. The results obtained are shown in Table 1.

From the data in Table 1 it is seen that when radioactive $\text{Na}_2^{35}\text{SO}_4$ was given alone, 6% of the ^{35}S appeared as E^{35}S in the 72 hr. period following dosing, and this indicates that a small amount of the administered inorganic sulphate was used in the synthesis of ethereal sulphates normally present in the urine. When 2-naphthylamine was administered together with the radioactive $\text{Na}_2^{35}\text{SO}_4$ there was a marked increase in ES. This was to be expected, for it is known that in the rat 2-naphthylamine is converted to 2-amino-1-naphthylsulphuric acid (Manson & Young, 1950). Under these conditions it was found that 48% of the ^{35}S given was excreted in the urine as E^{35}S and this suggests that the administered inorganic sulphate participated in the formation of 2-amino-1-naphthylsulphuric acid. It is interesting to note that the rate at which the ^{35}S was excreted in the urine as T^{35}S was not influenced significantly by the extent to which it took part in ethereal sulphate formation.

An experiment similar to that just described, except that the 2-naphthylamine was administered by subcutaneous injection, was carried out. Two rats were used and each received 0.4 ml. of radioactive $\text{Na}_2^{35}\text{SO}_4$ solution containing 0.07 mg. of sulphate S by intraperitoneal injection. After 72 hr. this dose was repeated and each rat was also given a subcutaneous injection of 0.025 g. of 2-naphthylamine in 1.0 ml. of olive oil. The results obtained were similar to those shown in Table 1. In the 72 hr. after radioactive $\text{Na}_2^{35}\text{SO}_4$ had been given alone, 5% of the ^{35}S was excreted in the ethereal sulphate fraction of the urine, whereas when radioactive $\text{Na}_2^{35}\text{SO}_4$ and 2-naphthylamine were given simultaneously, 44% of the ^{35}S was excreted in the ethereal sulphate fraction in the 72 hr. after dosing.

Table 1. *The excretion of inorganic, ethereal and total sulphate S (IS, ES and TS) and the distribution of ^{35}S in the inorganic, ethereal and total sulphate fractions of the urine (I^{35}S , E^{35}S , T^{35}S) of two rats following the intraperitoneal injection of radioactive Na_2SO_4 (0.1 mg. of sulphate S per rat) alone or with 2-naphthylamine (0.050 g. per rat) given by a stomach tube*

Period after dosing (hr.)	Sulphate S (mg.)			^{35}S (% of ^{35}S administered)		
	IS	ES	TS	I^{35}S	E^{35}S	T^{35}S
	Radioactive Na_2SO_4 administered					
0-12	7.4	1.2	8.6	73	4	77
12-24	6.6	0.8	7.4	10	1	11
24-48	12.6	1.6	14.2	6	1	7
48-72	8.6	1.6	10.2	—	—	—
0-72	35.2	5.2	40.4	89	6	95
2-Naphthylamine and radioactive Na_2SO_4 administered						
0-12	1.6	3.4	5.0	39	38	77
12-24	0.6	5.0	5.6	1	9	10
24-48	8.2	2.4	10.6	4	1	5
48-72	8.4	1.6	10.2	—	—	—
0-72	18.8	12.4	31.2	44	48	92

Isolation of radioactive 2-amino-1-naphthylsulphuric acid from the urine of rats dosed with 2-naphthylamine and radioactive Na_2SO_4

Confirmation of the use of administered inorganic sulphate in the synthesis of 2-amino-1-naphthylsulphuric acid in rats dosed with 2-naphthylamine was obtained by the isolation of the radioactive sulphuric acid derivative from the urine of rats which had been dosed with 2-naphthylamine and radioactive Na_2SO_4 .

Twenty-four rats were each given 0.025 g. of 2-naphthylamine in 0.5 ml. of olive oil by subcutaneous injection and at the same time each was given 0.5 ml. of radioactive Na_2SO_4 solution containing 0.12 mg. of sulphate S by intraperitoneal injection. The animals were dosed again 24 hr. later. They were housed in metabolism cages and the urine was collected daily for 3 days after the first dosing. As a measure of protection against the oxidation of urinary constituents, about 0.1 g. of sodium hydrosulphite was placed in each of the urine collection bottles at the start of each day's collection of urine. The collected urine was stored in the refrigerator. The total volume of urine obtained was 900 ml. Measured portions of the urine were analysed for I^{35}S and T^{35}S and the results were used to calculate the E^{35}S content.

The results obtained, expressed as percentages of the ^{35}S administered, were as follows: I^{35}S , 43; E^{35}S , 34; T^{35}S , 77 %. The remainder of the urine was used for the isolation of 2-amino-1-naphthylsulphuric acid by a method which closely resembled that described by Manson & Young (1950).

The urine was first extracted for a total of 30 hr. in a continuous extractor with 500 ml. of peroxide-free ether to remove 2-naphthylamine and 2-acetamido-6-naphthol. The ether extract was discarded. The ether dissolved in the ether-extracted urine was removed under reduced pressure at room temperature, and the urine was then acidified to pH 2 by the addition of conc. HCl. It was then extracted with one 200 ml. portion and five 100 ml. portions of *n*-butanol saturated

with water. The emulsions which formed during these extractions were broken by centrifuging. The butanol extracts were combined, made alkaline with ammonia and evaporated to dryness under reduced pressure at a temperature which did not exceed 45°. The residue was taken up in small portions (total vol. 60 ml.) of a mixture of 90 parts of ethanol and 10 parts of conc. HCl (v/v). This gave a purple-coloured solution which yielded a light-coloured precipitate after standing overnight in the refrigerator. The precipitate was filtered and washed with several 2 ml. portions of the acid-ethanol mixture. The residue was dried *in vacuo* and it was then dissolved in 15 ml. of *N*-KOH. The solution was filtered and acidified with conc. HCl. The precipitate which formed was filtered and washed with several 2 ml. portions of distilled water. A light-brown product was obtained which, after drying over P_2O_5 *in vacuo*, weighed 0.071 g. This material darkened at 216° and melted with decomposition at 221-222° (this and other melting points reported herein are uncorrected unless otherwise stated). Wiley (1938) reported a m.p. (corr.) of 224° for 2-amino-1-naphthylsulphuric acid isolated from the urine of dogs dosed with 2-naphthylamine. (Found: C, 49.9; H, 3.6; S, 13.1. Calc. for $\text{C}_{10}\text{H}_9\text{O}_4\text{NS}$: C, 50.2; H, 3.8; S, 13.4 %.)

A sample of the compound was analysed for its ^{35}S content and the remainder was dissolved in *N*-KOH in a centrifuge tube. The solution was acidified with conc. HCl and the precipitate was separated by centrifuging. The precipitate was washed with two 1 ml. portions of distilled water and after each washing it was separated by centrifuging. The compound was then dried over P_2O_5 *in vacuo* and samples were taken for the determination of the melting point and the measurement of the ^{35}S content.

The remainder of the compound was dissolved in 5 ml. of warm water and the solution was left overnight in the refrigerator. The precipitate which formed was separated by centrifuging and was washed in the centrifuge tube with two 1 ml. portions of distilled water. The product was dried over P_2O_5 *in vacuo* and its melting point and ^{35}S content were determined.

In the form in which its melting point was first determined the compound melted at 221-222°.

After being recrystallized it melted at 221–222°, and after further recrystallization the melting point was 222–223°. The corresponding ^{35}S contents of the compound (expressed as percentages of the administered ^{35}S per mg. of S in the compound) were 0.310, 0.310 and 0.307 %, respectively. As the specific activity of the compound was not changed significantly by recrystallization, it is concluded that the compound isolated from the urine of rats dosed with 2-naphthylamine and radioactive sodium sulphate was radioactive 2-amino-1-naphthylsulphuric acid.

Isolation of radioactive 2-naphthylsulphuric acid from the urine of rats dosed with 2-naphthol and radioactive Na_2SO_4

In the experiment just described it is probable that 2-naphthylamine was first converted in the rat to 2-amino-1-naphthol, and that this compound was then converted to 2-amino-1-naphthylsulphuric acid. Elson, Goulden & Warren (1946) have suggested, however, that *p*-aminophenylsulphuric acid may be formed from aniline in the rat by a process which is independent of the formation of *p*-aminophenol. If this is so, the conversion of a non-phenolic carbocyclic compound, such as 2-naphthylamine, to an ethereal sulphate *in vivo*, might not involve the intermediate formation of a phenol. It was decided, therefore, to determine whether administered inorganic sulphate can participate in the rat in the formation of an ethereal sulphate from an administered phenolic compound. With this object in view, 2-naphthylsulphuric acid was isolated from the urine of rats dosed with 2-naphthol and radioactive Na_2SO_4 . The 2-naphthylsulphuric acid was isolated in the form of its *p*-toluidine salt, and the method used was based on that described by Berenbom & Young (1951).

Each of twenty-four rats (weights 170–200 g.) received by subcutaneous injection 0.100 g. of 2-naphthol dissolved in 1.0 ml. of corn oil, and at the same time they were each given by intraperitoneal injection 0.6 ml. of an aqueous solution of radioactive Na_2SO_4 containing 0.1 mg. of sulphate S. The animals were dosed in this way once a day on three successive days. They were kept in metabolism cages, and the urine was collected for 88 hr. from the time of the first dosing and was stored in the refrigerator. The volume of the urine was 950 ml. Measured portions of the urine were analysed for I^{35}S and T^{35}S , and the values obtained were used to calculate the content of E^{35}S .

The results obtained, expressed as percentages of the ^{35}S administered, were as follows: I^{35}S , 16; E^{35}S , 46; T^{35}S , 62 %. The high proportion of the ^{35}S which appeared as E^{35}S suggested that radioactive 2-naphthylsulphuric acid was present in the urine.

After the removal of samples for analysis, the remainder of the urine was adjusted to pH 8–9 by the addition of 20 %

(w/v) NaOH solution and it was then concentrated at 50–60° under reduced pressure to one-tenth of its original volume. The concentrated urine was transferred to a 250 ml. centrifuge bottle and was extracted with five 100 ml. portions of *n*-butanol. The urine was shaken with each portion of butanol for 15 min. on a mechanical shaker and the emulsions which formed were broken by centrifuging. After each extraction, 10 ml. of water were added to the urine to maintain its volume. The butanol extracts were combined and evaporated to dryness at 50–60° under reduced pressure.

The residue was dissolved in 40 ml. of water at 60–70° and 3.0 g. of *p*-toluidine hydrochloride were added with stirring. The solution was left in the refrigerator overnight and was then filtered. The precipitate (*P*-1) consisted mostly of glucuronide, but it gave a slight positive test for ethereal sulphate. The filtrate (*F*-1) gave a strong positive test for ethereal sulphate. The precipitate, *P*-1, was dissolved in a minimum of boiling water, charcoal was added, and the solution was filtered. The filtrate was left in the refrigerator and gave a precipitate (*P*-2) which consisted of glucuronide and gave no test for ethereal sulphate. The filtrate (*F*-2), which contained ethereal sulphate, was combined with *F*-1, and the combined solutions were evaporated to 40 ml. at 50–60° under reduced pressure. This concentrate gave a precipitate (*P*-3) after it had stood in the refrigerator. This precipitate gave no test for ethereal sulphate. The filtrate (*F*-3) was evaporated to dryness at 50–60° under reduced pressure.

The residue was extracted with 60 ml. of boiling ethanol and the extract was cooled. The precipitate (*P*-4) which formed consisted of NaCl and *p*-toluidine hydrochloride. The filtrate (*F*-4) was evaporated to dryness under reduced pressure at 50–60°, and the residue obtained was dissolved in a minimum of boiling water and treated with charcoal. The solution was filtered, and after standing in the refrigerator it yielded a precipitate (*P*-5) which gave a strongly positive test for ethereal sulphate and a weakly positive test for glucuronide. After being recrystallized twice from hot water this material was free from glucuronide. The product weighed 0.307 g.; m.p. 156–158° with previous darkening and subsequent decomposition. (Found: C, 61.7; H, 5.1; S, 9.5. Calc. for $\text{C}_{17}\text{H}_{17}\text{O}_4\text{NS}$: C, 61.6; H, 5.1; S, 9.7 %.)

Potassium 2-naphthylsulphate was synthesized by the method of Feigenbaum & Neuberger (1941), and 1.0 g. of this compound when converted to *p*-toluidine 2-naphthylsulphate by the method of Berenbom & Young (1951) yielded 0.570 g. of purified product. (Found: C, 61.5; H, 5.0; S, 9.5. Calc. for $\text{C}_{17}\text{H}_{17}\text{O}_4\text{NS}$: C, 61.6; H, 5.1; S, 9.7 %.) The synthetic compound melted at 156–158° with decomposition, and the melting point was unchanged when the compound was mixed with the product isolated from urine.

A sample of the *p*-toluidine 2-naphthylsulphate isolated from urine was analysed for its ^{35}S content. The remainder of the compound was recrystallized twice from water, and after each crystallization it was dried over P_2O_5 *in vacuo* and samples were taken for the determination of the melting point and the measurement of the ^{35}S content. The melting point of the compound was unchanged by recrystallization. The ^{35}S contents of the compound after

successive crystallizations (expressed as percentages of the administered ^{35}S per mg. S in the compound) were 0.216, 0.210 and 0.220 %. The variations from the mean value were within the limits of error of the analytical method used, and it is concluded that the compound isolated from the urine was radioactive *p*-toluidine 2-naphthylsulphate.

Investigation of the possible interchange of inorganic sulphate S and ethereal sulphate S in urine

The conclusion that administered inorganic sulphate can participate in the formation of ethereal sulphate *in vivo* would be invalidated if the sulphur of radioactive inorganic sulphate in the urine were to exchange with ethereal sulphate S. Experiments were therefore carried out to investigate this possibility for urine containing radioactive inorganic sulphate and 2-amino-1-naphthylsulphuric acid or 2-naphthylsulphuric acid.

On each of two successive days six rats were each given a subcutaneous injection of 0.025 g. of 2-naphthylamine dissolved in 0.5 ml. of olive oil. The urine was collected for 48 hr. after the first dosing. The volume of the urine was made up to 100 ml. and measured portions were analysed for IS and TS. An ES/TS value of 0.51, indicative of the presence of ethereal sulphate derived from 2-naphthylamine, was found. A solution (2 ml.) of radioactive Na_2SO_4 containing 2.6 mg. of sulphate S was added to the urine. The urine was allowed to stand at room temperature and after 24 and 96 hr. samples were taken and analysed for I^{35}S and T^{35}S . The results obtained are shown in Table 2.

Another experiment was carried out in which each of six rats was injected subcutaneously with 0.100 g. of 2-naphthol dissolved in 1.0 ml. of corn oil. The urine was collected for 48 hr. after dosing. Analysis of the urine showed that its ES/TS value was high, namely 0.50. To the urine were added 5.0 ml. of a solution of radioactive Na_2SO_4 containing 3.3 mg. of sulphate S, and the volume was made to 100 ml. by the addition of water. The urine was allowed to stand at room temperature and samples taken after 24 and 72 hr. were analysed for I^{35}S and T^{35}S . The results obtained are shown in Table 2.

Table 2. I^{35}S and T^{35}S in urine at various times following the addition of radioactive Na_2SO_4 to the urine of rats dosed with 2-naphthylamine or 2-naphthol

Time (hr.)	Urine from rats dosed with 2-naphthylamine		Urine from rats dosed with 2-naphthol	
	I^{35}S	T^{35}S	I^{35}S	T^{35}S
	Percentage of ^{35}S present at 0 hr.			
0	100	(100)	100	(100)
24	100	98	99	99
72	—	—	97	97
96	101	100	—	—

If ^{35}S had passed from the inorganic sulphate fraction of the urine to the ethereal sulphate fraction the $\text{I}^{35}\text{S}/\text{T}^{35}\text{S}$ value would have fallen. The results

given in Table 2 show that this did not take place even though the experimental conditions (e.g. concentration of ethereal sulphate in the urine, duration of the experiments) were such that they gave ample opportunity for the occurrence of an exchange reaction. It is reasonable, therefore, to conclude that in the experiments described in the present paper, the appearance of ^{35}S in the ethereal sulphate fraction of the urine and the presence of ^{35}S in ethereal sulphates isolated from urine were not the result of exchange reactions taking place in the urine, but were the outcome of reactions occurring within the organism.

DISCUSSION

Baumann (1876), who was the first to show that formation of ethereal sulphates takes place in the body, was of the opinion that these compounds arise *in vivo* as a result of the union of sulphuric acid with a phenolic compound. Many years later, Sherwin and his co-workers (Rose, Shiple & Sherwin, 1924; Shiple, Muldoon & Sherwin, 1924) obtained results which led them to suggest that ethereal sulphates can arise by the oxidation of mercapturic acids in the body. No evidence of increased ethereal sulphate excretion after the administration of mercapturic acids was obtained, however, by Callow & Hele (1927), Coombs & Hele (1927) and Lawrie (1931). As a result of these and other studies on ethereal sulphate formation, Hele (1931) was led to conclude that in the organism ethereal sulphates are 'formed by the union of the sulphate ion with phenolic compounds'. More recently, work on the metabolism of phenols in tissue slices and homogenates (Bernheim & Bernheim, 1943; Storey, 1950; De Meio & Tkacz, 1952) has lent support to the view that inorganic sulphate is involved in ethereal sulphate formation *in vivo*.

If synthesis of ethereal sulphates takes place in the body in the manner envisaged by Baumann and by Hele, there arises the question of whether administered inorganic sulphate can take part in this process. The literature on this subject contains a number of conflicting observations. Whereas Baumann (1876) and Hele (1924, 1931) found that administered sodium sulphate could give rise to increased ethereal sulphate excretion by animals dosed with phenolic compounds, Tauber (1895), Rhode (1923), and Shiple *et al.* (1924) were unable to obtain evidence that this occurs.

As a result of the present work it is apparent that inorganic sulphate administered to rats by intraperitoneal injection can participate in ethereal sulphate synthesis. When sodium sulphate labelled with ^{35}S was administered alone, 5–6 % of the ^{35}S was excreted in the ethereal sulphate fraction of the urine. When an ethereal sulphate precursor,

2-naphthylamine, was given by stomach tube or injected subcutaneously and radioactive sodium sulphate was given by intraperitoneal injection at the same time, the excretion of ethereal sulphate was increased and 44–48 % of the administered ^{35}S appeared in the ethereal sulphate fraction of the urine. Furthermore, radioactive 2-amino-1-naphthylsulphuric acid and radioactive 2-naphthylsulphuric acid (as the *p*-toluidine salt) were isolated from the urine of rats which received radioactive sodium sulphate and were dosed with 2-naphthylamine and 2-naphthol, respectively. In our experiments the amount of radioactive sodium sulphate administered to each rat was less than 2 % of the normal daily excretion of total sulphate in the urine and the animals were maintained on a normal diet, whereas in a number of the balance experiments carried out by the earlier workers, large amounts of sulphate were administered and the protein intake of the animals was restricted.

Since the publication of the preliminary account of the present work (Laidlaw & Young, 1948), two papers bearing on the subject have appeared (Dziewiatkowski, 1949; Binkley, 1949).

Dziewiatkowski (1949) found that rats dosed by stomach tube or by intraperitoneal injection with sodium sulphate labelled with ^{35}S excreted some of the ^{35}S as ethereal sulphate sulphur in the urine. The amount of ^{35}S excreted in this form was greatly increased when phenol was administered, and this occurred when the phenol and radioactive sodium sulphate were both given by stomach tube and also when the phenol was given by this route while the radioactive sulphate was administered by intraperitoneal injection. These findings are similar to those reported by us.

Binkley (1949) described experiments in which he found that the addition of inorganic sulphate to the diet of rats receiving *p*-bromophenol neither produced a resumption of growth nor gave an increase in ethereal sulphate excretion. On the other hand, addition of cystine to the diet of these animals led to growth and an increased excretion of ethereal sulphate. Binkley considers that it is probable that no significant increase in the level of sulphate in the tissues is achieved by feeding sodium sulphate in the diet, whereas a single injection of sulphate, coincident with the administration of the phenol, would provide a more ad-

vantageous situation for conjugation. He is of the opinion that his findings and ours are not inconsistent if the mode of administration of the sulphate is taken into consideration.

While the extent to which administered inorganic sulphate takes part in ethereal sulphate formation in the animal body will clearly be influenced by a variety of factors, the experiments with radioactive sulphate have made it apparent that administered inorganic sulphate can participate in this process.

SUMMARY

1. Quantitative studies have been made of the excretion of radioactive sulphur (^{35}S) in the inorganic and total sulphate fractions of the urine of rats following the intraperitoneal injection of ^{35}S -labelled sodium sulphate alone, or simultaneously with the administration of 2-naphthylamine by stomach tube or by subcutaneous injection.

2. When radioactive sodium sulphate was given alone, 5–6 % of the ^{35}S administered was excreted in the ethereal sulphate fraction of the urine in the 72 hr. after dosing.

3. When 2-naphthylamine was administered as well as radioactive sulphate, the ethereal sulphate content of the urine was increased and 44–48 % of the ^{35}S administered was excreted in the ethereal sulphate fraction of the urine in the 72 hr. after dosing.

4. Radioactive 2-amino-1-naphthylsulphuric acid was isolated from the urine of rats injected intraperitoneally with ^{35}S -labelled sodium sulphate and given 2-naphthylamine by subcutaneous injection at the same time.

5. Radioactive 2-naphthylsulphuric acid (as the *p*-toluidine salt) was isolated from the urine of rats which were given ^{35}S -labelled radioactive sodium sulphate by intraperitoneal injection simultaneously with the subcutaneous injection of 2-naphthol.

6. It is concluded that intraperitoneally administered sodium sulphate can participate in the formation of ethereal sulphates in the rat.

An account of the work described in the present paper formed part of a thesis presented to the University of Toronto (Laidlaw, 1947), and a preliminary report of the investigation was presented to the Biochemical Society on 12 March 1948 (Laidlaw & Young, 1948).

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The Effect of Dinitro-*o*-cresol on the Deposition of Liver Glycogen in the Rat

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During the course of some experimental observations on the toxicity of dinitro-*o*-cresol (DNOC) a histological examination was made of the tissues of rats that had received a series of daily injections of a toxic dose (20 mg./kg.). The histological examination was essentially negative except for a somewhat unusual appearance of the liver cells. Special staining showed the liver to be packed with glycogen (Parker, Barnes & Denz, 1951). This was an unexpected observation because it is generally believed that the immediate effect of an injection of DNOC is to deplete the liver of its glycogen.

The effect of single and repeated doses of DNOC on the liver glycogen was, therefore, examined. The experiments showed that while the immediate effect of DNOC is to deplete the liver glycogen, the process is later reversed. The 24 hr. fasting levels of liver glycogen are always higher than normal in rats treated with one or a series of daily injections of DNOC.

In the course of the work an attempt was made to reduce the individual variation in the liver glycogen of normal fasting rats by submitting the animals to regular feeding regimes.

The normal practice in the animal house was to place in the cage a supply of rat cubes that was more than adequate for the needs of the rat during the next 24 hr. If food is suddenly withdrawn from these animals for a 24 hr. fast, the animals may differ in the amount of food each will have in its stomach at the time the food is withdrawn and the actual fast of some rats might be longer than 24 hr.

A regular feeding regime of two feeds a day, each of 1 hr. duration, and a single feed of 1 hr. was instituted. There was no reduction in the range of variation in individual animals, but the general fasting levels differed significantly.

These observations are discussed.

METHODS

Determination of liver glycogen, blood sugar and lactate. The liver glycogen was determined by the method of Good, Kramer & Somogyi (1933) on pieces of liver removed as quickly as possible from rats killed by guillotine. Although care was taken not to excite the animals immediately before death, an experiment was done that suggested that such a precaution was probably unnecessary. Two groups of four normal well-fed rats were killed. One group was kept quiet and killed, and the rats in the other group were excited just before being caught and killed. The glycogen levels in the two groups were 6.92 ± 2.9 and $7.2 \pm 0.72\%$ respectively. Blood sugar was determined by the method of Somogyi (1945) and blood lactate by that of Barker & Summerson (1941). All the results are expressed as means, together with the standard error.

*Dinitro-*o*-cresol injections.* DNOC purified by recrystallization from 50% ethanol was injected subcutaneously as a 1% (w/v) aqueous solution adjusted to pH 7. The dose was usually 20 mg./kg. which produces severe symptoms of poisoning. In order to reduce the number of deaths when the animals were to be given a series of injections, the cages were placed in a cool draughty corridor immediately after the animals had been injected.

Feeding regimes

Albino rats weighing 150–250 g. were used.

'Continuous feeding.' The rats were given each day a supply of cubes (Medical Research Council diet 41) more than adequate for their needs so that they had continuous access to food during the 24 hr.

Two meal regime. The rats were given cubes from 9–10 a.m. and 2–3 p.m. each day and had access to water at all times.

Single meal regime. The rats were given cubes only from 9 to 10 a.m. each day.

In the experiments with DNOC the rats were either on 'continuous feeding' or trained to eat a single feed for 1 hr. each day. In the latter case all rats were placed on the single feed regime for at least 3 weeks before they were used for experiments with DNOC to allow them to become accustomed to this new feeding regime.