Measurement and Kinetic Study of the Formation of Adrenaline Sulphate *in vitro*

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The sulpho-conjugation of [^{14}C]adrenaline from inorganic sulphate and ATP or preformed adenosine 3'-phosphate 5'-sulphatophosphate was demonstrated in the high-speed supernatant prepared from the liver and small intestine of various animals. Hydrolysis with sulphatase indicated the sulphate nature of the conjugate. The overall sulphation reaction has a pH optimum of 9.0. Maximal activity was obtained with a ratio of ATP/Mg $^{2+}$ of 1 at 4-6 mM. Above their optimal concentrations, ATP and Mg $^{2+}$, separately or in combination, were inhibitory. Dithiothreitol at 3 mM stimulated the reaction by about 30%. The K_m for adrenaline, determined by the sulphotransferase reaction and by the three-step (sulphate-activating and sulphotransferase) reactions was 125 μM . The rate of synthesis of [^{14}C]adrenaline sulphate, expressed in pmol/min per mg of protein for the livers of dog, monkey, rat, guinea pig and rabbit were, respectively, 144, 77, 47, 11 and 6. The corresponding values for the small intestines of dog and monkey were 60 and 62. Brain and heart tissues showed no measurable activity.

The sulphation of adrenaline in man was first reported by Richter in 1940 and later confirmed by other workers (Richter & MacIntosh, 1941; Deichmann, 1943; Beyer & Shapiro, 1945; Häggendal, 1963). In spite of the above evidence for its sulphate formation *in vivo*, adrenaline was not included in the studies *in vitro* of Jenner & Rose (1973) and Foldes & Meek (1973a,b) on the sulphation of the catecholamines, dopamine (3,4-dihydroxyphenethylamine) and noradrenaline. The present paper describes a simple, sensitive and specific method for determining adrenaline sulphate synthesized *in vitro* from inorganic sulphate and ATP or preformed 'active' sulphate, adenosine 3'-phosphate 5'-sulphatophosphate. The kinetics of the reactions were systematically studied.

Materials and Methods

Chemicals

The following radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: DL-[7- ^{14}C]adrenaline DL-bitartrate (33 mCi/mmol, radiochemical purity of 98-99%) and Na $_2$ $^{35}\text{SO}_4$ (24 mCi/mmol). The other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. They are: ATP (sodium salt, 98% pure), dithiothreitol, *trans*-2-phenylcyclopropylamine, sulphatase (S9626, type H-1 from *Helix pomatia*, containing 20000 units/g; 1 unit will hydrolyse 1.0 μmol of *p*-nitrocatechol sulphate/h at pH 5.0 at 37°C) and 2,6-dichloroquinone-4-chloroimide (Gibbs reagent).

Preparation of enzymes

Adult animals of both sexes were used. The rats were of the albino Wistar strain, the monkey belonged to the *Macaca fascicularis* species and the other animals were local hybrids. A 20% (w/v) homogenate in 0.15 M-KCl was prepared from freshly excised small intestine, liver or brain, in a Polytron homogenizer. The content of the small intestine was thoroughly washed off before homogenization. These homogenates were centrifuged at 178000g for 30 min as described by Wong (1975). To the high-speed supernatant was added 10 mM-*trans*-2-phenylcyclopropylamine to inhibit the activity of monoamine oxidase found to be present in such preparations (Wong, 1976). These enzyme solutions were stored in 0.5 ml portions in the Revco freezer at -80°C.

Assay conditions

(a) *The overall reaction.* The incubation medium, with a final volume of 350 μl and a final pH of 9.0, contained the following: [^{14}C]adrenaline (0.31 μCi , 27.1 μM), ATP adjusted to pH 7.2 (6 mM), Na $_2$ SO $_4$ (7.1 mM), MgCl $_2$ ·6H $_2$ O (6 mM) in dithiothreitol (3 mM) and 200 μl of 0.5 M-glycine/NaOH buffer, pH 9.2. The reaction was started with 50 μl of the enzyme solution whose protein content, measured by the method of Lowry *et al.* (1951) with bovine albumin as standard, was between 0.6 and 1.3 mg for the various preparations of liver and small intestine. Controls were carried out by omitting ATP from the reaction mixture. The reaction was stopped after 15 min of incubation at 37°C by adding 50 μl each of

5% (w/v) ZnSO_4 and 0.3M- $\text{Ba}(\text{OH})_2$, which produced a final pH of 8.3–8.5 and a final volume of 400 μl .

(b) *The sulphotransferase reaction.* For this reaction, adenosine 3'-phosphate 5'-sulphatophosphate was first generated in the conditions described above, except that the aglycone was not included in the incubation mixture. After 60min of incubation, the reaction tubes were boiled and centrifuged. The supernatant so obtained was used as preformed 'active' sulphate in a second reaction, whereby it is transferred to [^{14}C]adrenaline. The same high-speed supernatant preparation of dog liver was used as a source of sulphotransferase, but the sulphate-activating system was inhibited by a 3min preincubation of this enzyme solution with half its volume of 0.1M-EDTA (Brunngraber, 1958); the latter had been adjusted to pH9.2.

Separation, chromatography and quantification of [^{14}C]adrenaline sulphate

The reaction mixture was adjusted to pH8.6 with usually less than 5 μl of 0.1M-HCl and centrifuged (3000 rev./min for 10min). A portion (300 μl) of the supernatant was then added to 200mg of alumina suspended in 0.1 ml of 0.2M-sodium acetate reagent adjusted to pH8.6. This is a modification of the procedure of Wong *et al.* (1973). Mercaptoethanol was omitted, as dithiothreitol, another reducing agent, was already present in the incubation mixture. The alumina had previously been washed with 3ml of the same sodium acetate reagent. After thorough mixing on a Vortex mixer for 1 min, the tubes were centrifuged at 3000 rev./min on an MSE Super Minor for 10min. The radioactivity present in 50 μl of the supernatant was counted in 10ml of scintillator, containing 0.4% 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) and 0.025% 2,5-diphenyloxazole (PPO) in 1 litre of toluene and 500ml of Triton X-100. This gave a preliminary rough estimation of the degree of sulphation. For quantification of [^{14}C]adrenaline sulphate synthesized, 25 or 50 μl of the supernatant was chromatographed on Whatman no. 1 paper and the chromatogram was developed in butan-1-ol/acetic acid/water (4:1:5, by vol.) overnight (16h) at room temperature (29–30°C). The radioactivity present in six 2cm sections (7–19cm from the origin) was counted in the scintillator without the addition of Triton X-100.

To determine the percentage recovery of [^{14}C]adrenaline sulphate, the supernatant after treatment with alumina (fraction 1) was removed and the alumina was washed twice successively with 0.4ml of sodium acetate reagent (fractions 2 and 3). Subsequent chromatography and quantification of radioactivity of [^{14}C]adrenaline sulphate present in these fractions were carried out in exactly the same manner as for fraction 1. At least 85–90% of the total radioactivity of [^{14}C]adrenaline sulphate was found in

fraction 1, with little or no radioactivity in fraction 3.

Isolation, hydrolysis and identification of [^{14}C]adrenaline sulphate

The radioactivity present in the peak material with R_F 0.31 was eluted with water, and this eluate was adjusted to pH5 with 0.2M-HCl. To one fraction was added 2mg of sulphatase preparation, which was omitted from the control. Both tubes were incubated at 37°C overnight. They were adjusted to pH8.6 and subjected to treatment with alumina as before. The alumina was washed with 2 $\times$ 10ml of water and [^{14}C]adrenaline was eluted with 2ml of 0.2M-acetic acid (Wong *et al.*, 1973). The supernatant obtained after the adsorption on alumina and the acetic acid eluate were chromatographed on paper as described above. Comparisons were made with authentic [^{14}C]adrenaline.

Gibbs test

This was performed by mixing small quantities of the sulphated material present in the supernatant, after treatment with alumina, with 2,6-dichloroquinone-4-chloroimide in alkaline solution by the procedure of Gibbs (1927). The chromatograms were sprayed with 1% (w/v) Gibbs reagent in ethanol and exposed to NH_3 vapour (Jenner & Rose, 1973). The 4-O- but not the 3-O-sulphate could be detected by the production of a blue colour.

Results

Formation and hydrolysis of [^{14}C]adrenaline sulphate

The supernatant, after treatment of the reaction mixture with alumina, on chromatography showed one major radioactive peak with an R_F of 0.31. On some chromatograms, a minor peak with R_F 0.53 may be observed. The radioactivity in this peak normally did not exceed 300 c.p.m. and it corresponded to [^{14}C]adrenaline that was incompletely removed by alumina. Because of this uncertainty, it was necessary to separate [^{14}C]adrenaline sulphate by paper chromatography and quantify its radioactivity so as to ensure that the unchanged aglycone does not interfere with its measurement. When $\text{Na}_2^{35}\text{SO}_4$ was used in conjunction with unlabelled adrenaline (1 mM), adrenaline [^{35}S]sulphate produced had the same chromatographic mobility as [^{14}C]adrenaline sulphate in the solvent system used.

Hydrolysis of the material eluted from the radioactive peak with R_F 0.31 by sulphatase released radioactivity in the acetic acid eluate. The chromatographic behaviour of this hydrolytic product on alumina and paper was identical with that of authentic [^{14}C]adrenaline.

Kinetic data

These were obtained with the dog liver preparation. The radioactivity present in the supernatant after treatment with alumina reflected closely that of

Fig. 1. Effect of pH on the formation of [¹⁴C]adrenaline sulphate measured in c.p.m. per 15 min in (○) 50 µl of the supernatant after treatment of the reacted mixture with alumina and (●) a 25 µl chromatographed portion developed in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase), the radioactivity of [¹⁴C]adrenaline sulphate with an R_f of 0.31 being measured (the values on the ordinate in parentheses)

Fig. 2. Effect of ATP on the formation of [¹⁴C]adrenaline sulphate at 2.87 mM-Mg²⁺ (○) and 7.14 mM-Mg²⁺ (●). Portions of volume 50 or 25 µl respectively were chromatographed; values for the latter set of data are given on the ordinate in parentheses.

[¹⁴C]adrenaline sulphate separated on paper (Fig. 1). However, because of the high background radioactivity of the control and the uncertainty of contamination by [¹⁴C]adrenaline that may not be completely removed, quantification of the sulphate synthesized necessitates the additional paper-chromatographic step. As adrenaline sulphate is not commercially available, calculations were made by comparisons with a standard of [¹⁴C]adrenaline similarly chromatographed and measured.

Effects of pH, time of incubation and enzyme concentration. The pH optimum of the overall sulphation of adrenaline was 9.0 (0.5 M-glycine/NaOH buffer; Fig. 1). The formation of [¹⁴C]adrenaline sulphate increased progressively with time of incubation and enzyme concentration.

Effects of Mg²⁺ ions and ATP. At two different Mg²⁺ concentrations (2.87 and 7.14 mM), maximal activity was observed when ATP was present at similar respective concentrations (Fig. 2), suggesting that a ratio of ATP/Mg²⁺ of 1 was optimal. At this 1:1 ratio, highest activity was found in the 4–6 mM range (Fig. 3). This was also observed for Mg²⁺ concentration measured at optimal ATP concentration (Fig. 4).

Fig. 3. Formation of [¹⁴C]adrenaline sulphate with ATP and Mg²⁺ present in equimolar concentrations, ranging from 2 to 16 mM

See the text for experimental details.

Fig. 4. Effect of Mg^{2+} concentration on $[^{14}C]$ adrenaline sulphate formation with ATP present at a concentration of 6 mM

See the text for experimental details.

ATP and Mg^{2+} , above 6 mM, separately or in combination, inhibited the reaction. Two other bivalent ions, Mn^{2+} and Ca^{2+} , at equimolar concentration to Mg^{2+} , were only 66 and 10% as active.

Measurement of K_m for adrenaline. This was determined by the sulphotransferase as well as by the overall three-step reaction; the latter included the sulphate-activating and sulphotransferring reactions. In both cases, a K_m of 125 μM was obtained for adrenaline (Fig. 5) by Lineweaver-Burk (1934) plots.

Effects of dithiothreitol. Dithiothreitol at 3 mM stimulated the reaction by about 30%. Omission of dithiothreitol in the incubation mixture resulted in the appearance of a minor peak with R_F 0.73 on the paper chromatogram. This product was not identified; it was presumably formed by the enzymic or spontaneous oxidation of adrenaline, and dithiothreitol prevents its occurrence.

Gibbs test. Gibbs test, performed on the supernatant after adsorption by alumina and on the paper chromatogram, was negative, suggesting that adrenaline 3-*O*- rather than the 4-*O*-sulphate was synthesized *in vitro*. This observation was the same as that of Jenner & Rose (1973) with respect to the sulphation of dopamine.

Fig. 5. Lineweaver-Burk plots of velocity (v , expressed in c.p.m. of $[^{14}C]$ adrenaline sulphate produced/15 min) against concentrations of $[^{14}C]$ adrenaline in μM

The K_m was determined by the sulphotransferase reaction (●) and the three-step reaction (○); the latter included the sulphate-activating and sulphotransferring reactions. For details of reaction conditions, see the text.

Measurement of $[^{14}C]$ adrenaline sulphate synthesized by different tissues

The high-speed supernatant of liver and small intestine of the dog and monkey showed high activity (Table 1). Such preparations of the small intestine, in contrast with hepatic preparations, lose their sulphating activity rapidly on storage at $-80^\circ C$ and were therefore assayed on the day of preparation. Other tissues, e.g. brain and heart, were unable to conjugate adrenaline with sulphate under identical conditions (Table 1). The addition of unlabelled adrenaline at 10 times the concentration of its labelled counterpart decreased the radioactivity of $[^{14}C]$ adrenaline sulphate considerably. Consequently only labelled adrenaline was used in the standard assays.

Discussion

This is the first presentation of the measurement of the sulpho-conjugation of adrenaline *in vitro*. The method is simple, sensitive and specific. The pH optimum of 9.0 obtained was similar to that reported for the sulphation of noradrenaline and dopamine (Foldes & Meek, 1973a,b). As the pH of the reaction is 9.0, it would appear that an uncharged NH_2 group is necessary for the sulphation of these catecholamines as their pK values are around 8.4 (Armstrong & Barlow, 1976).

In general, the catecholamines seemed to be excreted as the sulphate conjugates, e.g. adrenaline (Richter, 1940; Richter & MacIntosh, 1941; Deichmann, 1943; Beyer & Shapiro, 1945; Häggendal,

Table 1. *Biosynthesis of [¹⁴C]adrenaline sulphate by different tissues*

Results are expressed as pmol of [¹⁴C]adrenaline sulphate formed/min per mg of protein for a 15 min assay, as determined by the three-step (sulphate-activating and sulphotransferase) reactions. The values were obtained from an average of three experiments carried out on the same enzyme preparation of the respective tissues; the variations of these readings is less than 5%. Abbreviation used: NM, not measurable.

Animals	Tissues	Activity
Dog	Liver	144.3†
Monkey	Liver	76.8
Rat	Liver	47.2
Guinea pig	Liver	10.7
Rabbit	Liver	6.2‡
Dog	Small intestine*	60
Monkey	Small intestine	62
Guinea pig, rat, rabbit	Small intestine	NM
Dog	Heart	NM
Dog, rat	Brain	NM

* Fresh preparations of the small intestines were used in the assay, as loss of activity was observed after storage at -80°C.

† The 'active' sulphate generated by this preparation (measured 3 months after storage at -80°C) was of the order of 220 pmol/min per mg of protein.

‡ This value is almost the limit of identification of adrenaline sulphate formed under the experimental conditions used; it corresponded to 500 c.p.m. of [¹⁴C]-adrenaline sulphate.

1963), dopamine (Andén *et al.*, 1970; Jenner & Rose, 1974; Rutledge & Hoehn, 1973; Bronaugh *et al.*, 1975; Merits, 1976) and isoprenaline (Conway *et al.*, 1968; Morgan *et al.*, 1969; Conolly *et al.*, 1972) are excreted as sulphates by man and dog. Because of the similarity between man and dog in their disposal of these catecholamines, it is conceivable that the sulphate-conjugatory mechanism may be equally active in both species. The generation of adenosine 3'-phosphate 5'-sulphatophosphate by dog liver *in vitro* from inorganic sulphate and ATP seemed to be a very rapid reaction. The low K_m of the sulphotransferase for adrenaline may be another contributing factor to its high rate of sulphation. In man, 2-5 min after the infusion of adrenaline, the conjugate, believed to be the sulphate, was detected in the urine (Goodall & Alton, 1965). Besides the liver, the small intestines of the dog and monkey are active sites of sulphation of this catecholamine. A comparison is made with catechol *O*-methyltransferase and monoamine oxidase, two other enzymes that inactivate adrenaline. The K_m of sulphotransferase for adrenaline (125 μ M) is of the same order of magnitude as that of 120 μ M reported for catechol *O*-methyltransferase

(Axelrod, 1975). Competition of the two enzymes may occur, as they seem to attack the same hydroxy group. The K_m of monoamine oxidase for adrenaline has not been measured; the corresponding value for dopamine, which appeared to be a better substrate than adrenaline (Blaschko *et al.*, 1937; Blaschko, 1953) is much higher (1600-7400 μ M; Tipton, 1975). It can thus be concluded that sulphation of adrenaline could be another major pathway for terminating the action of this hormone.

A systematic study of the kinetics of the sulphation reaction from inorganic sulphate and ATP showed that the generation of adenosine 3'-phosphate 5'-sulphatophosphate at pH 9.0 required stoichiometric amounts of ATP and Mg^{2+} ions at an optimal concentration of 4-6 mM. Above this concentration, these reactants, present either singly or in combination in the incubation mixture, were inhibitory. An analogous situation was found in the study of the allosteric enzyme phosphofructokinase (Lowry & Passoneau, 1966). Inhibition by high concentrations of ATP has previously been demonstrated in the sulphation of harmol, and a regulatory function of this nucleotide on the sulphate-activating system had been proposed (Wong, 1974). This effect, however, was not found in all sulphating systems, being absent from the sulphate conjugation of 4-hydroxy-3-methoxyphenylglycol in brain (Wong, 1975). Although all sulphate-conjugatory mechanisms are believed to operate via the same adenosine 3'-phosphate 5'-sulphatophosphate intermediate, involving the ATP sulphurylase and adenosine 5'-sulphatophosphate kinase (Dodgson & Rose, 1970), regulation of sulphate-activation presumably occurs only in the liver.

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References

- Andén, N. E., Carlsson, A., Kerstell, J., Magnusson, T., Olsson, R., Roos, B.-E., Steen, B., Steg, G., Svanborg, A., Thieme, G. & Werdinius, B. (1970) *Acta Med. Scand.* **187**, 247-255
- Armstrong, J. & Barlow, R. B. (1976) *Br. J. Pharmacol.* **57**, 501-516
- Axelrod, J. (1975) *Handb. Physiol. Sect. 7* **6**, 669-676
- Beyer, K. H. & Shapiro, S. H. (1945) *Am. J. Physiol.* **144**, 321-330
- Blaschko, H. (1953) *Br. Med. Bull.* **9**, 146-149
- Blaschko, H., Richter, D. & Schlossmann, H. (1937) *Biochem. J.* **31**, 2187-2196
- Bronaugh, R. L., McMurtry, R. J., Hoehn, M. M. & Rutledge, C. O. (1975) *Biochem. Pharmacol.* **24**, 1317-1320
- Brunngraber, E. G. (1958) *J. Biol. Chem.* **233**, 472-477
- Conolly, M. E., Davies, D. S., Dollery, C. T., Morgan, C. D., Paterson, J. W. & Sandler, M. (1972) *Br. J. Pharmacol.* **46**, 458-472

- Conway, W. D., Minatoya, H., Lands, A. M. & Shekosky, J. M. (1968) *J. Pharm. Sci.* **57**, 1135–1141
- Deichmann, W. B. (1943) *Proc. Soc. Exp. Biol. Med.* **54**, 335–336
- Dodgson, K. S. & Rose, F. A. (1970) in *Metabolic Conjugation and Metabolic Hydrolysis* (Fishman, W. H., ed.), vol. 1, pp. 239–325, Academic Press, New York
- Foldes, A. & Meek, J. L. (1973a) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, abstr. 3272
- Foldes, A. & Meek, J. L. (1973b) *Biochim. Biophys. Acta* **327**, 365–373
- Gibbs, H. D. (1927) *J. Biol. Chem.* **72**, 649–664
- Goodall, McC & Alton, H. (1965) *Biochem. Pharmacol.* **14**, 1595–1604
- Häggendal, J. (1963) *Acta Physiol. Scand.* **59**, 255–260
- Jenner, W. N. & Rose, F. A. (1973) *Biochem. J.* **135**, 109–114
- Jenner, W. N. & Rose, F. A. (1974) *Nature (London)* **252**, 237–238
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Lowry, O. H. & Passoneau, J. V. (1966) *J. Biol. Chem.* **241**, 2268–2279
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Merits, I. (1976) *Biochem. Pharmacol.* **25**, 829–833
- Morgan, C. D., Ruthven, C. R. J. & Sandler, M. (1969) *Clin. Chim. Acta* **26**, 381–386
- Richter, D. (1940) *J. Physiol. (London)* **98**, 361–374
- Richter, D. & MacIntosh, F. C. (1941) *Am. J. Physiol.* **135**, 1–5
- Rutledge, C. O. & Hoehn, M. M. (1973) *Nature (London)* **244**, 447–450
- Tipton, K. F. (1975) *Handb. Physiol. Sect. 7* **6**, 677–697
- Wong, K. P. (1974) *Anal. Biochem.* **62**, 149–156
- Wong, K. P. (1975) *J. Neurochem.* **24**, 1059–1063
- Wong, K. P. (1976) *Biochem. J.* **160**, 491–493
- Wong, K. P., Ruthven, C. R. J. & Sandler, M. (1973) *Clin. Chim. Acta* **47**, 215–222