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SULPHATE CONJUGATION OF ISOPRENALINE BY LUNG, SMALL INTESTINE AND OTHER ORGANS

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Abstract—Isoprenaline, a sympathomimetic drug used in the treatment of asthma, was found to be sulphated by the bronchial tissues of the monkey and dog. Enzyme preparations of the liver, small intestine and kidney of various animals are also able to catalyze this sulphate conjugation reaction from ATP and inorganic sulphate and from a commercial preparation of adenosine 3′-phosphate 5′-sulphatophosphate ($PAP^{35}S$) or $PAP^{35}S$ generated from $Na_2{}^{35}SO_4$ *in vitro*. The K_m values for isoprenaline for the sulphotransferase of mouse liver and monkey lung, are respectively, 51.3 μM and 138 μM. The significance of this detoxication reaction is discussed in relation to (a) the importance of lung as a potential biotransformation site of isoprenaline, (b) asthma deaths supposed to be associated with the use of isoprenaline in the form of pressurised aerosols and (c) the ability of the different tissues to synthesize PAPS *in vitro*.

Several systematic investigations on the absorption and metabolism of isoprenaline in man, dog and rat have been reported [1–8]. From these *in vivo* studies, it was suggested that this drug is inactivated essentially by *O*-methylation and/or conjugation with glucuronic or sulphuric acid, depending on its route of administration. Conjugation of isoprenaline presumably occurs to a great extent in the gut as reflected by the excretion of an isoprenaline conjugate, believed to be a sulphate, after its oral administration [4–6]. In this study, the conjugation of isoprenaline with sulphate was therefore examined in the small intestine and other organs, including the lungs, as isoprenaline in pressurised aerosols had been extensively used in the treatment of asthma. The introduction of this form of therapy was thought to contribute to the rise of asthma deaths in past years [9–12].

MATERIALS AND METHODS

Chemicals. The following radioactive chemicals were purchased from New England Nuclear, Boston, MA, U.S.A.: $Na_2{}^{35}SO_4$ with specific radioactivity of 894 mCi/mmole and radionuclide and radiochemical purity of 99% and $PAP^{35}S$ (adenosine 3′-phosphate 5′-sulphatophosphate, tetrasodium salt) with specific radioactivity of 0.38 Ci/mmole and concentration of 0.39 mg/4.3 ml. Chemicals obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. include L-isoprenaline bitartrate, ATP (sodium salt, 98% pure) and sulphatase (S9626, type H-1 prepared from *Helix pomatia*, containing 18,000 units/g. One unit of sulphatase will hydrolyse 1.0 μmole *p*-nitrocatechol sulphate/hr at pH 5.0 at 37°).

Enzyme preparations. Adult animals of both sexes were used. Freshly excised tissue was homogenized in a Polytron in cold 0.15 M KCl to give a 20% (w/v) homogenate. This homogenate was centrifuged at 178,000 *g* for 30 min to give the high-speed supernatant fraction, as described previously [13]. These enzyme preparations were stored in small aliquots at −80° in the Revco freezer. They tend to loose their activity on thawing and were used within one week after their preparation.

Assay conditions. (a) Sulphation of isoprenaline from ATP and Na_2SO_4. The incubation medium, which had a final pH of 8.8, contained the following chemicals in a final volume of 0.25 ml; the final concentrations are given in parentheses: Na_2SO_4 (0.38 mM)—this is a mixture containing 1 vol. of 5.59 mM $Na_2{}^{35}SO_4$ and 9 vol. 10 mM unlabelled Na_2SO_4; ATP (5.6 mM) adjusted to pH 7.2; $MgCl_2.6H_2O$ (8.4 mM) in dithiothreitol (3 mM); isoprenaline bitartrate (0.8 mM) and 0.5M glycine/NaOH buffer, pH 9.0. The reaction was started with 50 μl of the high-speed supernatant enzyme preparation as described above. Protein determinations were carried out by the Lowry procedure [14] with bovine serum albumin as standard. The protein concentrations, expressed in mg/50μl for the enzyme preparations from different animals ranged as follows: liver 0.47–0.9; kidney 0.35–0.52; small intestine 0.28–0.38 and lung 0.4–0.46.

After incubation in a metabolic shaker at 37° for 10 min, the reaction was terminated by the addition of 50 μl each of 5% (w/v) $ZnSO_4$ (equivalent to 0.18M $ZnSO_4$) and 0.3M $Ba(OH)_2$. This was followed by the removal of proteins and insoluble materials by centrifugation at 3500 rpm for 15 min.

(b) The sulphotransferase reaction using preformed PAPS. The 'active sulphate', PAPS, was first generated under identical conditions as described above except that isoprenaline was omitted from the reaction mixture and the incubation time was extended to 30 min. The reaction was stopped by boiling for 1 min and 0.2 ml of the supernatant, after centrifugation at 3500 rpm for 15 min, was used as a source of PAPS. In this sulphotransferase reaction, 0.1M EDTA at pH 9.0 was used to inhibit the sulphate-activating system present in the soluble enzyme preparation. This modification of the procedure of Brunngraber [15] had been found to be suitable [16].

(c) The sulphotransferase reaction using a commercial preparation of PAPS. Fifty μl of the stock solution of $PAP^{35}S$ was evaporated to dryness under a stream of N_2 gas. This step was necessary as ethanol present in the commercial preparation of $PAP^{35}S$ interfered with the reaction (unpublished observation). The $PAP^{35}S$ was redissolved in glycine/NaOH buffer, pH 9.0 and was used in place of the preformed $PAP^{35}S$ generated *in vitro* as described in (b). No quantitative measurements were attempted with this commercial preparation of $PAP^{35}S$ in this study as it was intended as a confirmatory test.

Paper chromatography and quantitation. The supernatant fraction of the reacted mixture (10μl of (a) and 20 μl of (b) and (c) above) was spotted on 1.3 × 57 cm strips of Whatman No. 1 paper and the chromatograms were developed in isopropan-1-ol/ammonia/water (8:1:1, by vol.) overnight (16 hr) at room temp (29–30°). Six sections of 2-cm each of the chromatograms, beginning 23 cm from the origin were counted in vials containing 10 ml of scintillator made up of 0.025% 1, 4-bis-(5-phenyl-oxazol-2-yl)benzene (POPOP) and 0.4% 2,5-diphenyloxazole (PPO) in toluene [16]. These six sections encompass the entire isoprenaline sulphate peak, with more than 90% of the total radioactivity in the two central sections. Acidic solvent systems, namely butanol/acetic acid/water (4:1:1 and 4:1:5, by vol.) were examined but found to be unsatisfactory because of extensive streaking of the isoprenaline sulphate peak.

Hydrolysis of isoprenaline sulphate by HCl *and sulphatase.* Isoprenaline sulphate formed in the reaction appeared as a peak with an R_f of 0.71 in the solvent system, isopropanol/ammonia/water (8:1:1, by vol.). The material present in this peak was isolated from several chromatograms by elution with water. One fraction of this eluate was subjected to hydrolysis by 54 units of sulphatase at pH 5, overnight at 37°. A second fraction, which served as control, was treated in the same manner without the addition of the hydrolase. A third fraction was hydrolysed by boiling with 2N HCl at pH 1 for 20 min. Both the hydrolysates and the control were chromatographed in the same basic solvent system, and 2-cm sections of the whole chromatograms were counted.

RESULTS

Preliminary studies showed that in addition to the peak of $Na_2{}^{35}SO_4$ present at the origin, another radioactive peak of R_f 0.71 was observed on the paper chromatograms. This latter peak was not found in controls using boiled enzyme or when ATP or Mg^{2+} was omitted from the incubation mixture. This peak was also observed in the sulphotransferase reaction using either commercial $PAP^{35}S$ or $PAP^{35}S$ generated *in vitro*. Hydrolysis of the material isolated from this peak with HCl or sulphatase released $^{35}SO_4$ which was identified by paper chromatography. This suggested that isoprenaline-$^{35}SO_4$ was the product of the reaction. Furthermore, analysis of this sulphate conjugate by the alumina procedure [17] showed that it was not absorbed, thus suggesting that one of the hydroxyl groups might be sulphated. As the enzyme preparation of mouse liver showed high activity, this was used in the preliminary experiments to work out the optimum conditions for the assay. It was found that the pH optimum for the overall sulphation was 8.8 and the optimum concentrations for ATP and Mg^{2+} were 5.6 mM and 5.6–8.4 mM, respectively. A linear response was obtained with time of incubation up to 15 min and enzyme solutions containing 0.16 to 0.8 mg protein/assay. Similar data were obtained using monkey lung preparation. The K_m for isoprenaline, measured by the sulphotransferase reaction was determined by the Lineweaver–Burk plot [18]. It was 51.3 μM and 138 μM, respectively for the

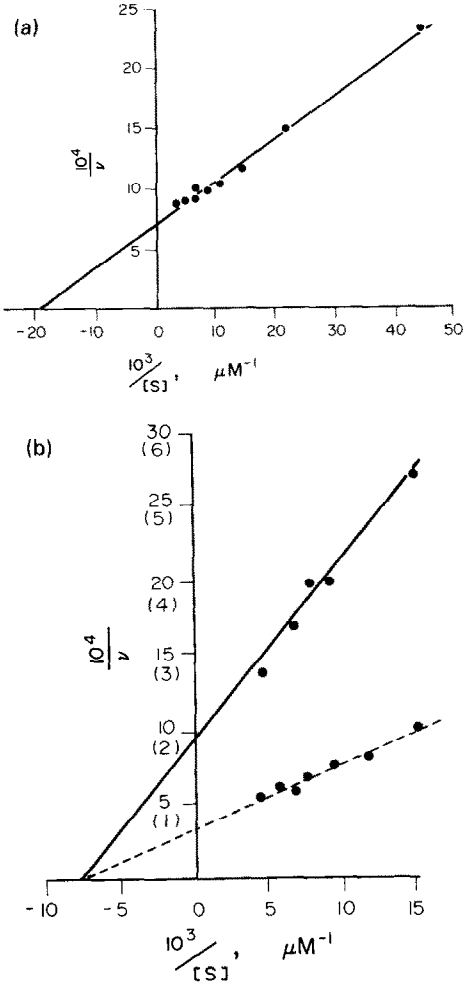

Fig. 1. (a) Lineweaver–Burk plot of velocity (v, expressed in cpm of isoprenaline-$^{35}SO_4$ formed/10 min; 25 μl of the reacted mixture was chromatographed) against the concentration of isoprenaline [S] in μM. $PAP^{35}S$ was generated *in vitro* using the high-speed supernatant fraction of mouse liver. The K_m for isoprenaline obtained from this plot was 51.3 μM for the sulphotransferase of mouse liver. (b) Lineweaver–Burk plot of velocity (v, expressed in cpm of isoprenaline-$^{35}SO_4$ formed/10 min; 20 μl of the reacted mixture was chromatographed) against the concentration of isoprenaline [S] in μM. $PAP^{35}S$ was generated *in vitro* using the high-speed supernatant fraction of rat liver (●----●) and rabbit small intestine (●——●) with figures in parentheses on the x-axis corresponding to the latter set of data. The K_m for isoprenaline from these plots was 138 μM for the sulphotransferase of monkey lung.

Table 1. Formation of isoprenaline sulphate by hepatic and extrahepatic tissues of various animals

Animals	Liver	Kidney	Small intestine	Lung
Mouse	665.7	1440	nil	nil
Monkey	78.9	146.4	116.4*	342.8 (air)
			160.1†	323.4 (N_2)
			113.1‡	
			160.1§	
Rat	283.3	nil	nil	nil
Rabbit	64.7	nil	nil	nil
Guinea pig	154.8	nil	357.7	18.5‖
Dog	404.5	447.7	92.7	225.2

Results are expressed as pmoles isoprenaline sulphate formed/min per mg protein in a 10-min assay, as measured by the three-step (sulphate-activating and sulphotransferase) reaction. Each value represents the average of triplicate experiments performed on the same enzyme preparation; the variation of these readings is less than 5%. The large intestine, heart and stomach of monkey were also examined but found to be devoid of activity.

* Data obtained from measurement of enzyme solution prepared from 60 cm of the intestine while †, ‡ and § were from three 20-cm sections of the intestine measured from the stomach, respectively.

‖ This represents the lower limit of measurement, corresponding to about 300 cpm of the isoprenaline-$^{35}SO_4$ peak on the paper chromatogram.

preparations of mouse liver and monkey lung (Fig. 1a and 1b).

A number of tissues were examined for their ability to form isoprenaline sulphate. The amount of sulphate conjugate synthesized was extrapolated from a standard curve of $Na_2{}^{35}SO_4$ chromatographed and similarly counted. The dilution of the radioactive sulphate by its cold cogener was taken into consideration in the calculations. The results obtained for the liver, kidney, small intestine and lung of various animals are shown in Table 1. The difference in the rates of sulphation of isoprenaline incubated in air and nitrogen was insignificant.

DISCUSSION

The importance of sulphation of isoprenaline by bronchial tissue lies in the fact that this is the target tissue of the drug. This conjugation reaction could represent a mode of its inactivation and the data in this study provided a comparison of hepatic and extrahepatic contributions to this detoxication pathway in several experimental animals. This is the first report which demonstrates the existence of a significant sulphate conjugation reaction in bronchial tissues. Previous studies showed either no or low activity of phenol sulphotransferase measured with *p*-nitrophenol as substrate [19, 20]. The data in Table 1 confirmed these results which were obtained with rabbit lung preparations. In contrast, the bronchial tissues of the monkey and dog possess all the enzymes responsible for the sulphate conjugation of isoprenaline from ATP and inorganic sulphate. Coupled to this, the low K_m for isoprenaline, measured by the sulphotransferase reaction, catalyzed by monkey lung suggested that this could indeed be an important metabolic pathway. The recent *in vivo* study of phenol conjugation by lung [21] attested to this importance. Of the bronchial tissues examined, only those of the monkey and dog exhibited this sulphating activity; low value was observed for guinea pig lung. In fact, when compared with the liver, small intestine and kidney of the monkey, the lung of this animal showed the highest activity. The sulphation of isoprenaline by lung, a hitherto unknown and unsuspected pathway, might offer an alternative explanation for the finding of Blackwell *et al.* [22]. They observed similar excretory pattern of isoprenaline and its sulphate conjugate after oral administration and administration of isoprenaline from pressurised aerosols and concluded that much of the drug given by the latter route was swallowed and thereafter conjugated by the gut. Sulphate conjugation of isoprenaline by the lungs could possibly yield similar results. It was estimated that 43 per cent of a dose of isoprenaline from an aerosol inhaler could be recovered from the mouth and pharynx [23], suggesting that more than 50 per cent would traverse the lungs. Apparently, each can of inhaler contained isoprenaline in sufficient quantity to cause death if this was absorbed systemically in a short time [9]. This seemed to be the case with phenol [21]. In the light of the present finding, the inactivation of isoprenaline by sulphation in the bronchial tissue would be crucial in the termination of the action of this drug. A deficiency of the sulphate-activating enzymes or the sulphotransferase or both might prolong its pharmacological action with detrimental effects. Whether this could be the cause of some of the asthma deaths from an overdose of isoprenaline remain unanswered. In this instance, Conolly *et al.* [8] believed that the gut was the major organ involved in its inactivation. There is no doubt that isoprenaline given orally would be sulphated by the gut. The entire length of the small intestine of the monkey, a primate closely related to man, was able to synthesize isoprenaline sulphate (Table 1).

The hepatic tissues of all the experimental animals

examined were able to form isoprenaline sulphate while the kidney and small intestine of some animals lack this ability. A strikingly similar trend had been observed in the sulphation of vanilylmandelic acid and homovanillic acid [24].

The mouse kidney was outstanding among the tissues studied in that it showed the highest isoprenaline sulphate conjugating activity (Table 1). This was also true with 4-hydroxy-3-methoxyphenylethanol as substrate [25]. One contributing factor to this predisposition could be the high activity of the sulphate-activating system of this organ [26]. The same explanation might be offered for the high rates of formation of isoprenaline sulphate by the small intestines of the guinea pig and monkey. The small intestine of the monkey had previously been demonstrated to readily form the sulphate conjugates of tyramine [27] and adrenaline [28]. On the contrary, the rabbit small intestine, though quite capable of generating PAPS *in vitro* (Ref. 26 and Fig. 1b) was unable to form isoprenaline sulphate. A deficiency of the sulphotransferase for isoprenaline in this tissue was therefore suggested. However, other factors, e.g. differences in the levels of sulphatase enzymes or presence of inhibitory factors have not been examined in this study, but are nevertheless important in a better understanding of this anomalous finding.

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