Assay of Adenosine 3'-Phosphate 5'-Sulphatophosphate in Hepatic Tissues

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A fluorimetric assay, based on the ability of boiled hepatic extracts to support the sulpho-conjugation of harmol, was used to demonstrate and quantify *PAdoPS* (adenosine 3'-phosphate 5'-sulphatophosphate) present in liver. A stoichiometric relationship was established between the sulphate conjugate formed and the 'active sulphate' utilized. Guinea-pig, rat, mouse and rabbit livers contain 3.3, 2.9, 0.8 and 0.5 μmol of *PAdoPS*/100 g wet wt. respectively.

Since the discovery of *PAdoPS* by Robbins & Lipmann (1956), this 'active sulphate' has never been demonstrated to exist as such in tissues, although it has been unequivocally established that it could be synthesized by hepatic tissues both *in vitro* (Robbins & Lipmann, 1957; Brunngraber, 1958; Pasternak, 1960; Jansen & van Kempen, 1973) and *in vivo* (Pasternak, 1960). Generation of *PAdoPS* is, however, not confined to animal tissues. Its biosynthesis from ATP and inorganic sulphate has been measured in extracts of yeast (Wilson & Bandurski, 1956; Robbins & Lipmann, 1958; Stanley *et al.*, 1975), plants (Mercer & Thomas, 1969; Burnell & Anderson, 1973; Burnell & Whatley, 1975; Stanley *et al.*, 1975), bacteria (Ibanez & Lindstrom, 1962; Burnell & Whatley, 1975) and algae (Hodson & Schiff, 1969). Its diversified roles in these various biological systems include detoxication, sulphate reduction and synthesis of sulphated mucopolysaccharides. This paper presents a very simple fluorimetric procedure to demonstrate and quantify *PAdoPS* present endogenously in liver.

Materials and Methods

Animals

Young adult animals of both sexes were used. The rats were of the albino Wistar strain, the monkeys belonged to the *Macaca fascicularis* species, the mice were of the mixed Swiss Webster strain and the other animals, namely the rabbit, dog and guinea pig, were local hybrids. The rats, mice and monkeys were purchased from the University Animal Centre. The rats and mice were fed on mouse pellet food, which contained 0.434% methionine, 0.32% cystine and 0.136% sulphur. The monkeys were fed on bananas and monkey pellets, which contained 0.387% meth-

ionine, 0.278% cystine and 0.058% sulphur. The above pellet foods were produced by the Gold Coin Co., Singapore. The guinea pigs and rabbits were bought from the local markets and these animals are normally fed on vegetables and sweet potatoes.

Extraction of *PAdoPS* from liver

Freshly excised liver, or freshly excised liver that was kept and transported in liquid air as in the case of the dog liver (1 g), was cut into small pieces and boiled in 2 ml of 0.5 M-glycine/NaOH buffer, pH 9.2, for 3 min. On cooling, it was homogenized in the Polytron for 1 min. The homogenate was centrifuged at 15000g for 30 min and the supernatant was made up to 2 ml with the same buffer. The mixture was shaken with 4 ml of chloroform to remove as much protein as possible. After centrifugation, the upper aqueous phase of this mixture was used as a source of *PAdoPS* in the subsequent sulpho-conjugation reaction, with harmol as substrate (Wong, 1974).

Sulphotransferase reaction with extracted *PAdoPS* as conjugating agent

Some 200 μl of the above boiled hepatic extract (corresponding to 0.1 g fresh wt. of liver) was incubated with 25 μl of 10 mM-harmol (Sigma) and 25 μl of 0.5 M-glycine/NaOH buffer, pH 9.2. The reaction was started with 100 μl of a mixture containing a 2:1 ratio of the high-speed (178000g) supernatant of dog liver and 0.1 M-EDTA; the latter had been adjusted to pH 9.2. A preincubation of this enzyme/EDTA mixture at 37°C for 3 min completely inhibited the sulphate-activating system (Wong, 1978; a modification of the method of Brunngraber, 1958). Incubation was carried out at 37°C for 30 min and the reaction was stopped with 100 μl each of 10% (w/v) ZnSO_4 and 0.3 M- $\text{Ba}(\text{OH})_2$. A further 0.5 ml of chloroform was added and the tubes were thoroughly shaken on a vortex-mixer. The proteins and insoluble

Abbreviation used: *PAdoPS*, adenosine 3'-phosphate 5'-sulphatophosphate.

materials were removed by centrifugation at 3500 rev./min for 15 min.

T.l.c. and fluorimetric analysis

The aqueous supernatant from above (15 or 30 μ l) was spotted on thin-layer plates coated with cellulose powder, MN300 (Macherey Nagel and Co., Düren, Germany). The plates were developed in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase) for 2.5 h. On drying, the plates were exposed to HCl fumes for 20 min to hydrolyse harmol sulphate. Complete hydrolysis of the conjugate by this treatment was confirmed by rechromatographing the spot (R_F 0.62) corresponding to harmol sulphate eluted from the plate. It subsequently appeared as harmol (R_F 0.77).

The fluorescent spots, which corresponded to harmol sulphate, were outlined under u.v. light and the material present therein was scraped from the plate and then extracted by shaking vigorously with 2 ml of 0.1 M-HCl on a vortex-mixer for 1 min. The tubes were normally left overnight in the dark. The fluorescence intensity of the supernatant was read on the Aminco-Bowman spectrophotofluorimeter with excitation at 320 nm and emission at 420 nm (Wong, 1974). No corrections were made for quenching. Calculations were based on a standard curve containing 20–200 pmol of harmol, which were first chromatographed in the same solvent system and subsequently eluted from the thin-layer plate with 2 ml of 0.1 M-HCl.

Sulphotransferase reaction with commercial PAdoPS as conjugating agent

PAdoPS, between 304 and 1520 pmol, corresponding to 0.87–4.35 μ M final concentration, was used instead of the boiled hepatic extract. This PAdoP³⁵S (tetrasodium salt) was purchased from New England Nuclear, Boston, MA, U.S.A.

Paper chromatography and radioisotopic analysis

The above incubated mixture (50 μ l) was spotted on Whatman no. 1 paper and the chromatograms were developed in butan-1-ol/acetic acid/water (4:1:5, by vol.) overnight (16 h) at room temperature (29–30°C). The radioactivity present in 2 cm sections of the chromatograms, beginning 1 cm before the origin, was counted by adding these strips directly to scintillation vials containing 10 ml of scintillator made up of 0.025% 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) and 0.4% 2,5-diphenyloxazole (PPO) in toluene.

Recovery experiment

Increasing amounts (3.04–15.2 nmol) of PAdoP³⁵S (specific radioactivity 0.38 Ci/mmol) were added to 2 ml of the buffer used in the extraction of the 'active sulphate' from liver. The entire procedure of boiling,

homogenization and centrifugation was performed as described above. As only one-tenth of the total volume was used in the subsequent sulphotransferase reaction, the amounts of harmol [³⁵S]sulphate formed were compared with those obtained by using only 0.3–1.52 nmol of PAdoP³⁵S added directly to the sulphotransferase reaction mixture. For this transferase reaction, the high-speed supernatant fraction of dog liver was most suitable, as the presence of PAdoPS was not demonstrated in this tissue. Besides, no degradation of harmol sulphate was found when it was incubated with this enzyme solution at 37°C for 30 min, and the preparation showed no loss of activity after 6 months in storage at –80°C. An alternative enzyme source lacking PAdoPS is monkey intestine, but the sulphotransferase activity with respect to harmol as substrate is only about half that of the dog liver preparation. These soluble enzyme preparations did not contain any glucuronyltransferase activity, as measured by the procedure of Wong (1969).

Stoichiometric experiment

A portion (10 μ l) of the stock solution of PAdoP³⁵S (specific radioactivity 0.56 Ci/mmol, concentration 0.28 mg/2.9 ml) was blown dry in a stream of N₂ gas and redissolved in 200 μ l of 0.5 M-glycine/NaOH buffer, pH 9.2. To this was added 25 μ l of 1 mM-harmol and 100 μ l of the dog liver supernatant/EDTA (2:1, v/v) mixture and incubated for 1 h at 37°C. The reaction was stopped by boiling, and, on cooling, 0.5 ml of chloroform was added and the tubes were shaken on the vortex-mixer and centrifuged at 3500 rev./min for 15 min. The supernatant (20 μ l) was spotted and developed by both thin-layer and paper chromatography for fluorimetric and radioisotopic analyses.

Results

Chromatography of the pure authentic PAdoP³⁵S in the solvent system used in this study showed that there was present in the commercial preparation a 'contaminant', which showed up as a radioactive peak with R_F 0.43; the R_F values for PAdoP³⁵S and harmol [³⁵S]sulphate were 0.05 and 0.63 respectively. This 'contaminant', which was not identified in this study, accounted for about 13% of the total counts, and it could be a degradation product formed during transport, storage and/or chromatography of PAdoP³⁵S. The observation that it represented 11–14% of the total radioactivity of the entire chromatogram in all the experiments suggested that it neither participated in the sulphotransferase reaction nor was derived from this enzymic reaction. No attempt was therefore made to remove it from the PAdoP³⁵S stock solution, since it did not seem to interfere with the reaction.

An allowance of 13% impurity of *PAdoP³⁵S* was made in all the calculations.

PAdoP³⁵S (0.87–4.35 μ M) formed harmol [³⁵S]sulphate in increasing amounts. No conjugate was produced in the control with boiled enzyme. A comparison of the formation of harmol [³⁵S]sulphate from *PAdoP³⁵S* subjected to the entire procedure of boiling, homogenization, extraction, centrifugation and sulpho-conjugation with the conjugates formed from *PAdoP³⁵S* added directly to the last (sulphotransferase) step demonstrated that the percentage recovery, when calculated in terms of the total radioactivity of *PAdoP³⁵S* and harmol [³⁵S]sulphate, was 91.7 ± 2.3 ($\pm$ S.E.M.) for five separate experiments. If the radioactivity of the contaminant peak was also included in the calculation, the percentage recovery was 92.6 ± 2.5 ($\pm$ S.E.M.). As the percentage conversion of *PAdoP³⁵S* to harmol [³⁵S]sulphate was very similar in both sets of experiments, namely 77.8 ± 0.73 ($\pm$ S.E.M.) for the entire procedure and 79.3 ± 1.35 ($\pm$ S.E.M.) for the sulphotransferase reaction, an average value of 78% was used in the calculations of all the results.

In the stoichiometric experiment, it was found that a 20 μ l portion chromatographed contained 49.5 ± 0.7 ($\pm$ S.E.M.) pmol of harmol [³⁵S]sulphate for six fluorimetric determinations. A 90% recovery of harmol sulphate by t.l.c. was also taken into consideration in the derivation of this value. The percentage recovery was calculated by comparing the intensities of fluorescence of 5–25 μ l (50–250 pmol) of harmol sulphate chromatographed and eluted from the t.l.c. plate with 0.1 M-HCl with those added directly to the acid; it was found to be 90 ± 1.5 ($\pm$ S.E.M.). The above value of 49.5 pmol of harmol [³⁵S]sulphate formed was compared with the amount of *PAdoP³⁵S* utilized, as determined by counting the radioactivity of the paper chromatograms; this was 52.4 pmol. It is noteworthy that this experiment again showed that 78.3 ± 0.56 ($\pm$ S.E.M.) of the added *PAdoP³⁵S* (1369 pmol) was converted into harmol [³⁵S]sulphate; a 13% impurity and a 13% deterioration, assuming 1% per month, were also taken into consideration in assessing the actual concentration of *PAdoP³⁵S* in the stock solution used in this experiment. The addition of EDTA to the enzyme solution to inhibit the sulphate-activating system ensured that the 0.8% inorganic sulphate, known to be present in this preparation of *PAdoP³⁵S*, did not interfere in the reaction. It can thus be concluded that there is a stoichiometric relationship between harmol [³⁵S]sulphate formed, measured fluorimetrically, and *PAdoP³⁵S* utilized, measured radioisotopically. As harmol possesses only one conjugable group, it seems reasonable to assume that the stoichiometric relationship between *PAdoP³⁵S* and harmol sulphate is 1:1 in this sulphotransferase reaction.

Boiled liver extracts of guinea pig, rat, rabbit and

mouse were able to form harmol sulphate in the presence of harmol and a sulphotransferase preparation of dog liver, suggesting that *PAdoPS* could be extracted from hepatic tissues. However, this was not observed with the liver extracts of monkey and dog nor with the boiled extracts of kidney, small and large intestines, heart and brain of the rat and guinea pig. The concentrations of *PAdoPS* present in the livers of guinea pig, rat, mouse and rabbit, expressed in μ mol/100 g, are respectively 3.3 ± 0.4 , 2.9 ± 0.2 , 0.78 ± 0.15 and 0.53 ± 0.05 . All values are means $\pm$ S.D. for four separate experiments, using different animals.

Discussion

PAdoPS is labile. However, it seems to withstand boiling at pH 9.2, as demonstrated in the study of sulpho-conjugation of tyramine (Wong, 1976) and adrenaline (Wong, 1978). On the basis of this observation, it was found that the 'active sulphate' could be extracted under alkaline conditions from liver, and quantified. Although *PAdoPS* is now commercially available (P-L Biochemicals, Milwaukee, WI, U.S.A.), its stability could not be assured, except by transport in solid CO₂, which imposes an additional cost. Even so, deterioration had been observed with the sample of *PAdoP³⁵S*. It is therefore not economical to use authentic *PAdoPS* for standardization in any assay. This problem is circumvented in this study by the establishment of a stoichiometric relationship between harmol sulphate formed and the *PAdoPS* utilized in the sulphotransferase reaction, thus allowing for an extrapolation of the concentration of *PAdoPS* from the measurement of the fluorescence of harmol sulphate. Nevertheless, authentic *PAdoPS* is required to determine the extent of conversion of the 'active sulphate' into harmol sulphate; this, of course, varies, depending on the activity of the sulphotransferase preparation. Curiously enough, a consistent value of 78% was obtained in all the experiments using the soluble fraction of dog liver. This possibly represents the maximum conversion, as it was obtained for both 30 and 60 min incubations. An important requirement for this fluorimetric procedure is a highly active sulphotransferase preparation, and so far, only the dog liver seems to be a satisfactory source.

The conjugation of harmol with glucuronic acid and H₂SO₄ have been extensively studied (Wong & Sourkes, 1967; Wong, 1974; Mulder & Hagedoorn, 1974; Mulder, 1975). A comparison of the hepatic concentrations of the conjugating agents that participate in these detoxication processes showed that UDP-glucuronic acid is found in higher concentrations (16–57 μ mol/100 g; Wong, 1977a) than *PAdoPS* (0.5–3.3 μ mol/100 g; this paper). Although there are hydrolases that act on both UDP-glucuronic acid (Wong & Lau, 1970) and *PAdoPS* (Dodgson &

Rose, 1970), numerous other active enzymes like 3'- and 5'-nucleotidases and deaminases also degrade *PAdoPS* (Dodgson & Rose, 1970). This factor, together with the chemical lability of *PAdoPS*, could account for its low concentration in the liver. Data obtained by Mulder & Scholtens (1978) suggested that the pool size of *PAdoPS* in liver is indeed very small. The rapid utilization of *PAdoPS* in various biosynthetic reactions, e.g. sulphation, may be responsible for the inability to demonstrate the presence of this 'active sulphate' in the hepatic tissues of some animals like the dog and monkey. This possibility seems to be supported by the observation of high rates of sulphation of tyramine and adrenaline in these tissues (Wong, 1976, 1978) while the high concentration of *PAdoPS* in guinea-pig liver was, in fact, associated with low sulphating ability (Wong, 1976, 1978). Pertinent to this discussion is the consistently high transglucuronidating activity exhibited by guinea-pig liver towards different substrates (Wong & Sourkes, 1967, 1968; Wong, 1969, 1977*b*), which may have a sparing action on *PAdoPS*. The endogenous concentration of *PAdoPS* measured in this study is therefore the result of the dynamic actions of a host of biosynthetic and degradative reactions, of which only sulphation has been discussed in this context.

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