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BIOSYNTHESIS OF PAPS *IN VITRO* BY HUMAN LIVER

MEASUREMENT BY TWO INDEPENDENT ASSAY PROCEDURES

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Abstract—The biosynthesis of 3′-phosphoadenosine-5′-phosphosulphate (PAPS) by extracts of human liver from inorganic sulphate and APS was assayed by transferring the "active sulphate" to two different acceptors, namely *N*-acetyldopamine (NADA) and 4-methylumbelliferone (4-MU). NADA-sulphate was quantified by an HPLC–ECD method while the decrease in 4-MU was monitored continuously by a fluorimetric procedure. The optimum pH was 8.0 for both the PAPS generation and APS-kinase reaction. The apparent K_m value for APS determined by the fluorimetric and HPLC–ECD procedures was 17.6 and 16.8 μM, respectively. PAPS-generation measured in 13 samples of human liver showed excellent correlation between the two assay procedures, with correlation coefficients (r) of 0.96 and 0.95 for PAPS generation from inorganic sulphate and APS, respectively. The fluorimetric assay was preferred as it is simple, equally sensitive and more reproducible. There was also a good correlation between the APS-kinase reaction and the two-step PAPS-generation from inorganic sulphate, with r = 0.97 and 0.91, as determined by the fluorimetric and HPLC–ECD procedures. The rate of PAPS generation from inorganic sulphate was only marginally less than that from APS in each of the 13 human liver samples, suggesting that the coupling of ATP-sulphurylase to APS-kinase facilitates "sulphate activation" and releases it from the constraints imposed by the unfavourable ATP sulphurylase reaction.

The formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS‡) from inorganic sulphate represents the first common pathway of sulphate conjugation, irrespective of the type of acceptor substrate or sulphotransferase involved. Its biosynthesis is fueled by two molecules of ATP which probably accounts for its generation only when required and explains its low intracellular concentration [1, 2]. Its formation is catalysed by ATP-sulfurylase (EC 2.7.7.4) and APS-kinase (EC 2.7.1.25) which are represented below:

$$ATP + SO_4^{2-} \rightleftharpoons APS + PP_i \quad (1)$$

$$APS + ATP \rightleftharpoons PAPS + ADP. \quad (2)$$

In this paper, "PAPS generation" represents the sum of both reactions (1) and (2) while APS-kinase refers to only reaction (2). The final product of these one-step and two-step reactions is PAPS or "active sulphate", which is the universal sulphate donor in all sulphate conjugation reactions. It is also the obligate co-substrate for the post-translational sulphation of proteins, which requires its transport into the Golgi apparatus [3, 4]. Recently, PAPS has also been implicated in post-translational phosphorylation [5] suggesting the endowment of an "active phosphate" at the other end of the PAPS molecule. In view of its "dynamic" nature and its participation in diverse metabolic processes, it is important to study its biosynthesis. Assay procedures for PAPS measurement were based on the transfer of its sulphate to different acceptors [1, 2, 6, 7], as its unstable nature made the direct quantification of PAPS [8] difficult. In this study, two independent but equally sensitive methods were developed to compare the PAPS-generating potential of extracts of human liver. The fluorimetric and HPLC–ECD procedures were modifications of the APS-kinase assay [9] and the assay developed for the measurement of overall sulphate conjugation of human liver [10] reported from this laboratory.

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‡ Abbreviations: APS, adenoside-5′-phosphosulphate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; 4-MU, 4-methylumbelliferone; PST, phenolsulphotransferase; NADA, *N*-acetyldopamine.

MATERIALS AND METHODS

Chemicals

Sodium ^{35}sulphate of specific radioactivity of 717.39, 574.58 and 541.33 mCi/mmol were purchased from New England Nuclear Corp. 3′-Phosphoadenosine-5′-phosphosulphate, lithium salt (PAPS) of 74% purity, adenosine-5′-phosphosulphate (APS), 4-methylumbelliferone (4-MU), *N*-acetyldopamine (NADA), dithiothreitol (DTT) and ATP were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Methanol of HPLC grade was from JT Baker. All other chemicals of analytical grade were from the usual commercial sources.

Preparation of enzyme extracts

The method of collection and storage of the human hepatic samples has been reported [10, 11]. To establish the optimum assay conditions, the 108,000 *g* cytosolic extract of a piece of frozen human liver was used. Similar cytosolic extracts of 17 other human samples were prepared, by homogenizing in

0.15 M KCl to give a 20% homogenate, followed by centrifugation at 108,000 *g* for 30 min. This same procedure was used to extract phenolsulphotransferase (PST) from livers of young Wistar rats and 2.5 mL of the cytosolic fraction was desalted by passing through a prepacked column containing Sephadex G-25 (PD-10, from Pharmacia, Uppsala, Sweden) according to the instructions of the supplier. The column was equilibrated and eluted with the same extracting solution (0.15 M KCl–3 mM DTT). After discarding the effluent and the first 2.5 mL, the next 1.5 mL was collected and stored in small aliquots. This desalted PST extract was found to be stable when stored at −80° for 3 weeks. Details for this step had been described previously [9].

Protein determination

The protein contents of the hepatic extracts were measured by the method of Lowry *et al.* [12] with bovine serum albumin as standard.

Assay conditions for PAPS biosynthesis from inorganic sulphate or APS

The incubating mixture, made up to a total volume of 300 μL with 50 mM phosphate buffer of pH 8.0, contained the following chemicals with their final concentrations in parentheses: (a) PAPS generation from inorganic sulphate: sodium sulphate (5 mM), ATP (5 mM), Mg^{2+} (1 mM) in DTT (3 mM); (b) APS kinase reaction: APS (50 μM), ATP (7 mM), Mg^{2+} (2 mM) in DTT (3 mM).

For each of the above assays, the reaction was started by adding 10–60 μL of the human liver supernatant, containing 66–219 μg protein. After incubating at 37° for 10 min, the reaction was terminated by boiling in a water-bath for 1 min. The tubes were centrifuged at 15,800 *g* in a microfuge for 1 min. Aliquots of the supernatant were used for the determination of PAPS by the following fluorimetric and HPLC–ECD procedures, which employed PST extracted from rat liver to transfer the sulphate from PAPS to 4-MU and NADA, respectively.

Continuous-monitoring fluorimetric assay. To 100 μL of the supernatant was added 0.18 μM 4-MU and 2 mM EDTA. The PST reaction was started with 100 μL of a mixture containing a desalted rat liver extract, 0.1 M EDTA and 0.1 M pyrophosphate in a ratio of 1:1:1 (by vol.); the protein content of this was about 170 μg. The final volume of incubate was made up to 2 mL with 50 mM phosphate buffer of pH 7.2. A standard curve was obtained under identical conditions, employing 0.3 to 2.2 nmol PAPS. A corresponding standard was carried out with prior boiling of PAPS for 1 min. This was to ascertain the stability of PAPS under our assay conditions. The continuous monitoring of the decrease in fluorescence of 4-MU for 6 min was followed on a recorder set at 2 mV. It has been established that this decrease in relative fluorescence units (RFU) reflects the formation of 4-MU-sulphate [9].

HPLC-ECD. In contrast to the above fluorimetric assay, the total final volume of the PST reaction with NADA (50 μM) as the acceptor substrate was only 100 μL; this included 50 μL of the supernatant from the first reaction step, i.e. from the two-step PAPS-generation or APS-kinase reaction. A longer incubation period of 30 min was necessary to produce sufficient NADA-sulphate for its measurement by HPLC–ECD. This reaction, however, had been shown to be linear up to 30 min and optimum chromatographic conditions had also been established previously [10].

The amount of PAPS formed by both assays was extrapolated from a standard curve corresponding to 0.3 to 2.2 nmol PAPS, carried out at the same time, under identical assay conditions. The 74% purity of the commercial preparation of PAPS has been accounted for in the derivation of the standard curves. Since ATP, among other nucleotides, inhibits the PST reaction [13], it was routinely added to the standards. The proportion of ATP and ADP would vary depending on the activities of ATP-sulphurylase and APS-kinase of each hepatic extract, but this cannot be predicted. Therefore, ATP equal to the concentration present initially in the first incubates was routinely introduced.

RESULTS

Both the PAPS generation and APS-kinase reaction showed an optimum pH of 8.0 when measured in human liver extracts by the fluorimetric or HPLC–ECD procedures. Two buffers, namely 50 mM phosphate (from pH 6.0 to 8.0) and 50 mM glycine–NaOH (from pH 8.4 to 9.6) were used. The amounts of PAPS generated from inorganic sulphate or APS were extrapolated from standard curves generated by the fluorimetric or HPLC–ECD procedures (Fig. 1a and b). It was also observed that under the conditions of assay, PAPS was relatively stable to boiling for 1 min (Fig. 1a). The reproducibility of both procedures was shown by the similar profiles observed in several independent sets of standards performed at different times, of which Fig. 1b is a representation of data obtained by the HPLC–ECD assay procedure. Similar observations were made with the fluorimetric assay procedure.

Kinetic data of PAPS-generation

As PAPS was generated from two enzymatic reactions, kinetic studies were carried out solely to establish the optimum concentrations of reactants for the two-step reactions. Suitable concentrations of the respective reactants in the assay incubate were described in Materials and Methods.

Kinetic data of APS-kinase reaction

Fluorimetric assay. Linearity was observed for the APS-kinase activity for up to 10 min of incubation, for enzyme protein less than 300 μg/assay incubate and for ATP concentration up to 10.5 mM. Analysis of data by the computer programme Enzpack [14] showed that the apparent K_m for APS was 17.6 μM (Fig. 2), similar to that obtained for APS-kinase of rat liver [9]. Two apparent K_m values for ATP were observed: they were 0.24 and 13.6 mM. The optimum concentration of Mg^{2+} ions was only 1 mM, and inhibition was observed above this concentration. There appeared to be no stoichiometry between the concentrations of ATP and Mg^{2+} ions.

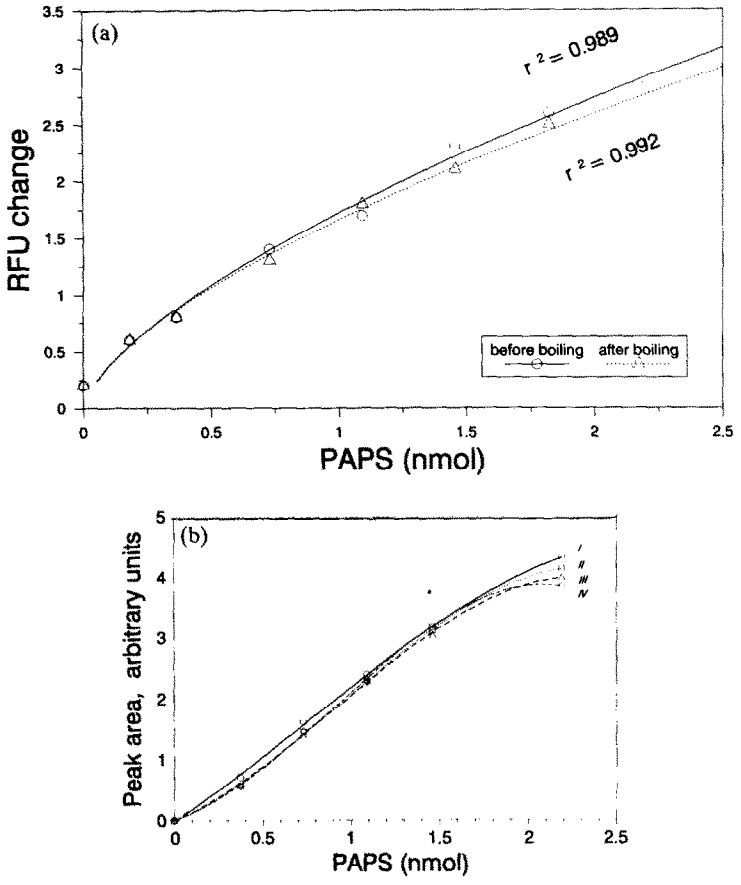

Fig. 1. Standard curves of PAPS obtained by (a) the continuous monitoring assay with 4-MU as acceptor. PAPS from 0–2.2 nmol was added before and after boiling for 1 min. The rate of sulphation of 4-MU was expressed as a change in relative fluorescence unit (RFU) in 6 min; (b) the HPLC–ECD procedure with NADA as acceptor. Four independent sets of data (I–IV) are presented to show the reproducibility of the assay procedure. The rate of sulphation of NADA was expressed in arbitrary units of peak area/30 min.

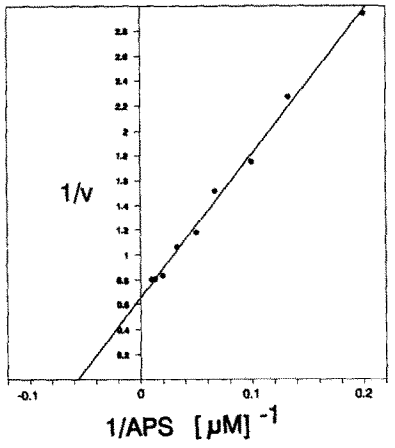

Fig. 2. Lineweaver–Burk plot of the APS-kinase activity of human liver where velocity, v, is expressed in nmol PAPS/10 min against 5–100 μM APS. Measurements were made by the fluorimetric procedure.

HPLC–ECD assay. An apparent K_m of 16.8 μM for APS and two apparent K_m values of 0.68 and 21.3 mM for ATP were obtained. The inhibitory effect of Mg^{2+} ions above 1 mM was again observed.

Specific activities of PAPS generation and APS-kinase in human liver extracts

Seventeen samples of human liver were used in this study of which four were below the limit of detection for both PAPS generation and APS-kinase activity. The specific activities of PAPS generation expressed in pmol PAPS/min/mg protein for the remaining 13 samples, measured by the fluorimetric and HPLC–ECD procedures (the latter set of values are given in parentheses) ranged from 165 to 1948, mean ± SD = 884 ± 502 (185 to 1897, mean ± SD = 923 ± 507). The corresponding values for APS-kinase, similarly expressed were 260 to 2743, mean ± SD = 1236 ± 672 (397 to 2770, mean ± SD = 1277 ± 748). A number of correlations, based on the orthogonal linear regression analysis were made between: (i) the two assay procedures for PAPS generation showing a correlation coefficient $r = 0.96$ (Fig. 3a) and for the APS-kinase activity with r =

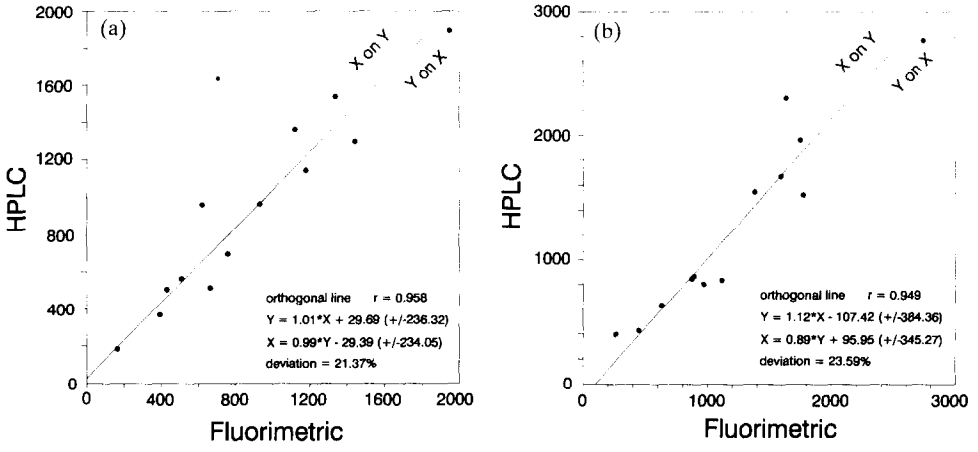

Fig. 3. Correlation between (a) PAPS generation from inorganic sulphate and (b) APS-kinase activity by the fluorimetric and HPLC–ECD procedures. Both activities were expressed in pmol/min/mg protein.

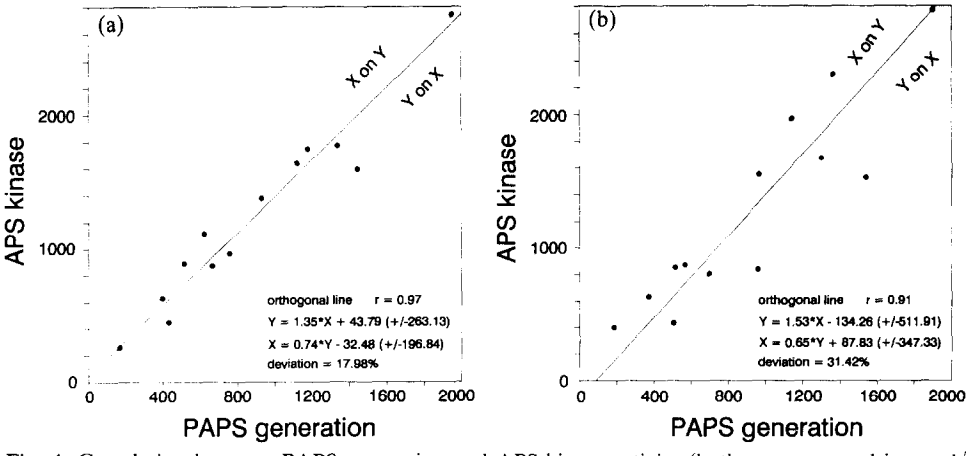

Fig. 4. Correlation between PAPS generation and APS-kinase activity (both are expressed in pmol/min/mg protein), by (a) the fluorimetric and (b) the HPLC–ECD procedures.

0.95 (Fig. 3b); (ii) APS-kinase activity and PAPS generation, showing a positive correlation of 0.97 and 0.91 by the fluorimetric and HPLC–ECD procedures, respectively (Fig. 4a and b); (iii) PAPS generation and the overall sulphate conjugation, the latter set of values had been determined in the same batch of human liver samples and reproduced from Ref 10. The correlation coefficients, $r = 0.97$ and 0.98 were obtained for values of PAPS-generation determined by the fluorimetric (Fig. 5a) and HPLC–ECD (Fig. 5b) procedures, respectively; and (iv) the overall sulphate conjugation and the PST activity, both measured by the HPLC–ECD procedure with NADA as substrate; the respective values were reproduced from Refs 10 and 15. The correlation coefficient, r was 0.31 (Fig. 6).

DISCUSSION

The earlier standard procedure for measuring PAPS generation by high-voltage paper electrophoresis following its separation from labeled inorganic sulphate [16] had been found to be impractical for routine analysis. The method based on the transfer of its sulphate, by PST, to an acceptor substrate such as harmol [7] with subsequent TLC-fluorimetric analysis of harmol sulphate had the requisite specificity and sensitivity and could conceivably be improved by the HPLC–fluorimetric assay developed recently in our laboratory for the measurement of harmol sulphate [17]. In this paper, PAPS formed by APS-kinase alone or in conjunction with ATP sulphurylase, was measured in a similar manner, but with 4-MU and NADA as acceptors. The assay for PAPS biosynthesized *in vitro*, in contrast to measurement of intracellular PAPS [1, 2] is complicated by the presence of ATP, inorganic sulphate or APS, often introduced in excess, in the first step. These unreacted precursors could be utilized by PST employed in the second step. The

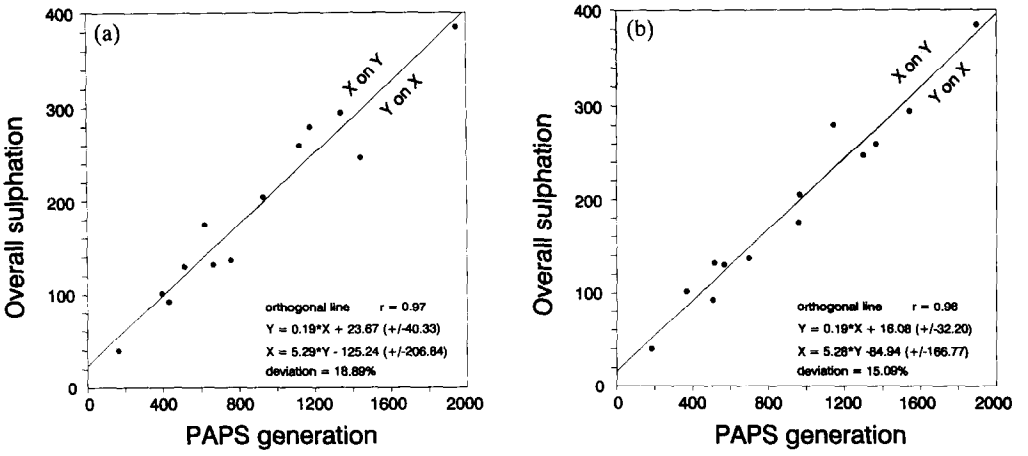

Fig. 5. Correlation between PAPS generation and the overall sulphate conjugation. The values for the overall sulphate conjugation of human liver were reproduced from Ref. 10. PAPS generation was determined by (a) the fluorimetric and (b) the HPLC–ECD procedures.

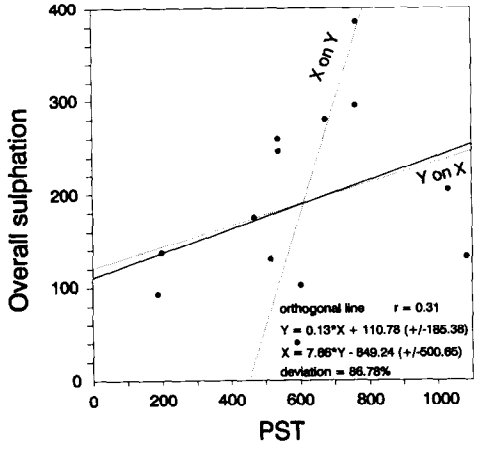

Fig. 6. Correlation between the overall sulphate conjugation and PST activity. Both measurements were carried out with NADA as acceptor substrate and by the HPLC–ECD procedures. The respective values were reproduced from Refs 10 and 15, respectively.

use of "desalted" extract of rat liver together with the addition of pyrophosphate and EDTA had resolved this problem. This approach had been detailed and discussed in our earlier publications [9, 10] and was followed in principle in this study.

Both the PAPS-generating system and the APS-kinase reaction of human liver extract showed an optimum pH of 8.0. These observations are consistent with the reported optimum pH values of ATP sulphurylase and APS-kinase measured in various other systems [18, 19]. Although it cannot be assumed that the enzymes which degrade PAPS and APS do not affect the rates of PAPS measurement as cytosolic fractions of human liver were used without any prior purification, it seems unlikely that the hydrolytic enzymes would influence the rates of PAPS generation because (i) the sulphate-activating and PAPS-degrading enzymes are present in different subcellular fractions [20–22] and (ii) degradation of PAPS and APS occurs optimally around pH 6 [20, 23–25], two pH units distant from the PAPS-generating system. Furthermore, the high concentration of ATP normally present in assay incubates for PAPS generation, particularly in phosphate buffer would inhibit the sulphohydrolases [23, 24].

The specific activities of PAPS-generation in human liver cytosolic fractions from inorganic sulphate or APS showed similar values when determined by the two independent assay procedures described in this paper, with correlation coefficients of 0.96 and 0.95 (Fig. 3a and b). The near perfect correlations reflect the reliability of the two assay procedures. The correlation of the overall sulphate conjugation with PAPS generation was high (Fig. 5a and b), an observation similar to that of the human platelets [26]. In contrast, there was little correlation between the overall sulphate conjugation and PST activity of the hepatic samples examined (Fig. 6).

The fluorimetric assay with 4-MU was preferred because of its simplicity; there was no extraction step and the continuous monitoring provided measurements of initial velocity. Many samples could be accommodated as the monitoring time was only 6 min. However, it did not determine the product formed, although the decrease in fluorescence of the substrate, 4-MU, had been shown to be directly related to the formation of 4-MU-sulphate [9]. On the other hand, the HPLC–ECD method measured specifically NADA-sulphate, but an intermediate step of adsorption was necessary to remove the unreacted substrate before HPLC-analysis. The operational difficulties inherent in ECD measurements made it less attractive as an assay for routine application. Both assays were equally sensitive; 0.3–2.2 nmol PAPS could be detected, a range which is lower than that reported using radioactive naphthol as acceptor [2].

The specific activities of PAPS-generation in human liver are of the same order of magnitude as values established previously in laboratory animals

[7, 27, 28]. The APS-kinase activities in the samples of human liver extracts were generally higher than those in rat and mouse liver [9]. A comparison of the two sets of values for PAPS generation from inorganic sulphate and APS showed that the latter were only marginally higher (1.4–1.5 times) than the corresponding values for PAPS generation from inorganic sulphate in each of the hepatic samples (Fig. 4a and b). It must be emphasized that the concentrations of inorganic sulphate and APS employed in the two assays differed by two orders of magnitude (being 5 mM and 50 μM, respectively). Higher concentrations of APS inhibit the APS-kinase reaction [9, 29–31], and therefore could not be used. The good correlation between APS-kinase activity and PAPS-generation (Fig. 4a and b) suggests that the formation of the "active sulphate" could be driven primarily by APS-kinase. Indeed the coupling of ATP-sulphurylase to APS-kinase seemed to neutralize the constraints imposed by ATP-sulphurylase, whose activity was reported to be seven orders of magnitude lower than that of APS-kinase [32]. It was therefore not surprising that two fractions, representing their respective activities were separated from yeast but only exhibited activity when combined [33]. These two enzymes in rat liver had defied separation [30] and appeared to exist as a single entity [29] as Nature had it that they act in unison and their functions be intimately coupled to achieve the common goal of sulphate activation.

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