Nucleotides Involved in the Enzymatic Conjugation of Phenols with Sulfate

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The demonstration by Bernstein and McGilvery (1) and DeMeio (2) that phenols are conjugated in fortified homogenates of liver led to the further discovery (1) that the enzymatic system is localized in the high speed supernatant fraction free of mitochondria and microsomes and that the reaction is ATP-dependent. Furthermore, Bernstein and McGilvery (3) demonstrated that the phenol is conjugated via an "active sulfate" intermediate. This was subsequently confirmed by Segal (4) and DeMeio et al. (5). The work of Wilson and Bandurski (6), Wilson et al. (7), and Robbins and Lipmann (8) established the over-all reaction sequence as

\[ \text{p-nitrophenol + m-aminophenol} \rightarrow \text{phenol sulfokinase} \rightarrow \text{PAP + m-aminophenyl sulfate} \]

A sensitive method for PAP assay is based on this exchange reaction. Its use permitted the demonstration that PAP will accumulate in the reaction medium along with the formation of m-aminophenyl sulfate if fluoride is present to inhibit a specific nucleotidase which attacks the 3'-phosphate of PAP. This nucleotidase differs from the rye grass 3'-nucleotidase of Wang et al. (10) since it will not attack 3'-adenylic acid.

MATERIALS AND METHODS

Chemicals—PAP and adenosine-2',5'-diphosphate were prepared from CoA and triphosphopyridine nucleotide by hydrolysis with a dinucleotide pyrophosphatase described by Wang et al. (10). ATP, ADP, A5P, and p-nitrophenyl sulfate were all products of the Sigma Company. A3P was obtained from Schwartz Laboratories. m-Aminophenol was a commercial preparation recrystallized twice from water and decolorized with Norit. The ATP used was a neutralized solution of the soluble sodium salt.

Rabbit Liver Supernatant Fraction—A rabbit was killed by a blow on the head, decapitated, and bled. Liver was excised, weighed, and homogenized for 3 minutes in a Waring Blender in a volume of isotonic potassium chloride 4 times the weight of the liver. The homogenate was filtered through glass wool and centrifuged in the Spinco model L ultracentrifuge for 1 hour at 44,000 \( \times g \). The high speed supernatant fluid thus obtained can be frozen and stored for future use.

Preparation of PAPS—The following procedure gave a final yield of 0.35 to 0.50 \( \mu \)mole of PAPS per ml. of the incubation medium described. Since the final step involving adsorption and elution from Norit results in some decomposition of PAPS to PAP and sulfate, the purity of the final product is of the order of 85 to 90 per cent. The yields and purity are better than in a previously described procedure (9).

The livers of fasted male rats were excised and homogenized by means of a Potter-Elvehjem homogenizer in a volume of isotonic potassium chloride 4 times their weight. The homogenate was centrifuged in the International centrifuge for 90 minutes at 20,000 \( \times g \), and the high speed supernatant fluid obtained was raised to 1.5 M ammonium sulfate by addition of

\[ 2 \text{ ATP} + \text{SO}_4^{2-} + \text{m-aminophenol} \rightarrow \text{PAP + ADP + PP + m-aminophenyl sulfate} \]

Robbins and Lipmann (9) isolated and characterized the active sulfate intermediate as PAPS. The sequence of reactions is unusual in that PAPS is not a coenzyme, for the reaction scheme does not provide for a recycling of PAP. The purpose of the investigation described in this paper is to define the role of PAP in sulfate metabolism. It was found that PAP will serve as a coenzyme in the transfer reaction

\[ \text{PAP + m-aminophenyl sulfate} \rightarrow \text{phenol sulfokinase} \rightarrow \text{PAP + m-aminophenyl sulfate} \]

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1 The abbreviations used are: PAP, adenosine-3',5'-diphosphate; PAPS, adenosine-3'-phospho,5'-phosphosulfate; APS, adenosine-5'-phosphosulfate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; A5P, 5'-adenylic acid; A3P, 3'-adenylic acid; PP, pyrophosphate; EDTA, ethylenedinitrilotetraacetic acid; CoA, coenzyme A.
The enzyme was added to an incubation medium so that the final concentrations were 0.015 M ATP, 0.004 M magnesium chloride, 0.02 M imidazole hydrochloride buffer, pH 7, and 4 to 6 mg. of protein per ml. The mixture was incubated for 2 hours at 38° and cooled to 3° in an ice bath. 1 ml. of 2 M perchloric acid per 6 ml. of incubation medium was then added, and the precipitated proteins were quickly removed by centrifugation. After increase in the pH of the supernatant fluid from the last step to 2 with the use of 2 M sodium hydroxide, 1 ml. of 20 per cent mercuric acetate in 0.1 M acetic acid per 11 ml. of original incubation medium was added to insure complete precipitation of the nucleotides. The copious white precipitate obtained was collected by centrifugation, washed three times with water to remove sulfate, and taken up in a volume of water 0.5 that of the original incubation medium. Hydrogen sulfide was introduced to decompose the precipitate, and the mercuric sulfide which formed was removed by centrifugation. The final clear nucleotide solution thus obtained was neutralized with 1 M sodium hydroxide. This solution was generally 1.2 to 1.4 X 10^{-3} M in PAPS, which represented 7 per cent of the total nucleotide in the solution. 77 to 80 per cent of the PAPS in the incubation medium (which is capable of accumulating 0.75 to 1 pmole PAPS per ml.) was recovered. The nucleotide solution is useful for routine use to assay phenol sulfokinase, provided that EDTA is present to block the activating enzymes.

The nucleotide solution was adsorbed on a Dowex 1-X10 chloride column, 200 to 400 mesh, which had previously been washed with 1 M HCl until the absorbancy at 260 m\(\mu\) was less than 0.005, and then the column was washed with water until neutral. A 2.5 X 15 cm. column will satisfactorily handle 1 mmole of adenine nucleotide as calculated from the absorbancy at 260 m\(\mu\). After adsorption of the nucleotides the column was washed with distilled water and elution begun with 0.5 M sodium chloride at a rate of 3 ml. per minute. The elution was continued until the absorbancy of the eluate was less than 0.01. The nucleotide solution was then completely eluted with 1 M sodium chloride. The concentration of material absorbing at 260 m\(\mu\) equals the concentration of sulfate-transferring activity as measured by the formation of m-aminophenyl sulfate. All of the PAPS placed on the column was recovered. The nucleotide was adsorbed on acid-washed Norit and eluted with a 40 per cent solution of alcohol containing 1 ml. of 1 M ammonium hydroxide per 100 ml. The alcohol was removed by evaporation under reduced pressure. An over-all yield of 50 per cent from the original incubation mixture was obtained.

**Analysis for m-Aminophenyl Sulfate**—The method for determining m-aminophenyl sulfate formed in incubation mixtures is a modification (1) of that reported by Levvy and Storey (12) and is based on a reaction (13) for the determination of sulfonamides.

**Assay for PAPs**—A final volume of 1 ml. that contained 0.039 M EDTA, pH 6.4, 0.001 M m-aminophenol, and 0.2 ml. of rabbit liver supernatant fluid, together with an aliquot containing PAPS, was incubated at 38° for 40 minutes. The reaction was terminated by the addition of perchloric acid and analyzed for the m-aminophenyl sulfate formed.

**Assay for PAP**—This assay is similar to the one reported by Gregory and Nose (14) and Gregory and Lipmann (15). A final volume of 1 ml. that contained 0.039 M EDTA, pH 6.4, 0.001 M m-aminophenol, and 0.2 ml. of rabbit liver supernatant fluid, together with an aliquot containing PAPS, was incubated at 38° for 40 minutes. The reaction was terminated by the addition of perchloric acid and analyzed for the m-aminophenyl sulfate formed.

**RESULTS**

**EDTA as Inhibitor for Sulfate Activation Reactions**—EDTA is a potent inhibitor of sulfate activation (Reactions 1 and 2) when supernatant fluid from lyophilized rat liver is used as the enzyme source. EDTA/Mg^{2+} in a concentration ratio of 2 in the incubation medium will inhibit the reaction 95 per cent. With ammonium sulfate fractions this ratio must be 12 in order to obtain the same amount of inhibition. By the utilization of EDTA it is possible to assay for either the activation or transferring systems in the presence of the enzymes of both systems. The enzyme preparation is incubated with all of the substrates except m-aminophenol, thereby allowing the accumulation of PAPS. EDTA and m-aminophenol are added simultaneously, and the reaction is incubated further to permit the enzymatic transfer of sulfate from PAPS to m-aminophenol. Kinetic curves obtained by variation of the time of incubation before the addition of EDTA and m-aminophenol with lyophilized supernatant fluid from rat liver as the enzyme source are illustrated in Fig. 1. The effect was first observed by McGilvery with fluoride as inhibitor. The time at which the peak of PAPS accumulation occurs appears to be a function of the enzyme concentration. PAPS is possibly being utilized to esterify some

**FIG. 1.** Accumulation of PAPS when lyophilized rat liver supernatant fluid is incubated with ATP, magnesium, and sulfate for various times before the addition of EDTA and m-aminophenol as measured by the amount of m-aminophenyl sulfate formed. 0.15 ml. of EDTA-phenol solution was added to terminate the sulfate activation reactions and to permit the transfer of sulfate from the accumulated PAPS to m-aminophenol to proceed. The final volume of 1 ml. contained 0.04 M imidazole hydrochloride buffer, pH 7.00, 0.005 M ATP, 2.5 X 10^{-3} M magnesium chloride, 3 X 10^{-5} M sodium sulfate, 1 X 10^{-3} M m-aminophenol, and 0.01 M EDTA. The following amounts of lyophilized rat liver supernatant fluid per ml. are represented by the indicated curves: A, 10 mg.; B, 20 mg.; C, 30 mg.; D, and 40 mg., B, 0.001 M m-aminophenol, 0.001 M p-nitrophenyl sulfate, and 0.1 ml. of rabbit liver supernatant fluid, together with the PAP to be determined, was incubated for 1 hour at 38° and analyzed for the m-aminophenyl sulfate formed. The incubation medium should contain no more than 0.8 mmole of PAP. Values of the m-aminophenyl sulfate formed are compared with a standard curve with use of known concentrations of PAP or PAPS. This assay does not distinguish between PAP and PAPS.

*Unpublished data.*
acceptor present in the enzyme preparation, or it is being destroyed upon prolonged incubation. With ammonium sulfate fractions the initial rate of PAPS formation was linear, with a subsequent leveling off. A decline in the amount of PAPS accumulated after a prolonged preincubation time was never observed. The ammonium sulfate fraction thus permitted the accumulation of PAPS in quantities suitable for preparative isolation.

The Exchange Reaction—PAP serves as a coenzyme in the exchange reaction by effecting a transfer of sulfate from p-nitrophenyl sulfate to m-aminophenol (Reaction 5) catalyzed by phenol sulfokinase. Fig. 2 represents the amount of exchange occurring when the concentration of PAP is in the range of 1 to $4 \times 10^{-4} \text{m}$. Concentrations beyond $2 \times 10^{-5} \text{m}$ will begin to inhibit this reaction. The nature of the inhibition is such that p-nitrophenol formation remains essentially constant, whereas m-aminophenol sulfate formation is depressed as the concentration of PAP is increased beyond $2 \times 10^{-5} \text{m}$. A3P, A5P, adenosine-2',5'-diphosphate, and ATP all failed to support the exchange reaction. ADP will catalyze some exchange due to the presence of PAP as an impurity. Samples of ADP used in these experiments contained 0.33 per cent of PAP.

Since such low concentrations of PAP serve to saturate the enzyme responsible for the exchange reaction, a sensitive method for detecting and estimating PAP was made available. The advantage of the method lies in the fact that incubation media suspected of containing PAP can be diluted many-fold, thereby diluting enzymes, salts, and other nucleotides in the sample beyond concentrations at which they may affect the reaction.

PAP as Inhibitor for Phenol Sulfokinase—Addition of an equivalent amount of PAP to a system containing PAPS inhibits the transfer of sulfate to m-aminophenol catalyzed by phenol sulfokinase to the extent of 50 per cent (Fig. 3). By virtue of its blocking action at the phenol sulfokinase level, PAP will inhibit the over-all reaction sequence involved in the formation of phenyl sulfate esters. The inhibitory effect is evident at concentrations as low as $2 \times 10^{-5} \text{m}$ PAP. Segal (16) and Bernstein and McGilvery (3) reported an inhibitory effect on phenol esterification by CoA and, furthermore, Segal (16) noted that dephosphorylated CoA was ineffective as inhibitor. The inhibition may be due to the PAP moiety of the CoA molecule or to the presence of PAP in the preparation. Whether PAP inhibits Reactions 1 and 2 is unknown.

Equivalence of Isolated PAPS and PAP Obtained by Hydrolysis of CoA—It was possible to demonstrate by means of the exchange reaction that PAPS obtained by the isolation procedure described is equivalent to PAP obtained by hydrolysis of CoA with the dinucleotide pyrophosphatase.

Whether PAP Accumulates in the System —The existence of the exchange reaction that effects the transfer of sulfate from p-nitrophenyl sulfate to m-aminophenol, in which PAP serves as a coenzyme, suggested the possibility that PAP may be involved in other exchange reactions. By such means PAP may serve as a true coenzyme, effecting transfer of sulfate from high energy donors (presumably APS) to acceptors. Berg's (17) postulation of the reactions

$$\text{acetate} + \text{ATP} \Rightarrow \text{adenylacetate} + \text{PP} \quad (6)$$

$$\text{adenylacetate} + \text{CoA} \Rightarrow \text{acetyl CoA} + \text{A5P} \quad (7)$$

provides an analogous reaction sequence in the acetate-activating system. The kinase function (Reaction 2) may serve as a means of providing PAP. If such a system were operative, PAP would not accumulate in systems which actively esterify phenols. In order to determine whether PAP is recycled, the appropriate substrates were incubated in the presence of the activating and transferring enzymes, and kinetic curves were obtained in which formation of m-aminophenol sulfate, PAP, and PAPS was determined.

![Graph](image-url)

Fig. 3. Kinetic curves demonstrating the effect of PAP on the transfer of sulfate from PAPS to m-aminophenol catalyzed by phenol sulfokinase, as measured by formation of p-nitrophenol (Curve A) and m-aminophenol sulfate (Curve B). 1 ml. of incubation medium, containing 0.039 M EDTA buffer, pH 6.4, 0.001 M m-aminophenol, 0.001 M p-nitrophenyl sulfate, and 0.1 ml. of rabbit liver supernatant fluid, was incubated for 1 hour at 38°.

![Graph](image-url)

Fig. 4A. The concentration of PAPS is increased beyond $2 \times 10^{-5} \text{m}$ and essentially remains at that
level. The presence of a nucletidase that destroys PAP was established by incubating PAP in the presence of the enzyme and Mg²⁺; 2 moles of phosphate were released per mole of PAP degraded. This nucletidase was Mg²⁺-dependent and was inhibited by a concentration of fluoride which permitted the sulfate activation steps to proceed, although at a 50 per cent reduction in rate. The kinetic curves obtained by incubation in the presence of 0.12 µl fluoride are represented in Fig. 4B. The amount of PAP accumulated parallels approximately the amount of phenol esterified. In view of the inhibitory effect of PAP on the over-all reaction, and the equivalent formation of m-aminophenyl sulfate and PAP, it appears that Reactions 1, 2, and 3 comprise the only pathway involved. The enzymes that catalyze the pathway of sulfate esterification thus outlined, together with the phosphatases and nucletidases known to be present in the enzyme preparation, would account for the 4 phosphate molecules released per mole of phenol esterified, as noted by Segal (16).

Nucletidase Activity—Table I illustrates some properties of the nucletidase that attacks PAP. The enzyme preparation, which contains an active 5'-nucletidase, fails to attack A₃P. It appears therefore that this is a unique 3'-nucletidase in that a charged group in the 5'-position is necessary for its activity. PAPS is also attacked, although at a slower rate. When 77 mmoles of PAPS were incubated in the presence of the enzyme and Mg²⁺, 41 mmoles of PAPS disappeared and 41 mmoles of phosphate were released. Under the same conditions, 117 mmoles of PAP released 252 mmoles of phosphate. This may account for the decline in PAPS accumulation noted in Figs. 1 and 44.

Anomalous Behavior Observed in Fluoride-Poisoned Systems—Bernstein and McGilvery (3) demonstrated the enzymatic activation of inorganic sulfate by ATP as a preliminary step for the conjugation of m-aminophenol by comparing the initial rate of the enzymatic system containing all the substrates and cofactors required for phenol conjugation with a control with a similar system which had first been preincubated with all the substrates with the exception of the phenol. The accumulation of an intermediate was manifested by an initial rapid conjugation when m-aminophenol was added to the preincubated system as compared to an initial lag in m-aminophenyl sulfate formation which characterized the control system. A curious result was obtained when the ATP concentration was lowered and fluoride included in the reaction medium. The addition of the phenol produced the expected rapid initial conjugation which in this case, however, slowed to an almost complete halt and then proceeded at about the same rate as the control. The existence of an inflection in the kinetic curves obtained in these experiments seemed to indicate a delay for building up the concentration of the active sulfate intermediate after the initial accumulation was depleted. It was believed that the intermediate contained a component which was added with the enzyme and, therefore, its concentration would limit the final level of intermediate accumulation. These conditions would be satisfied if the esterification of phenols depended upon the recycling of PAP. Since this explanation is no longer tenable, the inflection obtained in these curves constitutes an anomaly peculiar to the system.

These experiments were repeated in the course of this investigation in order to extend the analysis to the PAP and PAPS accumulation during the course of the formation of m-aminophenyl sulfate. The experiment was modified by including isolated PAPS in the original incubation medium instead of permitting an accumulation of PAPS by means of a preincubation in the absence of phenol. The inflection in the course of phenol esterification is evident in the lower curve in Fig. 5, and the effect is reflected in the curve that represents PAP accumulation. The concentration of PAPS was never limiting.

When this experiment was conducted by following the alternative procedure which utilized a preincubation in the absence

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**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Initial PAP</th>
<th>PO₄ released</th>
<th>PAP remaining</th>
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<tr>
<td>0.1 µl RF added</td>
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<tr>
<td>0.01 µl A₃P added</td>
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</tr>
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<td>210</td>
<td></td>
</tr>
<tr>
<td>Mg omitted and EDTA added</td>
<td>107</td>
<td>50</td>
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</table>

**Fig. 4.** The formation of m-aminophenyl sulfate, PAPS, and PAP when the appropriate substrates are incubated with an ammonium sulfate fraction of rat liver supernatant fluid in the absence of fluoride (A) and in the presence of fluoride (B). The incubation medium of 2.55 ml. (A) contained 0.047 m imidazole hydrochloride buffer, pH 7.00, 6 x 10⁻³ m magnesium sulfate, 6 x 10⁻³ m ATP, 1.2 x 10⁻³ m m-aminophenol, and 7 mg. of protein. The incubation medium of 2.55 ml. (B) contained 0.047 m imidazole hydrochloride buffer, pH 7.00, 3 x 10⁻³ m magnesium sulfate, 3.5 x 10⁻³ m ATP, 1.2 x 10⁻³ m m-aminophenol, 0.12 mg potassium fluoride, and 7 mg. of protein. The reaction was terminated in both cases by the addition of 0.4 ml. of 0.2 M EDTA, pH 7.00, and 1 ml. was immediately deproteinized with perchloric acid and subsequently analyzed for m-aminophenyl sulfate formed. To determine PAP and PAPS concentration, a 0.5 ml. aliquot was immediately assayed for PAPS and a 1 ml. aliquot was diluted to 100 ml. and assayed for PAP and PAPS. The difference in the two values represents the amount of PAP formed in the incubation mixture.
be excluded. The possibility of sulfate transfer to endogenous substrate cannot play a role in the metabolism of sulfate is suggested by the anomalous behavior observed under those conditions where contributing reactions may be inhibited by fluoride.

It was noted that when an enzymatic system containing phenol and all the cofactors essential for phenol esterification with sulfate is incubated, the kinetic curves obtained are characterized by an initial lag in the formation of m-aminophenyl sulfate (3, 4) (Fig. 4). Therefore, it was surprising that some preparations continued to exhibit this lag despite the fact that PAPS had been included in the original incubation medium. In contrast to the inflections in kinetic curves obtained in fluoride-poisoned systems, this effect was not reproducible. The effect was apparent only in some preparations of lower activity obtained from female rat livers.

Gregory and Lipmann (15) report a pH optimum between 7 and 8 for phenol sulfokinase from rabbit liver for the reaction effecting transfer of sulfate from p-nitrophenyl sulfate to phenol. This is in contrast to the pH optimum of 6 to 6.4 found in this investigation for the reaction which effects the transfer of sulfate from PAPS to m-aminophenol, utilizing EDTA and imidazole as buffers. The difference in findings can be explained on the basis of the differing substrates used, although the possibility of the existence of more than one enzyme responsible for activity towards phenols has not been excluded (15).

**DISCUSSION**

The observations reported in this paper indicate that PAP is a product of the enzymatic reactions that affect the biosynthesis of phenol sulfate esters, rather than a participant in the reaction. Its role as an inhibitor in the process and its accumulation as the reaction proceeds support such a view. The function of PAP as a coenzyme in the transfer reaction and its activity at extremely low concentrations, however, suggest that it may play a role in physiological processes.

The fact that the same enzyme preparation that contains enzymes leading to the synthesis of PAPS also contains a nucleotidase capable of degrading PAPS suggests a means of biological control whereby the level of PAPS available for sulfate esterification may be regulated. In this connection, it may be significant that the rat liver supernatant fluid is incapable of accumulating more than 60 μmoles of PAPS and actually exhibits a decline in the accumulation of PAPS if the incubation is prolonged.

The presence of other undisclosed enzymes or cofactors which play a role in the metabolism of sulfate is suggested by the anomalous behavior observed under those conditions where contributing reactions may be inhibited by fluoride.

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**SUMMARY**

1. A method for isolating 85 to 90 per cent pure adenosine-3'-phosphate, 5'-phosphosulfate (PAPS) in substrate amounts from reaction mixtures is described.
2. A sensitive method for estimating adenosine-3', 5'-diphosphate (PAP) was provided by an exchange reaction catalyzed by phenol sulfokinase whereby sulfate is transferred from p-nitrophenyl sulfate to m-aminophenol, requiring PAP as a coenzyme. The enzyme is saturated when the concentration of PAP is 2 X 10⁻⁵ M. Concentrations beyond 2 X 10⁻⁵ M are inhibitory.
3. Evidence is presented for the existence of a new nucleotidase which attacks PAP by hydrolysis of the 3'-phosphate linkage. PAPS is attacked at a slower rate. The enzyme does not attack 3'-adenylic acid.
4. PAP serves as an inhibitor in the over-all sequence leading to the esterification of phenols by virtue of its inhibitory effect on phenol sulfokinase. A concentration as low as 2 X 10⁻⁵ M will exert an inhibitive effect.
5. It was found that PAP accumulates in a system accomplishing phenol esterification with sulfate when the nucleotidase attacking PAP is inhibited by fluoride. The amount of PAP which accumulates is approximately equivalent to the amount of m-aminophenol esterified.
6. Some anomalies of the system accomplishing sulfate esterification of phenols under certain conditions suggest the presence of other undisclosed enzymes or cofactors involved in phenyl sulfate biosynthesis.

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**REFERENCES**

Nucleotides Involved in the Enzymatic Conjugation of Phenols with Sulfate
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