On the "active sulfate" intermediate*

The activation of sulfate as a preliminary step in the synthesis of phenyl sulfate was demonstrated by BERNSTEIN AND MCGILVERY[1] on the basis of kinetic studies. We[2] have confirmed their findings and present here some evidence for the nature of the intermediate.

Contrary to our previous conclusions[2] we find that the "active sulfate" intermediate is dialyzable as shown by the following experiment.

The activating enzyme preparation (obtained as indicated elsewhere[2]) was placed in a cellophane dialysis bag in an amount corresponding to 5 mg of protein. The bag contained a total volume of 3 ml of fluid and was immersed in 3 ml of outside fluid. The fluid containing the enzyme, and also the outside fluid, had the following composition: 15 μM Na_2H_2ATP, $4H_2O$**, ***; 45 μM K_2SO_4; 15 μM $MgCl_2$ and 150 μM potassium phosphate buffer pH 7. After one hour incubation at 37.5° C, with continual stirring of the fluid in the bag, the outside fluid was tested for the presence of intermediate by adding an amount of transferring enzyme (obtained as described elsewhere[2]) corresponding to 10 mg of protein, 60 μM of potassium phosphate buffer pH 7, 0.72 μM *p*-NP in a final volume of 3 ml and incubating for one hour at 37.5° C. The amount of intermediate formed corresponded to 14.8 μg of bound *p*-NP. If ATP, Mg^{++} or $SO_4^{=}$ was omitted during the dialysis-incubation period no formation of intermediate was detected. These results constitute definitive evidence that the "active sulfate" intermediate is dialyzable. Preparations submitted to prolonged dialysis after incubation of the active $(NH_4)_2SO_4$ fraction with ATP, Mg^{++} and $SO_4^{=}$, invariably showed an increased rate of formation of *p*-nitrophenyl sulfate when compared to the same fraction not submitted to preliminary incubation in the absence of substrate. The testing was carried out by incubating for one hour at 37.5° C, after addition of ATP, Mg^{++}, $SO_4^{=}$ and *p*-NP. These apparently contradictory results may mean simply that even prolonged dialysis does not eliminate all of the accumulated intermediate or that dialysis eliminates an inhibiting factor.

Preparation of the "active sulfate" intermediate

The fraction from rat liver "microsome-free" solution that precipitated between 1.7 and 2.3 M ammonium sulfate was dialyzed against 0.1 M potassium phosphate buffer pH 7.4 for 7½ hours and immediately after dialysis was brought to a concentration of 0.03 M with K_2SO_4 (this protected the enzyme from inactivation). In addition to this enzyme preparation, added in an amount corresponding to 36 mg of protein, the medium contained: 30 μM ATP; 90 μM $K_2{}^{35}SO_4$§ (specific activity 0.09 μc per μM); 30 μM $MgCl_2$ and 528 μM potassium phosphate buffer pH 7 in a total volume of 6 ml. After one hour incubation at 37.5° C, 0.12 ml of neutralized saturated ammonium sulfate and neutralized absolute alcohol to a final concentration of 75% were added. The protein precipitate was centrifuged off and discarded and the supernatant concentrated at 30° C by distillation under reduced pressure. The residue was dissolved in triple distilled water and brought to a final volume of 3 ml. Suspended material was eliminated by centrifugation and the fluid tested for the presence of intermediate by the technique indicated above. The amount of intermediate produced per vessel under these conditions corresponded to 100–150 μg of bound *p*-NP.

Paper electrophoresis

The fluid containing the intermediate and obtained as indicated above was streaked in amounts varying from 25 to 50 λ across 5 Whatman No. 1 paper strips 3.8 cm wide or in 1 ml quantity across Whatman No. 3 paper sheets 19 cm wide. The paper was soaked with 0.167 M ammonium acetate pH 4 and blotted in the usual manner, before applying the sample. The electrophoresis was carried out for 16½ hours at 100 volts (length of paper 50 cm) and a density of current of 2 mA for Whatman No. 1 paper or 4.5 mA for Whatman No. 3 paper.

At the end of electrophoresis the paper was dried and scanned for ultraviolet absorption with the "Mineralight"§§ and for radioactivity with a Tracerlab end-window counter attached to a Nuclear Scaler Model 163.

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** The following abbreviations will be used in this paper: AMP adenosinemonophosphate, ADP adenosinediphosphate, ATP adenosinetriphosphate, *p*-NP *p*-nitrophenol, *p*-NPS *p*-nitrophenyl sulfate.

*** Obtained from the Pabst Laboratories, Milwaukee, Wisconsin and neutralized with NaOH.

§ Supplied by the Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A., on allocation from the U.S. Atomic Energy Commission.

§§ In two cases the ultraviolet absorption was tested in the Beckman Model DU spectrophotometer using the adapter for direct photometry of paper chromatograms described by TENNENT *et al.*[3]

We consistently observed that the radioactive zone (indicated by readings of 4 to 28 times background) coincided with an ultraviolet absorbing zone and that the material eluted from this portion of the paper, with distilled water, showed the presence of intermediate when tested with the transferring enzyme as indicated above. The radioactivity could not be due to inorganic sulfate because ^{35}S-labelled inorganic sulfate ran off the paper under the same conditions of electrophoresis. That the ultraviolet absorption was probably due to adenine was indicated by an absorption maximum at 2600 A observed for the eluted material.

Material eluted from a radioactive zone was subjected to a second electrophoresis and the solution of the eluted intermediate tested. We obtained with this material a strong positive test for ribose (Bial-orcinol test as modified by LING[4]) while adjoining sections of the strip were negative. A preliminary quantitative determination of ribose (using the Bial-orcinol test with the modified technique of LING[4]), calculated as ribose-5-phosphate, showed that it was in a ratio of 1:0.93 to AMP (32.5 μg ribose-5-phosphate to 45 μg of AMP). AMP was determined with the Beckman DU spectrophotometer at 2600 A.

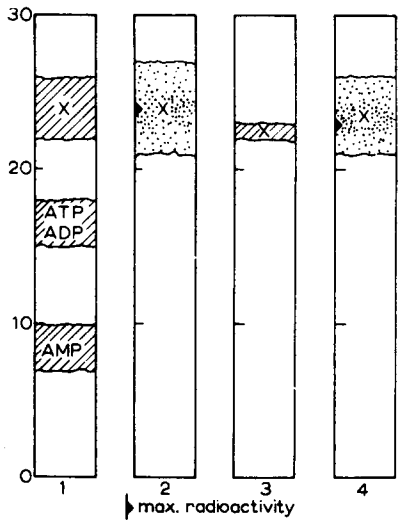

Fig. 1. X "active sulfate" intermediate. No. 1 UV absorption (shaded areas) and No. 2 radioactivity (dotted area) on the same strip. No. 3 UV absorption and No. 4 radioactivity of eluted intermediate submitted to a second electrophoresis.

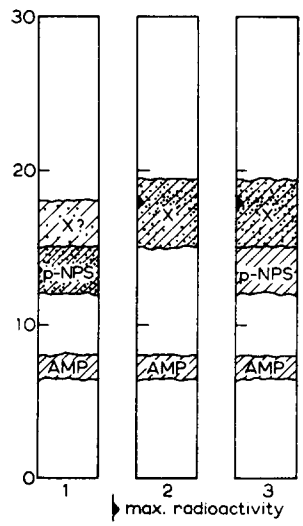

Fig. 2. No. 1. Extract containing "active sulfate" incubated with *p*-NP and transferring enzyme. No. 2 same as No. 1 but not incubated. No. 3 same as No. 1 but 0.4 μM of *p*-NPS added. X "active sulfate" intermediate. X'? unknown sulfuric acid ester. Shaded areas: UV absorption; dotted and shaded areas: UV absorption and radioactivity.

As indicated in Fig. 1, the "active sulfate" (X) intermediate had a faster rate of migration than AMP, ADP and ATP, and could be easily separated from them. Nos. 3 and 4 of a strip obtained by resubmitting to electrophoresis material eluted from the radioactive zone of a paper strip resulting from a first electrophoresis show that the UV absorption zone occupies the same position as the zone of maximum radioactivity.

The effect of incubation of the intermediate with transferring enzyme and *p*-nitrophenol (*p*-NP) is shown in Fig. 2. To strip No. 1 was applied a concentrated extract of the product of incubation, obtained as indicated above. It may be seen that the maximum of radioactivity shifts from the position occupied by the intermediate (as indicated by strips No. 2 and 3 obtained with non-incubated material) to the position corresponding to *p*-NPS (as indicated from the UV absorption zone produced by addition of *p*-NPS to the extract of the non-incubated mixture of intermediate and transferring enzyme). The strong radioactivity of strip No. 1 was accompanied by UV absorption due to the *p*-NPS. This same strip shows a weak radioactive and UV absorbing spot (X'?) probably representing some other unknown sulfate ester. The presence of *p*-NPS was additionally demonstrated by elution of the zone with distilled water, followed by hydrolysis and detection of *p*-NP.

The three strips show the presence of UV absorption in the position corresponding to AMP.

The material eluted from these zones was analyzed for AMP with the Beckman DU spectrophotometer. The difference between the value obtained for strip 1 (25.3 μg) and an average of the values obtained for strips 2 and 3 (21.2 and 21.4 μg) indicates liberation of AMP in the process of formation of *p*-NPS. The presence of AMP in strips 2 and 3 is probably due to decomposition of the "active sulfate" intermediate.

Eluted radioactive "active sulfate" intermediate was tested for phosphorus. A large amount of inorganic phosphorus was found, indicating that under the conditions employed the rate of migration of phosphate was similar to the rate of migration of the intermediate. As the difference between total and inorganic phosphorus was not significant no conclusion can be reached concerning the presence of phosphorus in the compound.

On the basis of our findings, viz. (a) coincidence of ultraviolet absorption and radioactivity (due to incorporated radioactive sulfate) in the zone of paper giving on elution "active sulfate" intermediate, (b) the presence of ribose in a ratio of 1:0.93 to AMP, (c) liberation of AMP in the process of formation of *p*-NPS, in conjunction with the fact that pyrophosphate is liberated in the process of formation of the "active sulfate" as shown by HILZ[5], the following tentative conclusion seems justifiable. The "active sulfate" is formed from ATP by splitting off pyrophosphate and binding of sulfate to AMP, probably through the phosphoric acid, forming a mixed anhydride. The "active sulfate" reacts with *p*-NP in the presence of transferring enzyme, transferring the sulfate with formation of *p*-NPS and liberation of AMP.

The activation step (formation of "active sulfate" intermediate) is probably the common first step in the biosynthesis of all sulfuric acid esters. Transfer of the sulfate from the "active sulfate" to the substrate (phenols, steroid phenols and alcohols, amino sugar containing substrates, and others) is probably mediated by specific transferring enzymes. An indication of this possibility is presented by HILZ AND LIPMANN[6] with *Neurospora sitophila* and by our findings[7] with the mold *Fusarium solani*, where formation of *p*-NPS was obtained only when the liver transferring enzyme was added to a cell-free preparation of the mold.

From the foregoing observations we may tentatively conclude that the "active sulfate" intermediate is adenylsulfate.

Our gratitude is expressed to Miss PHYLLIS SMITH and Dr. C. T. LING for the ribose determinations.

Note: A paper by HILZ AND LIPMANN[6] appeared while this work was in progress. Our findings are in agreement with theirs on many points.

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Effet du jeune protéique prolongé sur la répartition des ribonucléotides nucléiques dans diverses fractions cytoplasmiques du foie de rat

Au cours de recherches antérieures[1], nous avons constaté une réduction de la quantité absolue de l'acide ribonucléique (ARN) du foie de 30 à 50% à la suite d'un jeûne protéique prolongé (4 à 6 semaines). D'autre part, il s'est avéré que l'activité spécifique du P de l'ARN restant à la suite d'une dénutrition azotée se trouve accrue[2]. Nous nous sommes alors demandés s'il existe une différence de constitution entre l'ensemble de l'ARN du foie normal et celui qui reste à la fin du jeûne. Pour répondre à cette question, nous avons étudié la répartition des nucléotides dans diverses structures du cytoplasme du foie chez des animaux normaux d'une part, chez des animaux ayant perdu 30 à 45% de leur poids à la suite du jeûne protéique d'autre part.

Les animaux ont été sacrifiés après 18 heures de jeûne total, ce qui entraîne la disparition du glycogène hépatique. Le fractionnement a été effectué dans le saccharose 0.25 *M* selon DE DUVE[3]. Après sédimentation des noyaux, nous avons séparé les mitochondries lourdes à 30,000 *g* min*,

* La notation en *g* min a été proposée par DE DUVE ET BERTHET[4].