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PURIFICATION AND PROPERTIES OF THE ENZYME ATP-SULFURYLASE AND ITS RELATION TO VITAMIN A*

A. S. LEVI** AND G. WOLF

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. (U.S.A.)

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SUMMARY

1. The enzyme ATP-sulfurylase which catalyzes the formation of adenosine 5'-phosphosulfate (APS) was purified about 1000-fold over supernatant fraction by ammonium sulfate and acid precipitation and by elution from Sephadex G-200, agarose and hydroxyapatite columns.

2. The molecular weight of the enzyme was between 800 000 and 900 000; its optimum pH, at 8.0; its K_m with respect to ATP, $1.6 \cdot 10^{-3}$ M; K_m (sulfate), $1.0 \cdot 10^{-4}$ M; K_m (APS), $2.5 \cdot 10^{-4}$ M; K_m (PP_i), $3.7 \cdot 10^{-5}$ M. There was inhibition at high PP_i and APS concentrations and at low concentrations of ADP. Cu^{2+} and Co^{2+} were strongly inhibitory. *p*-Chloromercuribenzoate (PCMB) and EDTA also inhibited: the former inhibition was completely relieved by glutathione; the latter, by Mg^{2+}. The enzyme was irreversibly inactivated by deoxycholate. It was unstable in Tris–HCl buffer and decayed to about 15% activity in 24 h whether measured by ATP generation or molybdolysis. It was completely stabilized by phosphate but not by barbiturate or sulfate ions.

3. The mechanism of the reaction was explored by a study of the reaction kinetics and the following was observed: (a) the rate of exchange of $^{32}PP_i$ into ATP was greater in presence than in absence of sulfate and greater (even in absence of SO_4^{2-}) than the overall reaction of APS synthesis; (b) there was no exchange of $^{35}SO_4^{2-}$ into APS in presence or absence of PP_i; and (c) the rate of reaction of APS with PP_i at low APS concentrations was independent and at high concentrations was dependent on PP_i concentration. We conclude that the reaction proceeds through intermediate formation of an AMP–enzyme complex.

4. ^{14}C-Labeled vitamin A was administered to vitamin A-deficient rats, and ATP-sulfurylase was isolated after 3 days. Although radioactivity remained associated with the enzyme over some of the purification steps, highly active enzyme which had lost all radioactivity was obtained by fractionation on agarose columns. Similar results were obtained when the labeled vitamin was homogenized with rat embryo livers or

Abbreviations: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PCMB, *p*-chloromercuribenzoate.

* This is contribution No. 1249 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

** Present address: Department of Chemistry, Revelle College, University of California, San Diego, La Jolla, Calif., U.S.A.

injected into newborn rats. We conclude that neither vitamin A nor a metabolite of the vitamin is an integral or necessary part of the enzyme and suggest that since the enzyme is unstable, particularly under conditions of low phosphate concentration, vitamin A may help to stabilize the enzyme.

INTRODUCTION

In a previous report[1], we demonstrated that when dietary protein intake is maintained at normal levels, the activity of the enzyme ATP-sulfurylase (ATP–sulfate adenylyltransferase EC 2.7.7.4) is lower in livers of vitamin A-deficient rats than in those of pair-fed normal control rats. Other investigators[2,3] report that a metabolite of vitamin A or the vitamin itself can reactivate the enzyme of deficient animals to normal levels. We purified the enzyme in order to determine whether any radioactivity from previously fed radioactive vitamin A might be associated with the enzyme. During this work, we studied the properties of the enzyme also. A preliminary report of the purification procedure has been published[4]. A preliminary note of another purification procedure[5] has recently appeared.

METHODS

Animals and diet

Normal, Sprague–Dawley, albino male rats (120–125 g) (obtained from Sprague–Dawley, Inc., Madison, Wisc., or from Carworth Inc., N.J.) were housed in individual cages in a constant temperature room at 22° with a lighting period of 12 h per day. Animals ate laboratory chow until they were used. They were discarded if they reached a body weight above 200 g. Pregnant Sprague–Dawley rats, used in the embryo experiments, were from the Charles River Breeding Laboratories, Wilmington, Mass. The diet was that described by WOLF, LANE AND JOHNSON[6] and prepared by General Biochemicals Inc., Chagrin Falls, Ohio.

Materials

The materials used were: inorganic pyrophosphatase (Nutritional Biochemicals Corp., Cleveland, Ohio); luciferin–luciferase enzyme system was obtained from a firefly extract (Sigma Chemicals Co., St. Louis, Mo.); ammonium sulfate, recrystallized from EDTA solution, and *p*-chloromercuribenzoate (PCMB) (Sigma Chemicals Co., St. Louis, Mo.); ATP disodium salt, used as a standard for the luciferin–luciferase assay and for the molybdolysis assay[7] (D-L Biochemicals, Milwaukee, Wisc.); [^{35}S]sulfate as potassium salt and ^{32}P-labeled PP_i (New England Nuclear Corp., Boston, Mass.); gel for gel filtration columns and desalting and DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden); DEAE-cellulose and CM-cellulose (Distillation Products, Rochester, N.Y.); and beaded agarose Bio-Rad A-15m and hydroxyapatite (Bio-Rad Laboratories, New York, N.Y.). The authors obtained the [6,7-$^{14}C_2$]retinol, [6,7-$^{14}C_2$]retinyl acetate, and [15-^{14}C]retinoic acid, dissolved in toluene, as a generous gift from Hoffman-LaRoche Co., Switzerland. Synthetic adenosine 5′-phosphosulfate (APS) was prepared according to the method devised by BADDILEY, BUCHANAN AND LETTERS[8].

Preparation of [6,7-$^{14}C_2$]retinyl acetate in Tween-80 solution

After the toluene was evaporated, we dissolved the vitamin in a mixture of 10% ethanol, 16.2% Tween-80 and 73.8% water as described by WOLF, KAHN AND JOHNSON[9].

Assay for enzymatic activity of the enzyme ATP-sulfurylase

(a) Enzymatic activity of the 100 000 × *g* supernatant solution was determined by measurement of the enzymatic synthesis of labeled APS and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) from ATP and $^{35}SO_4^{2-}$. The reaction was made to proceed in the direction of APS synthesis by the inclusion of pyrophosphatase, which hydrolyzed the PP_i formed and thereby prevented reversal of the reaction. Incubation mixtures contained: 10.0 μmoles ATP; 250.0 μmoles SO_4^{2-}; 5.0 μmoles $MgCl_2$; 200.0 μmoles Tris–HCl (pH 7.6); 0.10 enzyme unit inorganic pyrophosphatase; about 1 μC $^{35}SO_4^{2-}$, in trace amount; and enzyme (protein added depended on the purity of the enzyme).

The final volume of the incubation mixture was adjusted to 1 ml. Incubation was carried out for 20 min at 37°; the reaction was then stopped by immersion in boiling water for 90 sec. Portions were taken for paper electrophoresis (see below) to separate the radioactive APS and PAPS from the radioactive sulfate.

(b) Enzyme activity for all fractions of enzyme except that of the 100 000 × *g* supernatant solution was determined by the generation of ATP from PP_i and synthetic APS. For this assay the incubation mixture contained: 0.1 μmole APS; 0.2 μmole PP_i; 1 μmole $MgCl_2$; 10 μmoles Tris buffer; and enzyme (protein added depended on the purity of the enzyme).

The incubation volume was brought to 1 ml with glass-distilled water. Reaction mixtures were incubated at 37° for 20 min and the reaction was stopped by immersing for 90 sec in boiling water, then cooled and diluted with water to 10 ml. Portions of diluted incubation mixture were taken for ATP determination. Incubations were always made in duplicates.

Determination of ATP was carried out by the firefly (luciferin-luciferase) assay and liquid scintillation counting (model 720, Nuclear Chicago scintillation counter) as adapted from the methods of DIKSTEIN AND SULMAN[10] and ADDANKI SAMASUNDARAM SATOS AND REARICK[11] with the following modification: counting intervals were 0.1 min starting at 15 sec from the addition of the luciferin-luciferase mixture. A standard curve of ATP was made with each new batch of firefly enzyme received in order to correct for the variation of activity of different batches.

The validity of the assay for ATP-sulfurylase activity was checked: a trace amount of radioactive PP_i was included in the incubation mixture described above. The labeled ATP from the reaction of APS with the labeled PP_i was isolated by electrophoresis and counted, and thus gave the μmoles of ATP formed.

Isolation of radioactive ATP and radioactive APS and PAPS

Isolation of the labeled nucleotides was carried out by paper electrophoresis on 1.5 inch × 15 inch strips of Whatman paper No. 1 in ammonium acetate buffer (pH 6.0) (5.52 g ammonium acetate and 0.45 ml acetic acid in 1 l of water), at 400 V and 20 mA.

Strips were removed after 1½ h and dried, and the location of labeled nucleotides, [^{32}P]ATP or [^{35}S]APS, and [^{35}S]PAPS, was determined by the use of a paperstrip radioactivity scanner (Vanguard model 800). Areas corresponding to radioactivity

peaks were cut out and counted by the liquid scintillation counting system. Under these conditions, PAPS was found to migrate together with APS.

Source of enzyme

Livers of Sprague–Dawley rats were removed, washed in sucrose–KCl solution, weighed, and minced. They were homogenized by a motor-driven, loosely fitting, Potter–Elvehjem homogenizer with 3 vol. (w/v) of sucrose–KCl solution (0.25 M sucrose, 0.025 M KCl).

Homogenization and all further operations were carried out in the cold. Homogenates were centrifuged at 1000 × *g* for 10 min to remove the nuclei and cell debris. The supernatant solution was centrifuged further at 100 000 × *g* for 60 min in a Spinco model L centrifuge. Precipitates were discarded, and the supernatant solution was collected for further work. Mercaptoethanol was added to a concentration of 25 μl/100 ml.

Protein determination

Protein determinations on both 100 000 × *g* supernatant solution and ammonium sulfate precipitated fractions were carried out according to the method of SUTHERLAND *et al.*[12]. Protein was determined on all more purified fractions by the method of Warburg and Christian as modified by LAYNE[13].

Addition of ammonium sulfate

Ammonium sulfate was added as solid salt according to the table of saturation given by GREEN AND HUGHES[14] while the solution was at 0°. Ammonium sulfate crystals were ground, and the proper amount was added to the protein solution slowly with continuous mechanical mixing to prevent local excess of the electrolyte. The suspension was left for about 30 min to equilibrate after the complete addition of ammonium sulfate to bring the solution to the desired saturation.

Addition of organic solvent

Cold acetone and butanol (−20°) were added separately to the 100 000 × *g* supernatant solution at −5° with a gradual decrease in temperature to −20°. Organic solvents were added slowly with continuous mechanical stirring to prevent excess local concentrations.

Preparation of acid precipitate

Cold 0.5 M acetic acid was added slowly to the enzyme solution kept in an ice bath with continuous stirring. The pH was not permitted to drop below 5.5.

RESULTS AND DISCUSSION

Purification

Much of the interest in the sulfate-activating enzymes arose from reports of their dependence on vitamin A for activity[2,15,16] and in particular, the recent finding by SUNDARESAN[3] of a radioactive butanol extract (from rats dosed with labeled vitamin A) which could reactivate lowered ATP-sulfurylase from deficient animals to the normal level. Therefore, we decided to purify the enzyme and test its relation to the

vitamin. ROBBINS AND LIPMANN[17] report a procedure for purification of the enzyme from yeast. Attempts to follow their procedure in the isolation of the enzyme from rat liver were unsuccessful. Therefore, a new procedure was developed. Before beginning purification, a satisfactory assay had to be found.

Previous investigations have determined the enzymatic activity of ATP-sulfurylase by the molybdolysis assay originated by WILSON AND BANDURSKI[7] in which the molybdate anion substitutes for sulfate.

There have been several objections to the molybdolysis assay: (a) the molybdate ion is activated by an enzyme different from the sulfation enzyme (evidence against this objection will be presented later); (b) the presence of the molybdate ion in the reaction mixture liberates phosphate groups from the enzyme protein and therefore interferes with the accuracy of the assay; and (c) the molybdate ion inhibits ATPase and thus produces an erroneously high blank value (*i.e.*, in the absence of the molybdate ion).

To avoid these objections, enzyme activity was assayed in the present work by measurement of the generation of ATP resulting from the reaction of synthetic APS with PP_i (see METHODS, Assay b). The reaction is known to be thermodynamically favored in this direction[17]. Determination of enzyme activity of the 100 000 $\times$ *g* supernatant fraction with this assay was difficult due to the presence of high levels of ATPase and other ATP-utilizing enzymes. Enzyme activity for this fraction was therefore determined by measurement of the enzymatic synthesis of labeled [^{35}S]APS and [^{35}S]PAPS from $^{35}SO_4^{2-}$ and ATP (see METHODS, Assay a).

The purification steps were the following (Table I). The 100 000 $\times$ *g* supernatant solution from rat liver was prepared as described. This solution was then fractionated by ammonium sulfate (see METHODS). Maximum activity was found in the fraction between 50 and 65% saturation, although to get better yields of the enzyme, the 40–65% fraction was used in routine purification.

The ammonium sulfate precipitate was then dissolved in phosphate buffer (ionic strength, 0.1; pH 7.6) and desalted by passage through a short column (1 cm $\times$ 5 cm) of Sephadex G-200. This eluate could not be further purified by chromatography on DEAE-Sephadex, CM-cellulose or CM-Sephadex columns. Therefore, the next step was addition of 0.5 M acetic acid to acidify the eluate to pH 6.5 (see METHODS). A precipitate formed which contained most of the enzyme activity. The precipitate was redissolved in phosphate buffer and centrifuged to remove insoluble material. The specific activity of the fraction, 12–16 μmoles ATP formed/h per mg protein, represented an approximate increase of 40-fold over the previous step.

The enzyme, dissolved in phosphate buffer, was next applied to a Sephadex G-200 column and eluted with the same phosphate buffer (Fig. 1). The enzyme appeared in the outer volume of the gel. A hemoglobin marker (mol. wt. 70 000) was retained on the column. At this stage, a pigmented protein (λ_{max} 406 nm) was associated with the enzyme but disappeared during the last stage of purification. This step consisted in batch-wise treatment with hydroxyapatite gel.

30 ml of the enzymatically active fraction from the column (Fractions 7–11, Fig. 1; total protein, 300 mg; specific enzymatic activity, 90 nmoles ATP/mg protein per h) were treated with hydroxyapatite suspension with stepwise slow addition and continuous mixing. The mixture was allowed to equilibrate in the ice bath for about 15 min after each addition. Enzymatic activity was assayed repeatedly after each

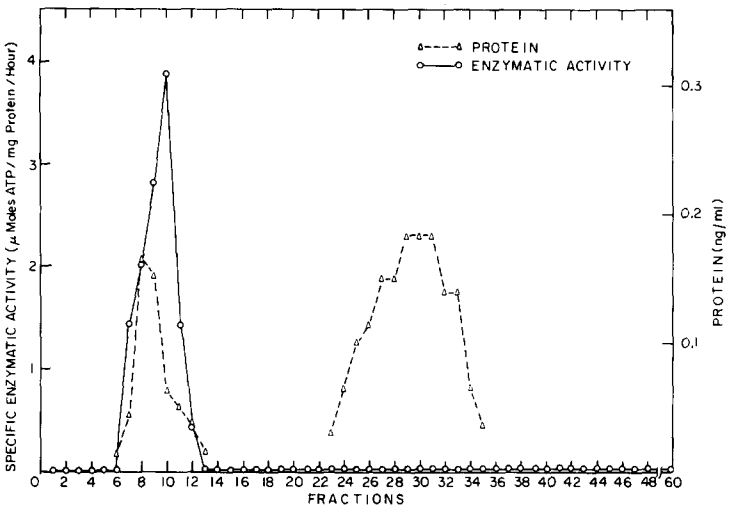

Fig. 1. Fractionation on Sephadex G-200 of ATP-sulfurylase purified by ammonium sulfate and acid precipitation. The 2 cm × 45 cm column was packed with the gel after equilibration with phosphate buffer (pH 7.4), ionic strength 0.02. Assay b (see METHODS) was used.

equilibration and centrifugation to remove the hydroxyapatite. Results of such an experiment are shown in Table II. The amount of hydroxyapatite needed varied with the degree of enzyme purification and with the amount of protein. Consequently, a pilot experiment was made with small portions to determine the amount of hydroxyapatite required.

As already mentioned, enzymatic activity of the whole homogenate or 100 000 × *g* supernatant fraction could not be measured by the generation of ATP from APS and and PP_i (Assay b) because of high ATPase activity and the presence of several other ATP-utilizing enzymes. Activity was measured here by the generation of radioactive [^{35}S]APS and [^{35}S]PAPS using ^{35}S-labeled sulfate in the presence of excess ATP and pyrosphosphatase (Assay a). Conditions for the assays are described in the METHODS section.

The ammonium sulfate fraction was used to measure both the generation of APS (Assay a) and the generation of ATP (Assay b). With the same preparation, a specific enzymatic activity of 3.8 nmoles APS/mg protein per h in Assay a* had a specific activity of 540 nmoles ATP/mg protein per h in Assay b. From the specific activities so obtained, a conversion factor from Assay a to Assay b could be determined (540/3.8). This conversion factor makes possible a calculation of the specific activities of the enzyme in homogenate and 100 000 × *g* fraction in terms of ATP generation (Assay b), by multiplying the specific activity obtained with Assay a by the conversion factor (540/3.8).

Table I shows that, using the calculated specific enzyme activity for the supernatant fraction**, the purification procedure resulted in over 1000-fold purification

* The ammonium sulfate-precipitated enzyme contained no kinase, so that APS free of PAPS was formed in this reaction. This was shown by incubation with exogenous sulfatetransferase and *p*-nitrophenol[1]. No *p*-nitrophenylsulfate was formed.

** The value for this specific activity is of the same order of magnitude as that observed by

TABLE I

PURIFICATION OF THE ENZYME ATP-SULFURYLASE

Assay b or Assay a (see METHODS) was used.

Fraction	*Specific enzymatic activity*		
	Assay b (μmoles ATP/h per mg)	*Assay a (nmoles APS/h per mg)*	*Total protein (mg)*
Homogenate	0.0013*	0.0125	—
100 000 × *g* supernatant	0.042*	0.3	7.450
40–60% $(NH_4)_2SO_4$** precipitate	0.42	3.0	564.3
pH 6 precipitate	16.20	—	33.3
Sephadex G-200	25.90	–	7.7
Hydroxyapatite	45.00	—	3.8***

* Calculated value.

** Was dissolved in phosphate buffer (pH 7.6), ionic strength, 0.1; all further steps were carried out in the same buffer.

*** With net recovery of 14% of original activity present in the 100 000 × *g* supernatant.

from the supernatant fraction and in over 10 000-fold purification from homogenates.

PANIKKAR AND BACHHAWAT[5] report a final specific activity for their pure enzyme of 96 μmoles PP_i consumed/h per mg protein, compared to our 45 μmoles ATP formed/h per mg protein. They report this, however, to represent a 200-fold purification, whereas in our case a 1000-fold purification was attained. The discrepancy may be due to a

TABLE II

BATCH-WISE ADSORPTION AND ELUTION OF ATP-SULFURYLASE ACTIVITY BY HYDROXYAPATITE

Assay b (see METHODS) was used.

Protein (mg/ml)	*Total protein*	*Remaining enzyme activity in supernatant (nmoles ATP/mg protein per h)*	*Remarks*
10	300	90	Activity in original solution (Sephadex G-200 eluate)
	77.3	420	Activity in supernatant after addition of hydroxyapatite suspension* (equivalent of 4.195 g dry weight). Supernatant I
		0	Activity after addition of hydroxyapatite suspension to Supernatant I (equivalent of 1.966 g dry hydroxyapatite). Precipitate II
		0	Activity in supernatant after elution of precipitate II with phosphate buffer (pH 7.4; ionic strength, 0.1)
3.75	37.5	660**	Activity in supernatant after elution of precipitate II with phosphate buffer (pH 7.4; ionic strength, 0.5)

* Hydroxyapatite suspended in 0.001 M phosphate buffer, (pH 7.4).

** With net recovery of 91% of original enzymatic activity and 7-fold purification.

LEVI *et al.*[1] (0.022 μmole sulfate activated/h per mg protein) and by MUHKERJI AND BACHHAWAT[18] (0.015 μmole PAPS formed/h per mg protein) with widely differing assays.

much higher concentration (about 10 times as high, by calculation) of the enzyme in sheep compared to rat liver.

Properties

Molecular weight. From the elution curve of the enzyme, in an experiment to be described later (see Fig. 14), it was possible to calculate a molecular weight of the enzyme of between 800 000 and 900 000 from standard elution curves (Bio-Rad Laboratories, Pamphlet BG-5, 1967) (Agarose A-15m column of total bed volume, 241.5 ml, void bed volume, 91.8 ml, and elution volume of the most active fraction, 159.5 ml). It was not unexpected, therefore, that the enzyme was eluted in the void volume of a Sephadex G-200 column.

pH dependence. SUBBA RAO, SESHADRI SASTRY AND GANGULY[19] report that the highest activity of the overall reaction of sulfate activation and sulfate transfer as measured in the 100 000 × *g* supernatant fraction was obtained at pH 7.6–8.0. In later work[2], these authors reported that the optimum pH for the activation step is 6.5 and for the transfer reaction is 7.1. The overall reaction of sulfate activation steps (synthesis of PAPS) in mast-cell tumors is reported by RICE *et al.*[20] to show an optimum at pH 7.7. To test the effect of pH on the activity of ATP-sulfurylase (first step of activation), the optimum pH was determined for a partially purified enzyme by the use of universal buffer to ensure the elimination of specific effects of certain buffer components present only in one buffer at certain pH values. Although the enzyme is

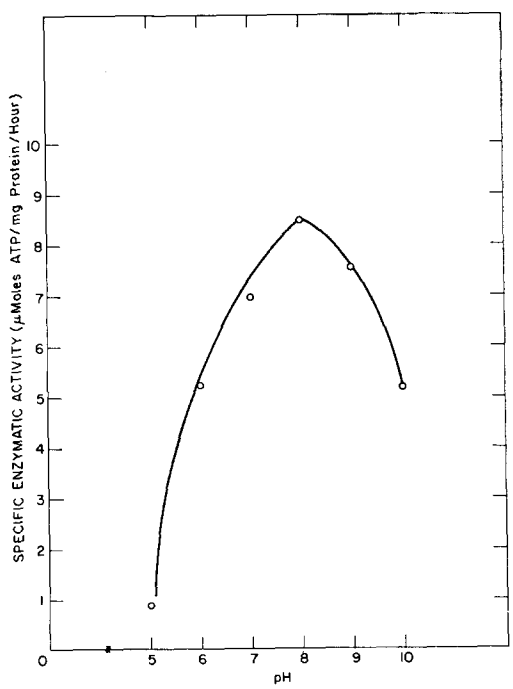

Fig. 2. Effect of change in pH on the activity of the enzyme ATP-sulfurylase as measured by Assay b (see METHODS). Universal buffer was used throughout the whole pH range.

active over a wide pH range (7–9) in agreement with that reported for the yeast enzyme by ROBBINS AND LIPMANN[17], rat liver enzyme had optimum activity at pH 8.0, a value in agreement with that reported for rat retina enzyme[21] (Fig. 2). PANIKKAR AND BACHHAWAT[5] report an optimum at pH 8.5.

Time and substrate dependence. ROBBINS AND LIPMANN[17] have shown that yeast ATP-sulfurylase activity is linear with time. Linearity of the reaction extended over 20 min, the span of the experiment. A similar series of studies by SUBBA RAO AND GANGULY[2] showed that in rat liver supernatant systems the rate of PAPS synthesis was linear with time for 3 h. It is known[17] that APS synthesis is the rate-limiting step in PAPS formation. We carried out a similar experiment with partially purified rat liver enzyme (specific enzyme activity, 19 μmoles ATP/mg protein per h) extended over 60 min (Fig. 3). Since the rate of reaction was constant over a period of 40 min, a 20-min incubation was always used to assay for the activity of ATP-sulfurylase.

In previous work, authors have reported on the affinity of the enzyme ATP-sulfurylase for its various substrates. Studying the synthesis of PAPS, HALL AND STRAATSMA[21] calculated the K_m value for sulfate ($1.5 \cdot 10^{-4}$ M) but not for the other substrates. These authors consider APS synthesis to be the limiting factor in PAPS synthesis. In order to complete available information, we studied the effect of increasing substrate concentration on enzyme activity of ATP-sulfurylase. The effect of increasing ATP concentration on the activity of the enzyme was measured by the molybdolysis assay[7] and is shown in Fig. 4, from which the K_m value was determined to be $1.6 \cdot 10^{-3}$ M.

The effect of increasing sulfate concentration, as measured by the sulfation of *p*-nitrophenol in the presence of excess amounts of APS-kinase and sulfatetransferase by the method of Carroll (see LEVI *et al.*[1]), was studied with the 100 000 $\times$ *g* supernatant fraction. Fig. 5 shows the result of double reciprocal plotting of substrate *vs.* products.

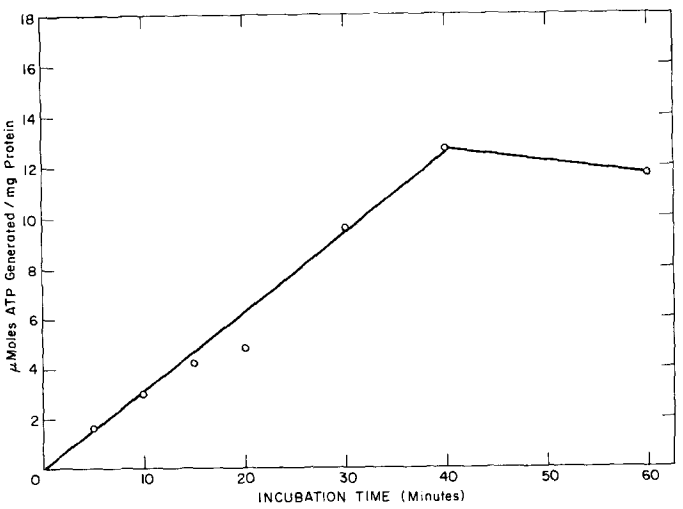

Fig. 3. Effect of incubation time on the activity of the enzyme ATP-sulfurylase as measured by Assay b (see METHODS).

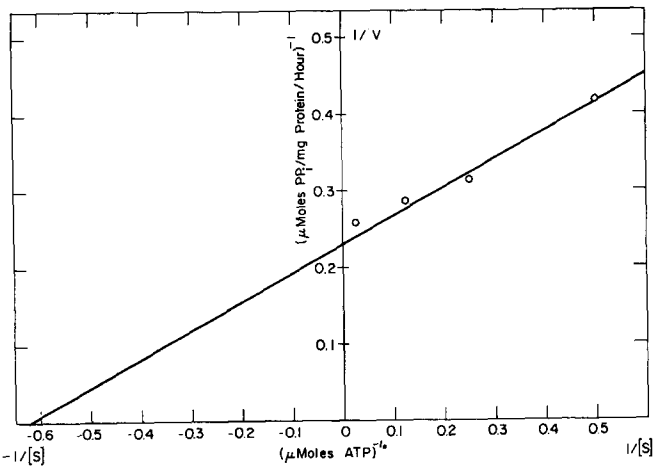

Fig. 4. Double reciprocal plot of ATP-sulfurylase activity with increasing ATP concentration. Activity was measured by the molybdolysis assay of WILSON AND BANDURSKI[7]. Calculation of K_m for ATP resulted in a value of $1.6 \cdot 10^{-3}$.

The K_m value was $1.0 \cdot 10^{-4}$ M, a value comparable to that reported by HALL AND STRAATSMA[21]. Supernatant fraction from vitamin A-deficient rat liver showed the same K_m for sulfate.

A partially purified enzyme (specific activity, 13 nmoles ATP/mg protein per h,

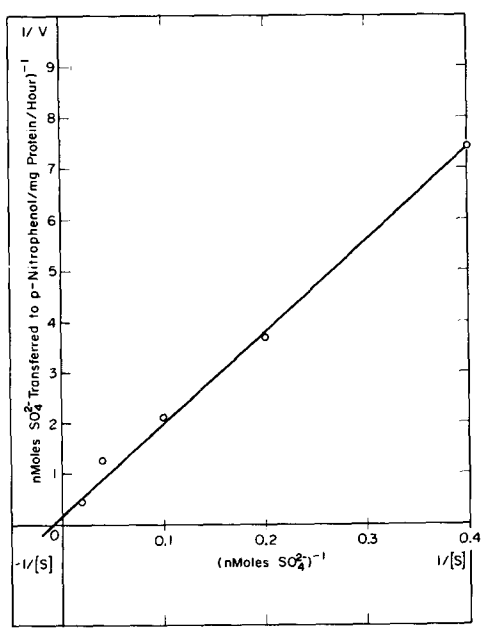

Fig. 5. Double reciprocal plot of ATP-sulfurylase activity with increasing amount of sulfate labeled with ^{35}S as measured by the sulfation of *p*-nitrophenol[1]. Calculation of K_m resulted in a value of $1 \cdot 10^{-4}$. Assay used was that of Carroll (see LEVI *et al.*[1]).

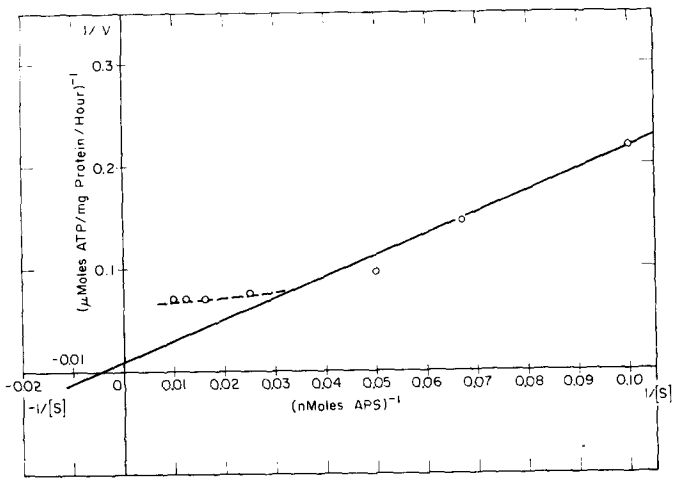

Fig. 6. Double reciprocal plot of ATP-sulfurylase activity with increasing APS as measured by Assay b (see METHODS). Calculation of K_m for APS resulted in a value of $2.5 \cdot 10^{-4}$.

300-fold purified) was used to test the effect of increasing APS concentration on the activity of the enzyme as measured by ATP generation with PP_i. Fig. 6 shows the double reciprocal plot; the K_m value for APS ($2.5 \cdot 10^{-4}$ M) indicated a comparable affinity of the enzyme for APS and sulfate. The upward deviation of the plot indicates a substrate inhibition at a high concentration. APS inhibition has been observed also in the case of the yeast enzyme[22]. Fig. 7 shows the effect of increasing PP_i concentration on the activity of ATP-sulfurylase. Calculation of K_m for PP_i from Fig. 6 gives a value of $3.7 \cdot 10^{-5}$ M, considerably lower than that for the other substrates. The enzyme,

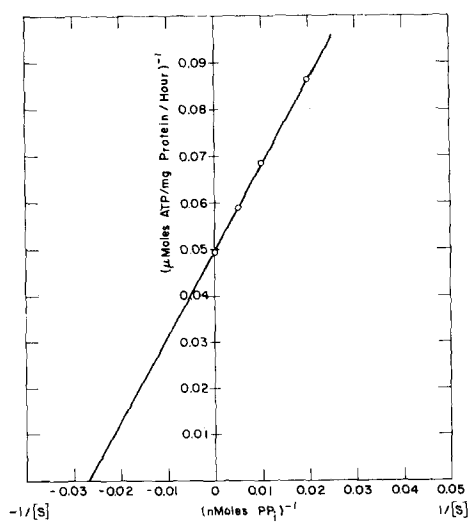

Fig. 7. Double reciprocal plot of ATP-sulfurylase activity and substrate as a result of increasing PP_i concentration. Assay b (see METHODS) was used. Calculation of K_m for PP_i resulted in a value of $3.7 \cdot 10^{-5}$.

again, was inhibited by high PP_i concentrations (38% inhibition at $3 \cdot 10^{-4}$ M). This observation agrees with that reported by SEGAL[23] and with the observation that the enzymatic synthesis of APS may only be observed in the presence of pyrophosphatase which not only hydrolyzes the PP_i and thereby displaces the equilibrium of the reaction toward the generation of APS, but also prevents inhibitory action of PP_i on the enzyme.

APS synthesis, or sulfate activation in general, is an energy-requiring reaction. From an evolutionary point of view, therefore, one would expect to observe both APS and PP_i inhibition; in other words, the product inhibition of an energy-requiring reaction.

The specificity of the enzyme toward its substrates, APS and PP_i, was demonstrated by the complete absence of ATP formation when substituting P_i for PP_i or ADP or AMP for APS. There was no reaction in the absence of APS or PP_i.

Inhibitors. The effect of heavy metals and other sulfhydryl-group inhibitors was tested. Both cobalt chloride and copper chloride are inhibitors. At a concentration of 2 mM complete inhibition occurs with cobalt chloride, but at the same concentration,

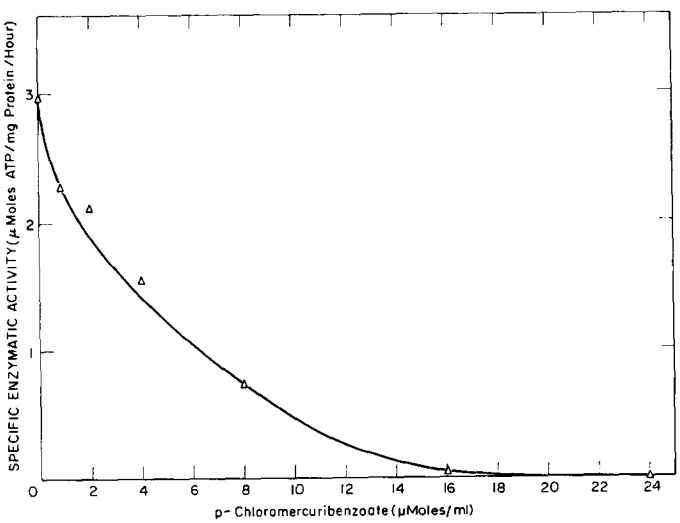

Fig. 8. Inhibitory effect of PCMB on the enzymatic activity of ATP-sulfurylase. Assay b (see METHODS) was used. A blank was carried out to correct for the effect of added PCMB on the luciferin–luciferase assay system. PCMB was dissolved in 0.25 ml of 0.1 M NaOH solution, then brought to the proper value with phosphate buffer (pH 7.8; ionic strength 0.1). pH of the final solution was 7.9

copper chloride has inhibited the enzyme only to the extent of 76.5%. PCMB was used to test for the involvement of sulfhydryl groups in the enzyme activity of ATP-sulfurylase. Fig. 8 shows the results of such an experiment. At a concentration of 16 mM, a 100% inhibition occurs. The PCMB inhibition was prevented by the addition of increasing amounts of reduced glutathione. Increasing concentrations of glutathione were added to an incubation mixture made 16 mM in PCMB, a concentration at which 100% inhibition of ATP-sulfurylase enzyme activity occurs. Fig. 9 shows the effect of glutathione addition to a completely inhibited enzyme. The essentiality of the

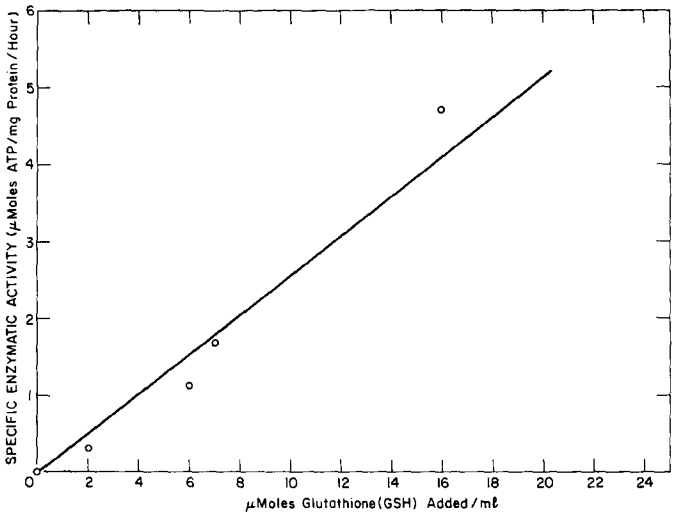

Fig. 9. Reactivation of PCMB-inhibited enzyme by the addition of glutathione. Assay b (see METHODS) was used.

reduced state of the enzyme was suggested from the fact that at a concentration of 16 mM of glutathione (equivalent to that of PCMB) the specific enzyme activity value was higher than for the uninhibited enzyme.

The essentiality of Mg^{2+} was shown by the inhibition of activity with addition of EDTA and subsequent reversal of inhibition by the addition of magnesium chloride. This agrees with the work of ROBBINS AND LIPMANN[17] and PANIKKAR AND BACHHAWAT[5].

Stability of the enzyme. The importance of hydrophobic bonding in the tertiary structure (or quarternary structure if subunits exist) in the active conformation of the enzyme was tested by treatment of the enzyme with deoxycholic acid at a concentration of 1%. The solution was left in an ice bath for 1 h afterwards and was then dialyzed against phosphate buffer (ionic strength, 0.1; pH 7.6) for 1–1½ h. The effect of added vitamin A on restoration of the enzyme activity was also tested by dialysis in the presence of retinol (20 μg/ml buffer). The complete loss of enzyme activity after deoxycholic acid treatment possibly indicated a rupture in the hydrophobic bonding which produced conformational changes and loss of enzyme activity. If vitamin A were present in the original enzyme and removed by the deoxycholic acid treatment and consequently caused the loss of activity, it could not restore the activity of the enzyme when re-added.

BALASUBRAMANIAN, SPOLTER AND RICE[24] reported an inhibitory effect of Tris buffer on the enzymatic incorporation of $^{35}SO_4^{2-}$ into PAPS. We noticed during the purification that the enzyme lost all its activity when eluted from the Sephadex G-200 column with Tris buffer instead of the usual phosphate buffer. The elution which caused inactivation of the enzyme was carried out in 0.1 M Tris–HCl buffer, and activity was assayed in an incubation mixture of 0.02 M Tris–HCl buffer. Dialysis of the enzyme in Tris buffer against phosphate buffer did not restore activity. These facts suggest that the effect of Tris buffer reported by BALASUBRAMANIAN, SPOLTER AND RICE[24] is

TABLE III

EFFECT OF DIFFERENT BUFFERS ON THE DECAY OF ATP-SULFURYLASE ENZYMATIC ACTIVITY WITH TIME

Enzyme fractions of various degrees of purity were stored at 4° for times and in buffers as indicated. Assay b (see METHODS) was used except in Expt. D for which the molybdolysis assay of WILSON AND BANDURSKI[7] was used. The specific activity was expressed in μmoles ATP/mg protein per h in Expts. A, B and C; in Expt. D in μmoles PP_i/mg protein per h.

Fraction	*Buffer*	*Time in buffer (h)*	*Specific activity*	*% of zero-time activity*
Expt. A				
G-200	phosphate;	3	18.8	100
eluate	pH 7.6; ionic	23	19.6	100 (103)
	strength 0.1	47	18.5	100
Expt. B				
$(NH_4)_2SO_4$	phosphate;	3	5.75	100
precipitate	pH 7.6; ionic	23	6.5	110
	strength 0.1	47	6.0	104
Expt. C				
$(NH_4)_2SO_4$	Tris–HCl	3	5.0	100
precipitate	0.1 M; pH 7.6	24	0.75	15
		48	0.10	2
Expt. D				
$(NH_4)_2SO_4$	Tris–HCl	3	1.7	100
precipitate	0.1 M; pH 7.6	24	0.27	15.7
		48	no activity detected	

an effect on the stability of the enzyme rather than an inhibitory action of the buffer. To test this hypothesis 2 fractions of the enzyme, (a) the enzymatically active ammonium sulfate-precipitated fraction and (b) the enzymatically active Sephadex G-200 fraction were kept in the 2 buffers. Enzymatic activity was assayed at various times. Results are shown in Table III. Neither fraction lost activity with time when kept in phosphate buffer, and the decay of activity in Tris buffer as measured by the generation of ATP or by the molybdolysis reaction was almost complete after 24 h. The equal rate of decay of activity of ammonium sulfate fraction in Tris–HCl buffer as measured by the generation of ATP or molybdolysis suggests that both reactions, ATP generation from APS and molybdolysis, are catalyzed by the same enzyme. The molybdolysis assay could not, of course, be carried out in phosphate buffer because it depends on the assay of phosphate formed.

In another experiment the dialysis of an enzymatically active fraction from a Sephadex G-200 column was carried out against various buffers. Enzymatic activity was tested at different times. The effect of phosphate buffer on the enzyme was also tested. Fig. 10 shows that the enzyme completely lost its activity upon dialysis against 0.1 M Tris–HCl for 4 h. To test the hypothesis that sulfate can stabilize the activity of the enzyme, dialysis was carried out against Tris–sulfate buffer 0.1 M (pH 7.8). Fig. 10 shows that such an hypothesis is not valid.

Dialysis of the enzyme against barbiturate buffer did not cause as rapid a decay

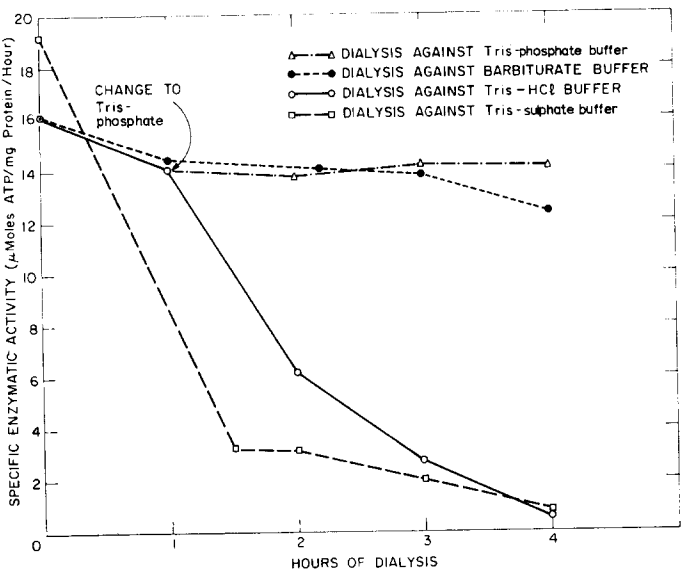

Fig. 10. Effect of different buffers on the enzymatic activity of ATP-sulfurylase as measured by Assay b (see METHODS). The enzymatically active fraction from a Sephadex G-200 column in phosphate buffer was dialyzed against the following: (a) Tris–HCl 0.1 M (pH 7.8) for 4 h; (b) Tris–HCl 0.1 M (pH 7.8) for 1 h, then Tris–phosphate 0.1 M (pH 7.8) for 3 h; (c) barbiturate buffer 0.1 M (pH 7.8) for 4 h; and (d) Tris–sulfate 0.114 (pH 7.8) for 4 h.

of enzyme activity as in the other two buffers, but an increase in the rate of the decay was evident after 3 h of dialysis. Removal of a portion after dialysis of the enzyme for 1 h against Tris–HCl and continued dialysis for the remaining 3 h in Tris–phosphate did not cause any further loss of activity.

This experiment conclusively shows the stabilizing effect of phosphate ions on the enzyme and may explain why attempts to purify mammalian enzyme in the absence of phosphate have failed. PANIKKAR AND BACHHAWAT[5] make no comment on the stability of their enzyme. They used Tris buffer throughout. Possibly the sheep liver enzyme is more stable in Tris buffer than the rat enzyme.

TABLE IV

DECAY OF ATP-SULFURYLASE ACTIVITY IN Tris BUFFER (0.1 M, pH 7.6)

Enzyme from Sephadex G-200 column eluate (see under *Purification*) was used. Molybdolysis was done by the method of WILSON AND BANDURSKI[7]. ATP generation by Assay b.

Time in buffer (h)	*Molybdolysis assay (μmoles PP_i/h per mg protein)*	*ATP generation (μmoles ATP/h per mg protein)*	*% of zero-time activity*	
			Molybdolysis	*ATP generation*
0	2.27	6.15	100	100
12	1.91	4.43	84	72
24	1.0	2.09	44	34
36	0.261	0.91	11	14
48	0	0	0	0

The decay rate of the enzyme activity in Tris buffer could also be used to determine whether ATP-sulfurylase and the enzyme which effects molybdolysis[7] are identical. As shown in Table IV, the rate of decay is quite similar for both reactions; therefore, catalysis is probably by the same enzyme.

Mechanism of the reaction. The earliest attempt to study the mechanism of the ATP-sulfurylase-catalyzed reaction was made by SEGAL[23]. SEGAL studied the enzymatic exchange between radioactive PP_i, P_i, and ATP in the presence and absence of sulfate in the "high-speed" supernatant fraction from rat liver and found that there is sulfate-dependent PP_i exchange as well as a P_i exchange independent of sulfate.

To clarify further the mechanism of the reaction, a series of experiments was carried out to study the initial rate of exchange between labeled PP_i and ATP in the presence and absence of sulfate. We found (Fig. 11) that $^{32}PP_i$ was incorporated into ATP in the absence of sulfate, and that the initial rate of this reaction (63 μmoles/mg protein per h) was greater than that of the overall reaction between ATP and sulfate to form APS (0.43 μmoles/mg protein per h calculated from Table I). Since the rate of the partial reaction is faster than that of the overall reaction, we postulate a two-step mechanism:

$$ATP + E \rightleftharpoons E\text{–}ATP \rightleftharpoons E\text{–}AMP + PP_i \quad \text{(fast)} \qquad (1)$$

$$E\text{–}AMP + SO_4^{2-} \rightleftharpoons E\text{–}APS \rightleftharpoons APS + E \quad \text{(slow)}. \qquad (2)$$

If the overall reaction took place by a direct exchange between sulfate and PP_i on the enzyme, the rate of the exchange reaction should be comparable to that of the overall reaction.

Though sulfate is not required for the exchange reaction, its presence increased the rate (Fig. 11) for reasons so far unexplained, but possibly due to a conformational change or an allosteric effect.

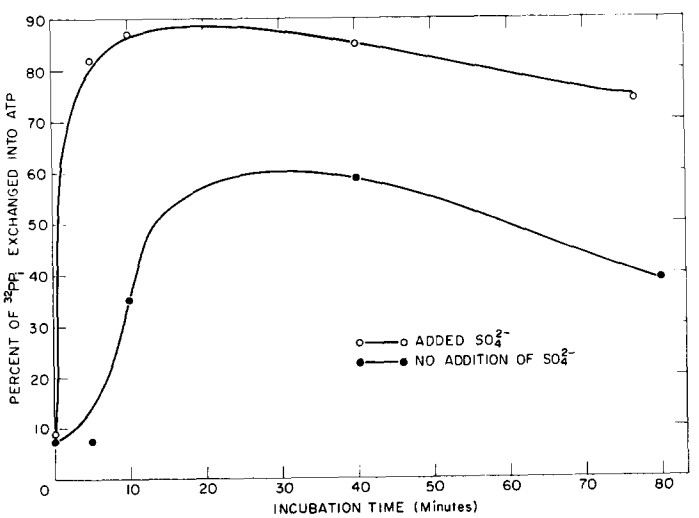

Fig. 11. Exchange of $^{32}PP_i$–ATP with time in the presence and absence of sulfate. At the time indicated, portions were removed; the reaction was stopped by immersing in boiling water for 90 sec. Sulfate was added up to a final concentration of 250 μmoles, $^{32}PP_i$, 0.2 μmole. Separation of ATP and PP_i was done electrophoretically; radioactivity in ATP was assayed as described in METHODS.

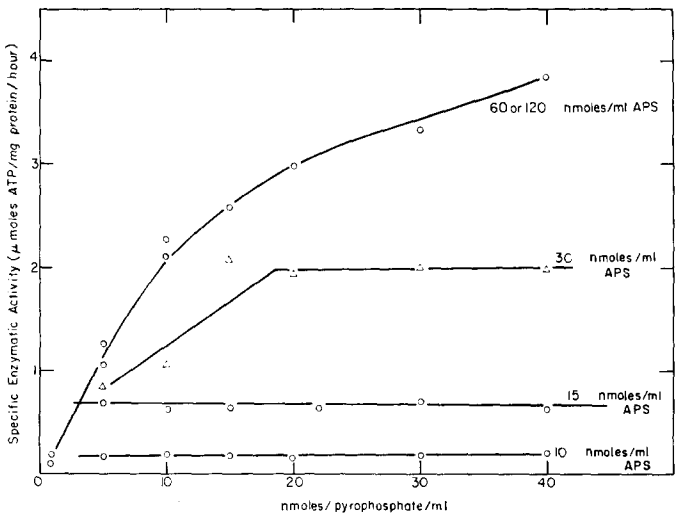

Fig. 12. Effect of increase in PP_i concentration at different levels of APS concentration on the activity of the enzyme ATP-sulfurylase. Enzymatic activity was assayed by Assay b (see METHODS).

No exchange could be observed between APS and labeled SO_4^{2-} with the enzyme, in the presence or absence of PP_i, no doubt due to the slow rate of reaction (2), above, and the inhibitory action of APS on the enzyme (see Fig. 6).

An investigation of the dependence on PP_i concentration of the rate of reaction of APS with PP_i was made at various APS concentrations. A family of curves was obtained (Fig. 12) illustrating and supporting the two-step reaction mechanism, with one step slower than the other.

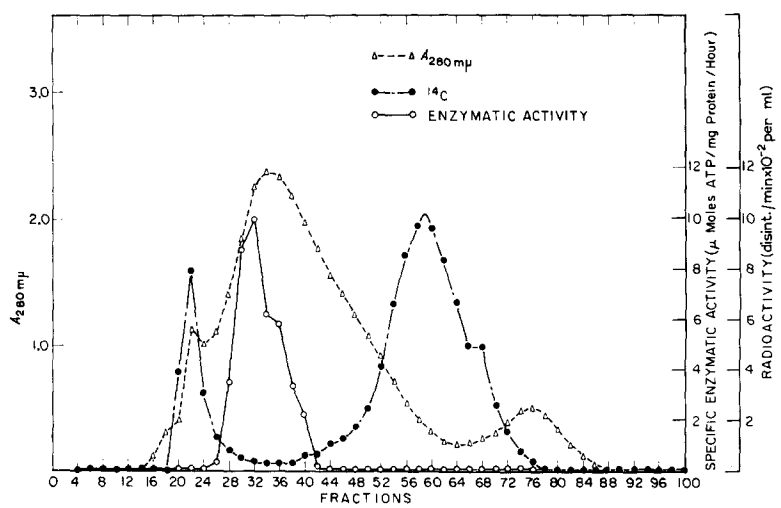

Fig. 13. Fractionation of an enzymatically active fraction precipitated with ammonium sulfate from livers of vitamin A-deficient rats given [6,7-$^{14}C_2$]retinyl acetate for 3 days. A Sephadex G-200 3 cm × 5 cm column was used. Proteins were eluted by the use of phosphate buffer (ionic strength 0.1; pH 7.7). Fractions of 5.5 ml were collected. Assay b (see METHODS) was used.

Fig. 12 shows that at low APS concentrations (10 and 15 nmoles/ml), the rate of reaction is independent of PP_i concentration and dependent on APS. We interpret this to mean that the rate depends only on the amount of APS that reacts with the enzyme and is converted to *E*–AMP. This complex reacts fast with any available PP_i.

We postulate that at high APS concentrations (60 nmoles/ml) all the enzyme sites available have been converted to *E*–APS and hence *E*–AMP complex and that the reaction is completely dependent on available PP_i. At even higher concentrations of APS (120 nmoles/ml), for instance, the same rates of reaction were obtained as at 60 nmoles/ml.

At intermediate APS concentrations (30 nmoles/ml) the rate is dependent on PP_i concentrations when these are low. We interpret this to mean that as much of the enzyme is in the *E*–AMP complex as can react with PP_i. When the PP_i concentrations are high (greater than 20 nmoles/ml), there is insufficient APS to form the *E*–AMP complex to react with all the available PP_i, and the reaction becomes dependent on APS concentration.

Relation of vitamin A to the enzyme ATP-sulfurylase

The direct coenzymatic involvement of vitamin A in the activity of the enzyme ATP-sulfurylase is a controversial issue. It has been proposed by SUBBA RAO AND GANGULY[2] and by SUNDARESAN[3], but could not be confirmed by PANIKKAR AND BACHHAWAT[5]. The present purification of the enzyme presented an advantage for a test of the direct involvement of the vitamin in ATP-sulfurylase activity.

In earlier experiments, we treated vitamin A-deficient rats with radioactive vitamin A as follows: [15-^{14}C]retinoic acid and [6,7-$^{14}C_2$]retinyl acetate were fed to vitamin A-deficient rats until cured (9–17 days). The ATP-sulfurylase was then isolated and purified from the livers of these animals by the method described above. Although some radioactivity remained associated with the enzyme in the purest fractions, specific enzyme activity increased, whereas specific radioactivity decreased throughout the purification. The results were therefore inconclusive.

Some of the radioactive enzyme was treated with *n*-butanol, but no radioactivity was extractable from it by this solvent.

To get more conclusive results, purification was carried out through the usual steps (Table I) but with the use of larger Sephadex G-200 and agarose columns, which permitted true fractionation rather than elution. The results are described in Expts. I through III.

Experiment I. [6,7-$^{14}C_2$]Retinyl acetate (specific activity 25.3 μC/mg) was injected subcutaneously in a Tween-alcohol solution into 4 vitamin A-deficient rats. A dose of 400 μg/rat on the first day was followed by 200 μg/rat on the second and third days. The total radioactivity was 20.24 μC/rat. The rats were killed on the third day, 4 h after the last administration of the vitamin. Livers were dissected and homogenized, and the 100 000 $\times$ *g* supernatant was prepared; then ammonium sulfate fractionation was carried out. The ammonium sulfate-precipitated, enzymatically active fraction was fractionated on a Sephadex G-200, 3 cm $\times$ 50 cm column. Fractions were assayed for both radioactivity and enzyme activity. Results are shown in Fig. 13. As can be seen, little radioactivity was associated with enzyme activity. Enzymatically active fractions were pooled, concentrated, and fractionated further on a 1.8 cm $\times$ 95 cm agarose column (agarose bio-gel A-15m with fractionation capacity of 40 000–

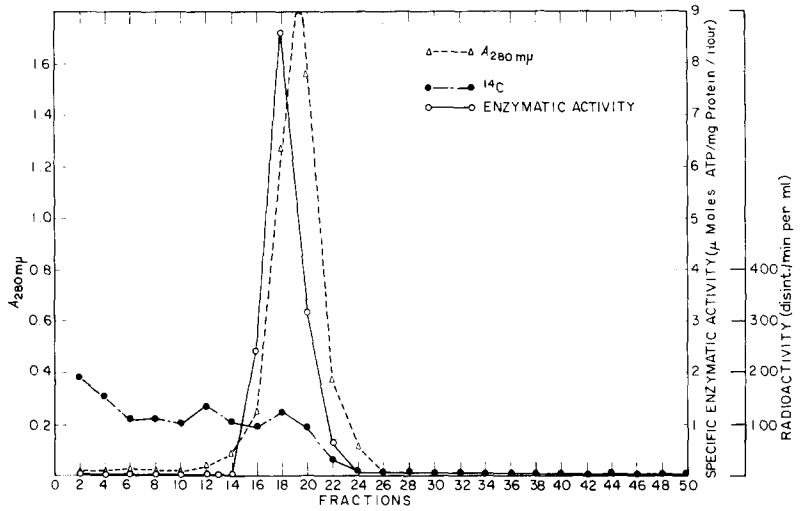

Fig. 14. Further fractionation of enzymatically active eluates from Sephadex G-200 on 1.8 cm × 95 cm agarose columns. Proteins were eluted from the column by phosphate buffer (ionic strength 0.1; pH 7.7). Fractions of 5.5 ml were collected. Assay b (see METHODS) was used.

15 000 000 mol. wt.). Results are shown in Fig. 14. A very low level of radioactivity was distributed over most of the fractions.

Assuming the molecular weight of the enzyme to be 900 000, as suggested by the elution behavior described, and knowing the specific radioactivity of the vitamin and the concentration of protein in the peak tube for enzyme activity (Tube 18,

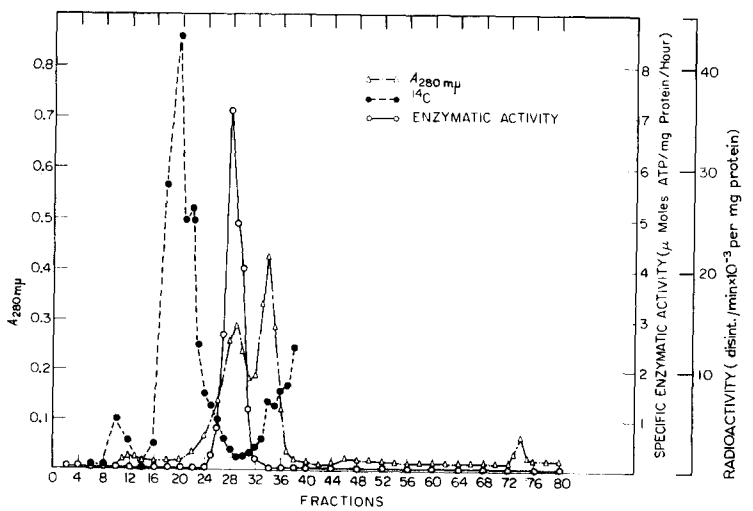

Fig. 15. Further fractionation of enzymatically active eluates from Sephadex G-200 columns after concentration to 3 ml. Fractionation on a 1.8 cm × 95 cm agarose A-15m column prepared in phosphate buffer (pH 7.6; ionic strength 0.1). Fractions of 5.5 ml were collected. Assay b (see METHODS) was used.

Fig. 14), one can calculate the radioactivity eluted with the enzyme if there exists a 1:1 molar association between enzyme and vitamin. Since the animals used were vitamin A-deficient, one can assume that there was little dilution of the radioactive with the nonradioactive stored vitamin. Tube 18 contained 1.45 mg protein/ml, which should then be associated with 31 000 disint./min of the label. However, though the stage of purity was 6600-fold over the crude homogenate, the enzyme in this tube was obviously not pure. Making a conservative estimate of only 10% purity, one should still expect 3100 disint./min of the labeled vitamin in this tube if an association exists. Actually, only 120 disint./min were detected; therefore, an association between the enzyme and vitamin A (or a metabolite) does not occur.

Experiment II. CARROLL AND SPENCER[16] found that the addition of the vitamin (retinoic acid or retinol) to embryonic sulfurylase (of low activity) prepared from rat embryo liver activates the enzyme to the normal adult level. We purified the enzyme from 100 000 × *g* liver supernatant fraction prepared from 17-day-old rat embryos after homogenizing them with 200 μg of [6,7-$^{14}C_2$]retinol. Enzymatically active fractions from Sephadex G-200 columns were pooled, concentrated and further fractionated on a 1.8 cm × 95 cm agarose column. Elution pattern of radioactivity and enzymatic activity is shown in Fig. 15. Both enzyme activity and radioactivity were eluted in different fractions with no coincidence of radioactivity and enzymatic activity. Fractionation of the enzyme solution on the agarose column resulted in a specific enzymatic activity of 7.15 μmoles ATP/mg protein per h. Pre-incubation of the embryo liver homogenates with labeled retinol for 10 min, before isolation of the enzyme, gave a similar result.

Experiment III. CARROLL AND SPENCER[16] report that the ATP-sulfurylase level in rats is low at birth and stays low unless the infant is suckled by its mother, the level of the enzyme activity corresponding to the level of vitamin A in the liver. Consequently, infant rats were removed from mothers immediately after birth and injected with radioactive vitamin (50 μg [6,7-$^{14}C_2$]retinyl acetate/rat; specific radioactivity, 25.3 μC/mg; 1.26 μC/rat). They were kept for 16–19 h in a humid atmosphere at 37° away from the mothers to prevent suckling. Their livers were dissected and homogenized, and enzyme purification was carried out. Three radioactivity peaks were eluted from Sephadex G-200, one of which corresponded to the enzymatic activity peak. Enzymatically active fractions were pooled and concentrated, and a portion fractionated further on an agarose column with no coincidence of enzymatic activity and radioactivity. Fractionation on the agarose column resulted in a 225-fold purification from the 100 000 × *g* supernatant.

Expts. I–III show that neither vitamin A nor a metabolite of the vitamin which still retains at least carbon atoms 6 and 7 is an integral or necessary part of the enzyme. The fact that some radioactive substance remains associated with the enzyme to a high degree of purity is puzzling and has not been explained. It must have a high molecular weight, comparable to that of the enzyme, since it can only be removed after careful fractionation on long Sephadex or agarose columns. Possibly, it is a lipoprotein or another type of vitamin A-carrier protein.

One possible relation between vitamin A and the enzyme, which would not require direct attachment of the vitamin to the enzyme, could be in stabilization. We found the enzyme to be extremely unstable in the absence of phosphate ions. Possibly, under conditions of low phosphate concentrations in the cell, the vitamin is required

to maintain stability of the enzyme. We carried out some of the purification steps in the presence of vitamin A and found no evidence of stabilization, but this problem merits more rigorous investigation with respect to concentrations and possible metabolites of the vitamin. It may be significant in this regard that the K_m of the enzyme with respect to sulfate of the deficient animals was identical to that of the normal; this phenomenon suggests that the lowered sulfate activation in the deficient rat is due to less enzyme rather than a lower rate of reaction of the same amount of enzyme in the absence of the vitamin.

ACKNOWLEDGMENT

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