BBA 12239

PYRIDINE NUCLEOTIDE-STIMULATED PRODUCTION OF 3'-PHOSPHOADENOSINE 5'-PHOSPHATE BY BEEF-CORNEA-EPITHELIAL EXTRACT

BERNARD WORTMAN*

Department of Ophthalmology,
Washington University School of Medicine,
St. Louis, Mo. (U.S.A.)

(Received December 11th, 1962)

SUMMARY

The production of 3'-phosphoadenosine 5'-phosphate (PAP) can be observed by coupling transfer reactions. The production of PAP by beef-cornea-epithelial extract is shown to be dependent upon reduced di- or triphosphopyridine nucleotides and a heat-stable, undialysable substance in epithelial extract. When 3'-phosphoadenosine 5'-phospho[^{35}S]sulfate is supplied to the reaction mixture a second peak of radioactivity can be detected after paper electrophoresis. Several reaction mechanisms are hypothesized to explain the accumulation of PAP.

INTRODUCTION

PAP content of corneal epithelium was estimated at approx. 4 times as much as that reported in liver or at least 68 mμmoles of PAP per gram of wet tissue[1]. The presence of large amounts of PAP complicated the earlier study of sulfotransferases because such high levels are inhibitory[2]. While dialysis of fresh epithelial extracts does not reduce the apparent PAP content appreciably, extracts aged at $-20°$ for several weeks behave as though they contain less than inhibitory amounts of PAP. Other preliminary experiments suggested that fresh epithelial extracts accumulate PAP while aged extracts do not. Concomitantly, there is no appreciable loss of sulfate-activating enzymes or of phenol sulfotransferase (3'-phosphoadenylylsulfate:phenol sulfotransferase, EC 2.8.2.1) activity**.

This communication will present evidence that beef-cornea-epithelial extract contains enzyme activities which may be similar to those of the sulfate and disulfide reduction systems described in yeast[3-5]. The epithelial enzyme system is dependent upon the presence of DPNH or TPNH and a heat-stable substance present in the extract for its activity. The enzyme activity is detected by the production of PAP. Beef cornea serves as a source of a homogenous population of epithelial cells and demonstrates that these enzyme activities are present in mammalian cells.

Abbreviations: PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAP, 3'-phosphoadenosine 5'-phosphate.

* Established Investigator of the American Heart Association.

** B. WORTMAN, unpublished observations.

EXPERIMENTAL PROCEDURE

Chemicals

DPNH, TPNH, *p*-nitrophenyl sulfate, *p*-nitrophenol, thioctic acid and thioctic acid amide were obtained from Sigma Chemical Company (St. Louis, Mo). PAP is present as a 2% contaminant in Pabst lot 602 ADP (see refs. 2 and 6).

Beef-cornea-epithelial extract

Epithelium was scraped from beef corneas, homogenized in 4 vol. (w/v) of 0.005 M Tris–HCl (pH 7.4) and centrifuged for 1 h at 34 800 × *g*. The supernatant fluid was analyzed for its protein concentration[7] and stored at −20°, as previously described[1,6]. All operations were conducted in the cold (0–4°), unless otherwise stated.

Boiled extract

Epithelial extract was heated in a boiling-water bath for 3 min. Heat-denatured proteins were removed by centrifugation for 10 min at approx. 500 × *g*. Protein content was reduced from approx. 16 to 0.8 μg/μl by this procedure.

Preparation of [^{35}S]PAPS

Epithelial extract was used as the source of sulfate-activating enzymes. [^{35}S]-PAPS was prepared and isolated as previously described[1,7].

Paper electrophoretic localization of radioactivity

Horizontal paper electrophoresis was performed as previously described[1]. Radioactivity was localized on paper strips with a Vanguard Model 880 Autoscanner (Vanguard Corporation, LaGrange, Ill.).

PAP assay

The production of PAP can be detected colorimetrically by use of phenol sulfotransferase, which is also present in crude epithelial extract[1,6]. The reaction sequence is as follows[2]:

$$\text{PAP} + p\text{-nitrophenyl sulfate} \leftrightharpoons \text{PAPS} + p\text{-nitrophenol} \quad (1)$$

$$\text{phenol} + \text{PAPS} \rightarrow \text{PAP} + \text{phenyl sulfate} \quad (2)$$

Reaction 1 as used in this study will detect PAP in concentrations greater than 10^{-6} M. Therefore, phenol is added to the reaction mixture to bring about enzymic cycling which will increase the sensitivity of the assay at least 10-fold[2]. *p*-Nitrophenol is measured in an alkaline medium at 400 mμ as previously described[1,2,6].

RESULTS

Reduced pyridine nucleotide dependency

The appearance of *p*-nitrophenol is dependent upon the presence of DPNH or TPNH of the enzymically reduced type (Sigma type II); both serve equally well.

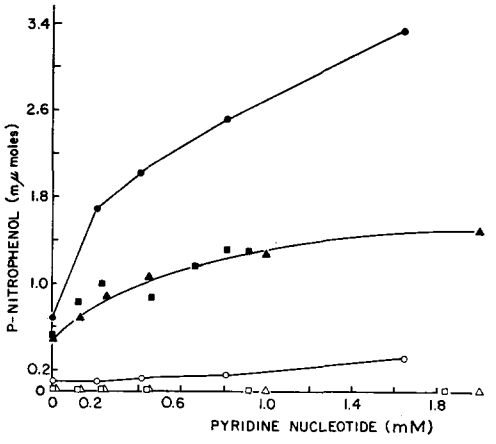

Fig. 1. Stimulation of activity by DPNH and TPNH. The complete reaction mixture contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 304 μg of epithelial extract protein; varying concentrations DPNH or TPNH in a total volume of 102 μl. Incubations were for 1 h at 38°, after which 5 μl of 5 N NaOH were added, absorbancy read at 400 mμ and compared with *p*-nitrophenol standards. Chemically reduced TPNH: complete system, ●—●; without phenol, ○—○. Enzymically reduced TPNH: complete system, ■—■; without phenol, □—□. Enzymically reduced DPNH: complete system, ▲—▲; without phenol, △—△.

TPN^+ and DPN^+ are inactive. The chemically reduced TPNH (Sigma type I) exhibits a higher level of stimulation of the enzyme system (Fig. 1).

Effect of protein concentration

Enzymic activity is linear in the presence of TPNH. In the absence of added TPNH, no significant amount of *p*-nitrophenol is measured until larger amounts of epithelial extract are added to the reaction mixture (Fig. 2).

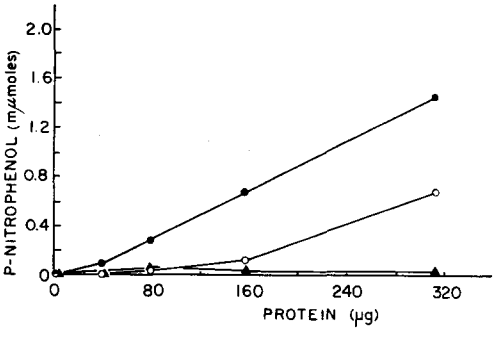

Fig. 2. Effect of increasing amount of protein on activity. The complete reaction mixture contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 95 mμmoles of TPNH; varying amounts of protein in a total volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1. ●—●, complete system; ○—○, without TPNH; ▲—▲, without phenol.

Effect of incubation time

The appearance of *p*-nitrophenol is practically linear over the period of time tested. Higher activity is observed in the presence of DPNH or TPNH than in their absence (Fig. 3).

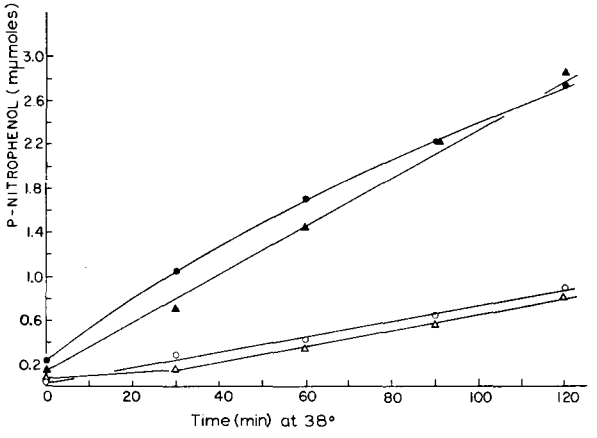

Fig. 3. Effect of incubation time on activity. The complete reaction mixture contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 88 mμmoles of TPNH or 119 mμmoles of DPNH; 375 μg of epithelial extract protein in a total volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1. ▲—▲, with DPNH; △—△, without DPNH; ●—●, with TPNH; ○—○, without TPNH.

Effect of boiled epithelial extract

Boiled extract will stimulate the appearance of *p*-nitrophenol in the absence of DPNH or TPNH. Dialysis of 0.5 ml of boiled extract *vs.* 1 l of water for 1 h did not alter the effect shown by the undialyzed boiled extract. Approx. 18 μg of boiled

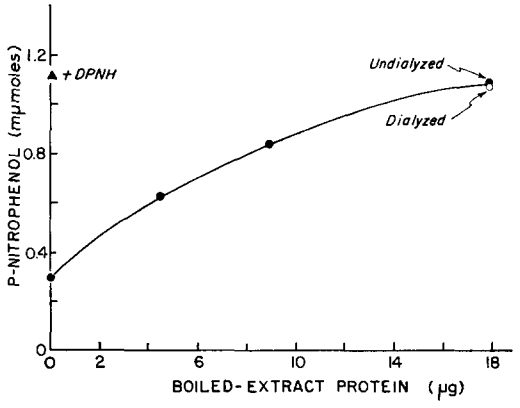

Fig. 4. Stimulating effect of boiled extract. The complete reaction mixture contained: 10 μmoles of phosphate buffer (pH 7); 351 μg of epithelial extract protein; 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; varying amounts of boiled epithelial extract protein (0.8 μg of protein/μl) in a total volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1. ●—●, undialyzed boiled extract; ○, dialyzed boiled extract; ▲, 114 mμmoles of DPNH without added boiled extract.

epithelial extract protein (equivalent to an additional 22.5 μl of epithelial extract) in the absence of DPNH produced a similar level of activity as did approx. 1.1 mM concentration of DPNH in the absence of boiled extract (Fig. 4).

Localization of radioactivity after paper electrophoresis

[^{35}S]PAPS with or without incubation demonstrates one peak of radioactivity (A and D, Fig. 5). [^{35}S]PAPS moves anodally to TPNH and ATP in electrophoresis, as used in this study. In the absence or presence of TPNH and epithelial extract, a second peak of radioactivity with an electrophoretic mobility similar to that of TPNH is observed (B and C, Fig. 5).

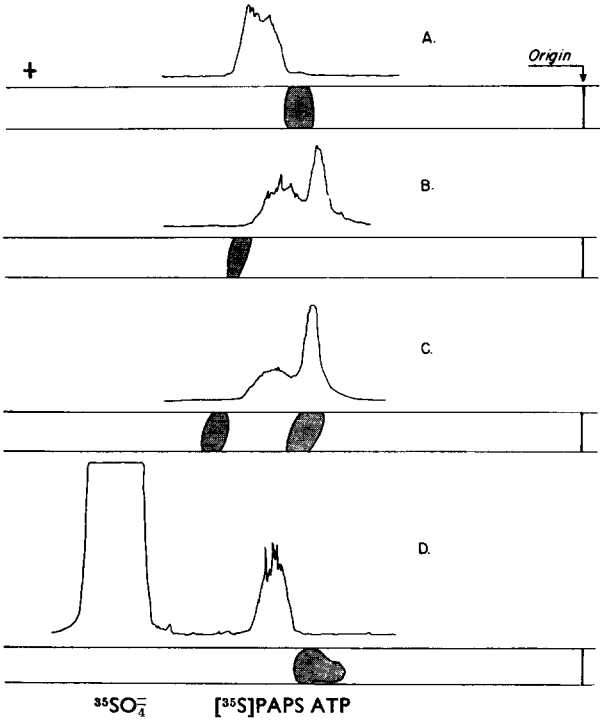

Fig. 5. Localization of radioactivity after electrophoresis. Reaction mixtures contained: 10 μmoles of phosphate buffer (pH 7); 402 μg of epithelial extract protein; 88 mμmoles of TPNH; 25 μl of [^{35}S]PAPS ($1.6 \cdot 10^3$ counts/min/μl) in a total volume of 108 μl. A, incubated at 0–4°; B, incubated at 38° in the absence of TPNH; C, incubated at 38°; D, [^{35}S]PAPS, [^{35}S]sulfate and ATP markers. After 3 h at the above temperatures, the incubation mixtures were subjected to electrophoresis on Whatman 3 MM paper in 0.05 M citrate buffer (pH 4.7) at a voltage gradient of 12 V/cm and radioactivity localized. The shaded spots are areas of ultraviolet quenching.

Effect of thiol and disulfide compounds

Thioctic acid and thioctic acid amide depress PAP production but have no significant effects on phenol sulfotransferase activity (Table I). Thiol compounds are without effect, with the possible exception of reduced glutathione.

TABLE I

EFFECT OF DISULFIDE AND THIOL COMPOUNDS

Added compounds are in the final concentration shown. Reaction mixtures for the estimation of the PAP production contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 82 mμmoles of TPNH; 304 μg of epithelial extract protein in a final volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1. Reaction mixtures for the estimation of the phenol sulfotransferase activity contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 0.17 mμmole of PAP; 339 μg of epithelial extract protein in a final volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1.

Additions	*mM*	*Relative activity*	
		PAP production	*Phenol sulfotransferase*
None		1.00	1.00
Thioctic acid	1.1	0.83	0.87
Thioctic acid amide	0.7	0.68	0.93
Glutathione (reduced)	2.2	1.15	1.03
Cysteine	2.2	1.02	1.06
2-Mercaptoethanol	2.2	1.04	1.06

Effect of various inorganic compounds

Phenol sulfotransferase activity was reduced in the presence of $CdCl_2$. However, PAP production was significantly reduced in the presence of all compounds tested (Table II).

TABLE II

EFFECT OF VARIOUS INHIBITOR COMPOUNDS

All additions were in a final concentration of 0.1 mM. Reaction mixtures for the estimation of the PAP production contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 109 mμmoles of TPNH or 117 mμmoles of DPNH; 399 μg of epithelial extract protein in a final volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1. Reaction mixtures for the estimation of the phenol sulfotransferase activity contained: 10 μmoles of phosphate buffer (pH 7); 60 mμmoles of *p*-nitrophenyl sulfate; 60 mμmoles of phenol; 0.24 mμmole of PAP; 379 μg of epithelial extract protein in a final volume of 102 μl. Incubations were for 1 h at 38°, and then treated as in Fig. 1.

Additions	*Relative activity*		
	PAP production		*Phenol sulfotransferase*
	(TPNH)	*(DPNH)*	
None	1.00	1.00	1.00
$NaAsO_2$	0.70	0.74	1.01
Na_2HAsO_4	0.65	0.59	0.98
NaF	0.68	0.68	1.04
Na_2SO_4	0.65	0.72	0.99
Na_2SO_3	0.72	0.75	1.02
$CdCl_2$	0.40	0.26	0.83

DISCUSSION

An earlier report verified the feasibility of using phenol sulfotransferase as a feeder system for the production of PAPS which can be coupled with other sulfotrans-

ferases[1]. The latter enzyme activities result in the production of PAP. In another sense, phenol sulfotransferase can be used as a detector system.

The transfer of SO_4^{2-} or SO_3^{2-} from PAPS to an acceptor compound would result in the production of PAP which can be assayed according to Reactions 1 and 2. Neither the sulfate-activating enzymes[8] or phenol[2], steroid[9] and mucopolysaccharide[1] sulfotransferases have been shown to be stimulated by pyridine nucleotides, nor do they show a cofactor dependency[2,5]. Therefore, other speculative explanations for the stimulation of PAP production by DPNH and TPNH are proposed.

The reduction of sulfate in PAPS to sulfite and its subsequent incorporation into a larger molecule is known to involve the participation of two or more enzymes, *viz.*, PAPS reductase (3'-phosphoadenylylsulfate:thiol sulfitetransferase) and disulfide reductase ($NAD(P)H_2$:disulfide oxidoreductase)[3,4,10]. The reaction series is complex; it requires sulfate in the biologically active form of PAPS, DPNH or TPNH, and a sulfite-acceptor compound. This type of enzymic reduction and transfer has been studied in yeast and bacteria[11,12]. The possibility that it exists in animal cells is raised by studies of chick embryo which can synthesize [^{35}S]taurine when [^{35}S]sulfate is supplied in an inorganic form[13,14]. Recent reports demonstrate that cardiac muscle[15], as well as microorganisms[16], can synthesize [^{35}S]isethionic acid from [^{35}S]taurine. In addition, cornea can incorporate [^{35}S]sulfur from inorganic [^{35}S]sulfate into cystine and methionine[17]. Therefore, there may be an operative pathway by which inorganic sulfate can be reduced and incorporated into a larger molecule. Whether such a pathway is functioning in cornea-epithelial cells can not be decided from the present investigation but is offered as a possible mechanism.

Reduced pyridine nucleotides may function to bring about a valence change in sulfur by the reduction of sulfate in PAPS to sulfite according to the following reactions[5]:

$$PAPS + RSH \rightarrow RSSO_3 + PAP \quad (3)$$

$$RSSO_3 + TPNH \text{ or } DPNH \rightarrow RSH + SO_3^{2-} + TPN^+ \text{ or } DPN^+ \quad (4)$$

The question of whether measured sulfite was present as a free ion or in a larger molecule in the yeast system was left unanswered[3,4]. Free sulfite is probably not a final product in the epithelial system. If free sulfite resulted due to the presence of Reaction 4, a radioactive material with the same electrophoretic mobility as free [^{35}S]sulfate ions would have been anticipated and detected after incubation of epithelial extract with [^{35}S]PAPS. A new radioactive substance with an electrophoretic mobility similar to that of an ATP marker and not to that of a [^{35}S]sulfate marker was found.

If the acceptor compound were a disulfide which must be reduced to a thiol, then TPNH and DPNH may function in this manner due to disulfide reductase activity. The higher activity seen in the presence of a chemically reduced TPNH (Sigma type I) may be due to bisulfite contamination which could contribute to the reduction of a disulfide compound in the extract. This hypothesis is illustrated by the following reaction sequence[3]:

$$(RS)_2 + H^+ + DPNH \text{ or } TPNH \rightleftharpoons 2RSH + TPN^+ \text{ or } DPN^+ \quad (5)$$

$$RSH + PAPS \rightarrow RSSO_3 + PAP \quad (6)$$

Reactions 4 and 5 would be rate limiting if the endogenous pyridine nucleotides were oxidized or destroyed during aging of the extracts. Furthermore, these hypotheses

require the proposal that the newly formed compound contains [^{35}S]sulfite according to Reactions 3 and 6.

These hypotheses could in part offer an explanation for the accumulation of PAP in aged extracts after the addition of TPNH or DPNH and the stimulation observed after the addition of a heat-stable substance in the extract.

ACKNOWLEDGEMENTS

The author thanks Mrs. F. BLACK for technical assistance and Dr. D. LIPKIN, Department of Chemistry, Washington University, for an additional supply of PAP.

This work was supported in part by a research grant (NB 01911) from the National Institutes of Health and by the St. Louis Heart Association.

REFERENCES

1 B. WORTMAN, *J. Biol. Chem.*, 236 (1961) 974.
2 J. D. GREGORY AND F. LIPMANN, *J. Biol. Chem.*, 229 (1957) 1081.
3 L. G. WILSON, T. ASAHI AND R. S. BANDURSKI, *J. Biol. Chem.*, 236 (1961) 1822.
4 T. ASAHI, R. S. BANDURSKI AND L. G. WILSON, *J. Biol. Chem.*, 236 (1961) 1830.
5 J. D. GREGORY AND P. W. ROBBINS, in J. M. LUCK, F. W. ALLEN AND G. MACKINNEY, *Ann. Rev. Biochem.*, Vol. 29, Annual Reviews, Palo Alto, Calif., 1960, p. 347.
6 B. WORTMAN, *Am. J. Physiol.*, 198 (1960) 779.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 P. W. ROBBINS AND F. LIPMANN, *J. Biol. Chem.*, 229 (1957) 837.
9 Y. NOSE AND F. LIPMANN, *J. Biol. Chem.*, 233 (1958) 1348.
10 S. BLACK, E. M. HARTE, B. HUDSON AND L. WARTOFSKY, *J. Biol. Chem.*, 235 (1960) 2910.
11 H. HILZ AND M. KITTLER, *Biochem. Biophys. Res. Commun.*, 3 (1960) 140.
12 D. FUJIMOTO AND M. ISHIMOTO, *J. Biochem.*, 50 (1961) 533.
13 I. P. LOWE AND E. ROBERTS, *J. Biol. Chem.*, 212 (1955) 477.
14 F. CHAPEVILLE AND P. FROMAGEOT, *Biochim. Biophys. Acta*, 26 (1957) 538.
15 W. O. READ AND J. D. WELTY, *J. Biol. Chem.*, 237 (1962) 1521.
16 R. BRAUN AND P. FROMAGEOT, *Biochim. Biophys. Acta*, 62 (1962) 548.
17 C-H.DOHLMAN, *Acta Ophthalmol.*, 35 (1957) 115.