Adenosine 3′,5′-Monophosphate Phosphodiesterase: Multiple Molecular Forms

Abstract. *By starch-gel electrophoresis and a specific staining technique, seven different molecular forms of cyclic nucleotide phosphodiesterase have been demonstrated in supernatants from homogenates of rat and rabbit tissues. No tissue contains more than four components, but there are distinct differences in component patterns among the various tissues. Tentative molecular weights of the three most prevalent components of rat tissues have been estimated at 135,000, 150,000, and 167,000 by sucrose density gradient centrifugation.*

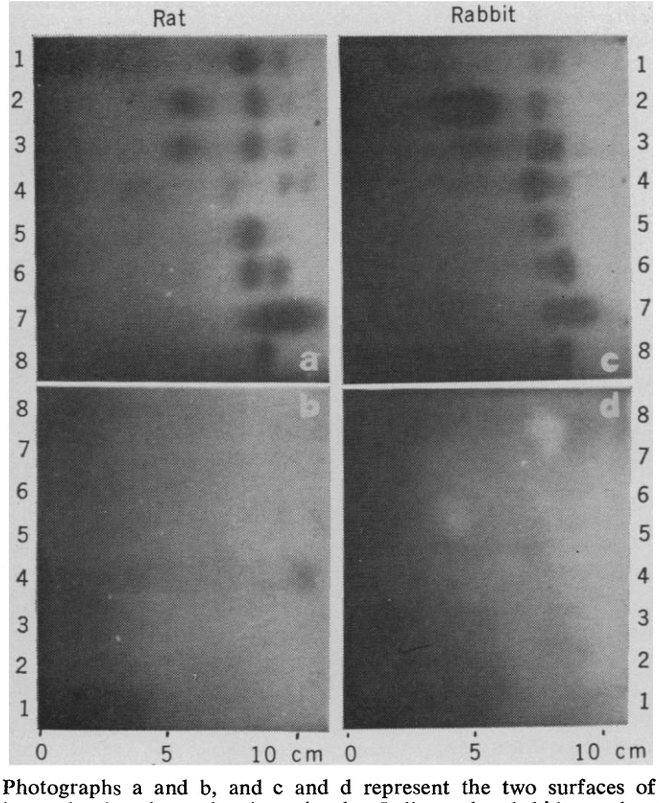
Adenosine 3′,5′-monophosphate (cyclic AMP) has been identified as the intracellular mediator of the action of multiple hormones (*1*). Tissue levels, and therefore the effective concentrations, depend not only on the rate of formation of the nucleotide (catalyzed by hormone-sensitive adenyl cyclases) but also on the rate of degradation effected by cyclic 3′,5′-nucleotide phosphodiesterase (PDE). Since the first demonstration of PDE (*2*), the enzyme has been partially purified and the enzymatic conversion of cyclic AMP to 5′AMP has been extensively studied. Conversion to 5′AMP is the only physiological mechanism known to terminate the action of the cyclic nucleotide (*3*).

There may be more than one molecular species of PDE in the supernatants of tissue homogenates of bovine brain (*4*), rat brain (*5*), rat kidney (*6*), frog bladder epithelium (*6*), and frog erythrocytes (*7*). Two lines of evidence have lead to this conclusion: (i) partial separation of activity into two or more peaks by gel filtration, and (ii) the demonstration of different kinetic parameters when the separate peaks are examined.

Starch-gel electrophoresis combined with histochemical staining designed to demonstrate specific enzyme activities has been extremely useful for the investigation of multiple molecular forms of many enzymes (*8*). This approach has been adapted to use with PDE, and a specific staining method has been designed which can be combined with electrophoretic techniques. By these means, seven different molecular forms of PDE have been demonstrated in both rat and rabbit tissues.

Adult Wistar rats and albino rabbits were killed by a blow to the head, and the organs were immediately removed. Fat and fibrous tissues were removed, and the tissue was blotted and homogenized for 1 minute in Potter-Elvehjem homogenizers in two to ten volumes (weight to volume) of ice-cold 50 m*M* tris-HCl solution at *p*H 7.4 containing 2 m*M* $MgSO_4$. The homogenates were centrifuged at 100,000*g* for 1 hour at 2°C, visible floating lipid was removed, and the remainder of the supernatant was used for analysis (*9*).

Staining for PDE activity after electrophoresis was accomplished by slicing the gel and overlaying the cut surface with a complex staining mixture in agar gel. The staining mixture was designed so that PDE converted cyclic AMP to 5′AMP, which was further converted to adenosine diphosphate (ADP) by adenosine triphosphate (ATP) and myokinase. The ATP was regenerated from the ADP so formed by the



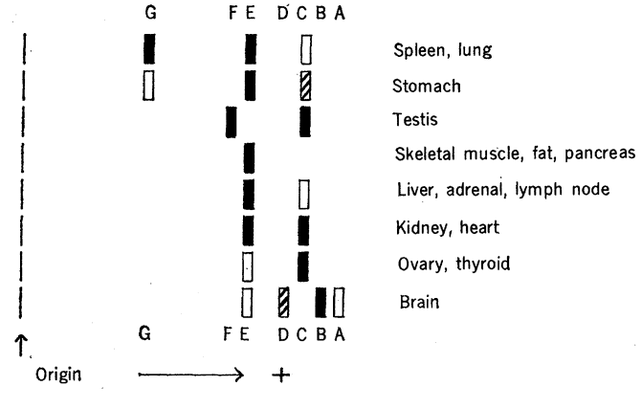


Fig. 1 (left) Starch-gel electrophoretic patterns of cyclic AMP phosphodiesterase in different tissues of one rat and one rabbit. After electrophoresis the gel was sliced, and a 0.5 percent agar gel overlay at 55°C was applied to the cut surface. The overlay gel contained 0.1*M* tris-HCl, *p*H 7.9, 0.004*M* cyclic AMP, 0.01*M* $MgSO_4$, 0.00075*M* ATP, 0.002*M* phosphoenolpyruvate, 0.05*M* KCl, 0.0003*M* $NADH_2$, 0.0005*M* $CaCl_2$ 1 unit of myokinase, 1 unit of pyruvate kinase, and 9 units of lactate dehydrogenase per milliliter. The gel was incubated at 37°C for 1 to 2 hours. The photographs were taken in longwave ultraviolet light on Polaroid film through a yellow filter. The two upper gels (a and c) were stained as described, but in the two lower gels (b and d) cyclic AMP was omitted from the staining mixture. Photographs a and b, and c and d represent the two surfaces of the same two gels, respectively. Slot 1, cardiac muscle; slot 2, lung; slot 3, spleen; slot 4, testis; slot 5, liver; slot 6, kidney; slot 7, brain; and slot 8, partially purified PDE from bovine heart (*13*). Fig. 2 (right). Rat tissue cyclic AMP phosphodiesterase activities as revealed by starch-gel electrophoresis and specific staining. The relative intensities of the bands in each tissue are indicated by the shading of the bands. Black bands indicate marked absorbance, crossed bands intermediate absorbance, and white bands weak absorbance.

conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase. Pyruvate was then reduced to lactate by lactate dehydrogenase, with concomitant oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$). When the gels were examined in ultraviolet light after incubation, the sites with PDE activity appeared as zones of absorption (NAD) on a fluorescent background ($NADH_2$).

When this technique was applied to rat and rabbit tissues, multiple forms of PDE were demonstrated (Fig. 1, a and c). Rat brain contained two major and two minor components. This agrees with previous work which demonstrated two main forms, one with high molecular weight, relatively low affinity for cyclic AMP, and somewhat higher affinity for cyclic guanosine monophosphate (GMP), and the other, a lower molecular weight species with high affinity for cyclic AMP and virtually no activity for cyclic GMP (*5*). Similarly, two forms of the enzyme have been reported for rat kidney (*6*), one of high molecular weight and low affinity for cyclic AMP, and the other of low molecular weight and high affinity for the nucleotide. For both tissues, the concentration of cyclic AMP used in the gel staining mixture was sufficiently above the K_m (Michaelis constant) of either enzyme so that both forms could be visualized. A crude particulate fraction from bovine heart apparently contained two forms of PDE, whereas a partially purified supernatant contained only a single PDE (*10*). This same partially purified preparation gives only a single band on gel electrophoresis.

A diagram of the PDE electrophoretic patterns observed in 15 different rat tissues is given in Fig. 2. Seven different bands with PDE activity have been observed and are referred to as A to G in the figure. Spleen and lung contain two major enzyme bands, E and G, and a minor band, C. Stomach contains the same three bands but with different absorbance. Testis contains two bands, C and F, with the F band appearing in no other tissue studied. Skeletal muscle, fat, and pancreas contain only a single band, E. The C and E bands are shared by many tissues, but with some differences in absorbance. The brain contains two major and two minor components. Homogenization of brain in buffer containing 0.32*M* sucrose did not change the number of bands present, and sonication of this material only increased absorbance, in agreement with previous observations (*5*). The formed elements of blood were also examined (*11*). No zones of PDE activity were observed in red cells, in accordance with previous observations (*3*). A homogeneous preparation of platelets contained only the G band, while a preparation of leukocytes contained the E band. It is thus possible that the G and E components observed in different tissues may have been due to contamination by these formed elements of blood.

The PDE patterns of identical tissues of rat and rabbit were generally similar, but with some variations in absorbance (Fig. 1). In rabbit lung, most of the activity was located within the slow component, whereas in rabbit spleen the slow component was quite weak in activity. The slow-moving component of rabbit testis was much more intense than the fast one, whereas in the rat they were of similar intensity. Similarly, rabbit kidney showed a weak slower moving component, while the rat showed equally intense absorbing bands.

To test for the specificity of the method, all gels were sliced, and half of each was overlaid with a staining mixture from which cyclic AMP had been omitted. When this was done, only very few nonspecific zones developed (Fig. 1, b and d). In rat heart there was a nonspecific band with slow mobility; in rat testis there were two nonspecific bands, one quite slow and the other faster than all others. In rabbit heart there were two slow moving, nonspecific bands. These nonspecific bands apparently represent adenosine triphosphatases that convert the ATP in the overlay gel to ADP or AMP, both of which can lead to oxidation of $NADH_2$ in the staining mixture. For this reason, all gels were stained in duplicate with and without substrate.

None of the bands appears to result from changes caused during electrophoresis. When rat spleen and rat brain were subjected to two-way electrophoresis, three and four components, respectively, were found in a straight line at a 45° angle to the directions of the runs. When strips containing bands A, B, C, D, E, and G were cut out, turned 90°, inserted into a new gel, and resubjected to electrophoresis, each component gave only a single band migrating to the original position.

When electrophoresis was performed under identical conditions but with *p*H 7.7 sodium phosphate (0.1*M* in the bridge and 0.005*M* in the gel) in place of tris-maleate, almost identical patterns occur. However, the D component of brain was less intense, and the rabbit isozymes C, E, and F were better resolved.

Estimation of the molecular weight of PDE components by gel filtration (*3*, *5–7*) have yielded values from 40,000 to 750,000, but in no case has complete separation of any component been achieved. Rat spleen and rat kidney supernatants were subjected to sucrose density gradient contrifugation (*12*) with rabbit muscle pyruvate kinase (molecular weight, 240,000) and hemoglobin (molecular weight, 68,000) as markers. After the run, fractions were collected and analyzed for PDE (*13*) and pyruvate kinase (*14*) activities and hemoglobin (*15*). Fractions showing PDE activity were subjected to electrophoresis. The components were not completely separated, but from the intensities of each of the bands it was possible to identify the fraction where a given band had its highest absorbance. Tentative molecular weights of 135,000, 150,000, and 167,000 have been assigned to the C, E, and G components.

The suggestion that multiple molecular forms of PDE exist has been confirmed by our studies. Seven different components have been observed, with no more than four present in any tissue. The fact that similar patterns have been observed in two different species suggests that this heterogeneity is general. With the growing recognition that cyclic AMP plays a major role in biological systems, knowledge of both its production and degradation are paramount to the understanding of many cellular processes.

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9. The supernatants were usually soaked into Whatman No. 3 paper inserts to be placed into the starch gel, but when low activity was encountered No. 17 paper was used instead. Horizontal starch-gel electrophoresis was carried out at 4°C for 18 hours at 4 volt/cm in a tray 22 by 13 by 0.8 cm. The gel con-

tained 13 g of hydrolyzed starch per 100 ml of buffer. The gel buffer was a 1 : 10 dilution of the bridge buffer; the bridge buffer was 0.1*M* tris, 0.1*M* maleic acid and 0.01*M* $MgCl_2$ adjusted to *p*H 7.7 with NaOH. Hydrolyzed starch was purchased from Connaught Medical Research Co., Toronto, Canada. Chemicals and agar powder type IV were obtained from Sigma Chemical Co., St. Louis, Missouri. All enzymes were obtained from Boehringer, Mannheim, Germany.

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11. Under ether anesthesia, heart puncture was performed and blood was drawn into ethylenediaminetetraacetate (EDTA) solution. The elements of blood were separated according to R. Silber, B. W. Gabio, F. M. Huennekens [*J. Clin. Invest.* **41**, 230 (1962)]. Blood cells were lysed by freezing and thawing twice.
12. Linear sucrose gradients with 17 to 38 percent sucrose in 50 m*M* tris-HCl, *p*H 7.4, and 2 m*M* $MgSO_4$ were prepared with the Beckman density gradient former. The gradients were made in polyallomer tubes (1.2 by 7 cm); total volume was 4.8 ml. Centrifugation was carried out in the SW65 K rotor of the Spinco L2-65 centrifuge at 2°C for 18 hours at 55,000 rev/min. The bottoms of the tubes were punctured with a needle, and fractions of approximately 0.17 ml were collected.
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Human Lymphocyte Antigen Reactivity Modified by Neuraminidase

Abstract. *Human lymphocytes treated with neuraminidase (from* Vibrio cholera*) are more susceptible to lysis with antiserums directed against HL-A antigens in the cytotoxicity test than are the corresponding cells incubated in buffer. Enzyme-treated cells are also lysed by antibodies other than those directed against HL-A, but control cells are not. The extra sensitivity to antibody disappears after 2 to 6 days in tissue culture.*

Blood and tissue cells of many types and from diverse species show a change in antigenicity after treatment with neuraminidase (sialidase), an enzyme known to cleave the 2,3- and 2,6-glycosidic linkages between terminal sialic acid residues and cell surface mucopolysaccharides (*1*). Currie *et al.* (*2*) showed that A_2G mouse trophoblast cells, treated with neuraminidase and injected into CBA mice, would elicit second-set responses when the mice were tested 14 days later with A_2G skin grafts. Untreated trophoblasts did not elicit this response. Currie and Bagshawe (*3*) found that four out of five methylcholanthrene-induced mouse tumors failed to grow in compatible hosts after treatment with neuraminidase, although exposure to neuraminidase did not kill the cells. Sanford reported that 6 percent of C3H mice were susceptible to the allogeneic TA3 tumor after neuraminidase treatment, compared with a 56 percent susceptibility to this tumor in mice injected with untreated cells (*4*). Gasic and Gasic (*5*) used a subline of the TA3 tumor, which has a heavy sialomucin coat at the cell surface, to demonstrate the removal of the coating after neuraminidase treatment. Kraemer (*6*) reported regeneration of the sialomucin layer of a Chinese hamster cell line 16 to 20 hours after neuraminidase treatment. This regeneration could be inhibited by the addition of puromycin to the culture medium. Although in vitro methods have been used to study the removal and regeneration of sialomucin, antigen studies have been largely inferential since they have relied heavily on in vivo demonstrations and changes in reactivity. We now report our use of the two-stage cytotoxicity test (*7*) to determine the in vitro reactivity of neuraminidase-treated human lymphocytes both before and after they were placed in tissue culture.

Lymphocytes taken for culture purposes were prepared aseptically (*7*), with buffered calcium saline (CBS) (*8*) used as a final suspension medium in place of barbital buffer.

Vibrio cholera neuraminidase (Behringwerke or Calbiochem) was used at a concentration of 2 units per 5×10^6 cells suspended in 1 ml of CBS (*9*). An equivalent volume of neuraminidase-free CBS was added to the control cells and both sets were incubated at 37°C for 20 minutes. The cells were then washed once in one volume of CBS and resuspended in barbital buffer at the original concentration of 5×10^6 cell/ml.

Both neuraminidase and sham-treated cells were also washed once in RPMI 1640 (Grand Island) before being cultured at a concentration of 1×10^6 cell/ml in this medium to which the following were added: heat-inactivated human serum (10 percent), 1 ml of glutamine per 100 ml, 25 mg of streptomycin per 100 ml, and 50,000 units of penicillin G per 100 ml. The reactions of the cells in the two-stage cytotoxicity test (*7*) were examined on day 0 and at intervals of 1 to 2 days during culture. In the cytotoxicity test, 1 μl of serum and 1 μl of lymphocytes were incubated together in a microtiter plate (Falcon) at 25°C for 15 minutes. The cells were then washed with barbital buffer, and 1 μl of rabbit serum, which had been absorbed with 0.1 ml of packed human lymphocytes or spleen cells, was added as a source of complement. The plates were incubated at 37°C for 15 minutes, trypan blue–ethylenediaminetetraacetate was added, and the percentage of dead cells was determined microscopically.

Over a wide range the amount of neuraminidase and the time of incubation required to increase lymphocyte sensitivity are not critical. Cells were tested for sensitivity by the two-stage cytotoxicity test at several dilutions of antibody. To test for nonspecific cytotoxicity the same test procedure was used except the cells were incubated in barbital buffer instead of in antiserums. One unit of neuraminidase produced severe damage to 5×10^5 lymphocytes within 20 minutes, as was evidenced by an increase in the number of cells staining with trypan blue in control tests. Although as little as 0.02 unit incubated for 1 minute would increase sensitivity in some tests, 0.2 unit for 20 minutes gave high sensitivity with little or no loss of viability and was adopted for regular use. The supernatant obtained after exposing 5×10^5 lymphocytes to 0.2 unit of neuraminidase was still active while enzyme heated at 37°C for 3 hours or in a boiling water bath for 10 minutes failed to increase lymphocyte sensitivity.

The type of suspension medium and the absorption of the complement were important to the stability of the cells. Variability in trypan blue exclusion was found when lymphocytes suspended in saline, Hanks solution, or barbital buffer were treated with neuraminidase. Good viability was more consistently obtained when the cells were prepared in CBS. The volume of suspending medium was not critical over the range of 10 to 200 μl of CBS per 5×10^5 cells when 0.2 unit of enzyme were used. Absorption of the rabbit complement with human leukemic lymphocytes or spleen cells was essential to avoid damage from a heterophile antibody in nor-