Characterization of Adenosine Deaminase from Normal Colon and Colon Tumors. Evidence for Tumor-Specific Variants†

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ABSTRACT: Differences in the molecular characteristics of the enzyme adenosine deaminase (EC 3.5.4.4) have been documented in colon tumors vs. normal colonic mucosal cells in both human and rat tissues. In normal human colon most of the adenosine deaminase exists as a high molecular weight form (mol wt > 100 000) (type A). Colon adenocarcinomas in distinction are characterized by a high proportion of species of apparent average molecular weight 35 000 (type C) and 72 000 (type B). The proportion of these forms is found to vary from tumor to tumor. Mean specific adenosine deaminase activity is also shown to be elevated in the tumor (0.0084 ± 0.0014) compared with the surrounding normal tissue (0.0052 ± 0.0011). Tumor enzymes show a strong similarity in relative substrate specificity, K_m values for adenosine, and sensitivity to inhibition by specific antibodies when compared with the normal enzyme. The type B enzyme, however, which has not been previously described in mammalian tissues, can be distinguished from the other forms by its diminished capacity to interact with antibodies to the normal human enzyme. Similar patterns of molecular weight distribution are characteristic of a kidney tumor. The kidney tumor is lacking a conversion factor protein which causes the type C enzyme to be converted to the type A enzyme, and which is found in relatively large quantities in the normal kidney. The capacity of colon and kidney tumor ADase to interact with this factor is shown to be normal. Chemically induced colon tumors in the rat demonstrate a new main electrophoretic variant of ADase (ADase I) which has the same apparent molecular weight as the normal enzyme (ca. 35 000) but a somewhat higher isoelectric point. These tumors display a specific enzymatic activity about one-half normal. ADase I, as well as a second rat tumor variant ADase II, can be distinguished from normal enzyme by substrate specificity, K_m values for adenosine and 9-β-D-arabinofuranosyl-6-aminopurine, and the nature of the antigenic determinants. ADase I and II in analytical isoelectric focusing resemble forms present in the normal rat red cell. Changes observed in the tumor ADase are discussed in terms of mechanisms involving altered or absent conversion factor, or changes in the regulation of enzyme biosynthesis.

Enzymatic deamination at the 6-amino position of a number of potent anti-tumor and anti-viral nucleosides interferes with their effectiveness as chemotherapeutic agents (Brink and Le Page, 1964, 1965; Ellis and LePage, 1965; Schabel, 1968; Plunkett and Cohen, 1975). In contrast to these analogues the 6-oxy derivatives are relatively ineffective as cytotoxic agents. The principal system for catalyzing this hydrolytic deamination is the enzyme adenosine deaminase (EC 3.5.4.4), which performs the function in vivo of catalyzing the deamination of adenosine to inosine and ammonia. Recent data strongly support the enhancement of the antimetabolic effects of these nucleosides by a combination chemotherapy involving ADase[1] inhibitors 2′-deoxycoformycin (LePage et al., 1976; Cass and Au-Yeung, 1976; Johns and Adamson, 1976) and 9-*erythro*-(2-hydroxy-3-nonyl)adenine (Plunkett and Cohen, 1975). It is therefore of obvious value to elucidate differences between ADase in normal and malignant cells which might be exploited in the rational design of cancer chemotherapy regimens involving ADase inhibitors.

Evidence has also been accumulating supporting a critical role for ADase in regulating the growth and differentiation of certain types of mammalian cells. In particular, it was first reported in 1972 that an autosomal recessive form of severe combined immunodeficiency disease is associated with a deficiency of this enzyme (Giblett et al., 1972; Dissing and Knudsen, 1972). A causal relationship between the apparent absence of this enzyme and the syndrome, which involves severe defects in both cellular and humoral immunity, has been amply documented (Meuwissen et al., 1975). Indeed, it is consistent with this conclusion that an immunosuppressive effect for ADase inhibitors has been demonstrated by their effect in prolonging the survival of allografts in mice (Lum et al., 1977; Chassin et al., 1977). Although the molecular mechanisms for these various anti-proliferative effects is not established, in vitro studies do support a high toxicity for low concentrations of adenosine against certain mammalian cell lines of lymphoid and fibroblastic origin (Ishii and Green, 1973; Green and Chan, 1973). These considerations further emphasize the importance of biochemical characterization of ADase in rapidly proliferating tissues.

A number of clearly distinguishable forms of ADase exist in nature that can be resolved on the basis of electrophoretic migration or molecular weight. In particular, three molecular weight ranges are characteristic of the enzyme in vertebrates: type A (mol wt ~ 200 000); type B (mol wt ~ 100 000); and type C (mol wt ~ 35 000) (Ma and Fisher, 1968, 1969, 1971, 1972). In man erythrocytes contain exclusively the type C form, whereas other tissues contain varying amounts of the higher molecular weight type A (Akedo et al., 1970, 1972; Edwards et al., 1971). In addition the ratio of the amount of high to low molecular weight form varies from tissue to tissue (Ma and Magers, 1975). A considerable amount of evidence supports the regulation of this ratio by a protein conversion factor, which can convert low molecular enzyme to the type

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[1] Abbreviations used are the following: ADase, adenosine deaminase; RNase, ribonuclease; *ara*-A, 9-β-D-arabinofuranosyl-6-aminopurine; formycin A, 7-amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine; APTR, 4-amino-8-(β-D-ribofuranosyl)pyrazolo[1,5-*a*]-1,3,5-triazine; MAM, methylazoxymethanol.

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A species (Akedo et al., 1970, 1972). Previous studies have suggested that an increase in the proportion of low molecular weight enzyme might be an essential feature of rapidly proliferating cells (Nishihara et al., 1973; Hirschhorn and Levytska, 1974).

A particular interest in our laboratory has been an elucidation of the biochemical characteristics of tumors of the colon, which represent one of the leading causes of cancer fatality in Western civilization (Doll et al., 1970). We describe here the appearance of new variants of ADase in surgical specimens of human colon tumors as well as in chemically induced tumors in the rat that can be distinguished from the normal enzyme by differences in size and charge, respectively. Significant but less striking differences between normal and tumor were also noted in relative substrate specificity and K_m values. In the human tumor specimens there was a large increase in the relative proportion of the lower molecular weight forms in comparison with the ratio characteristic of adjacent normal tissue. These data suggest a possible alteration in the synthesis or biological activity of the conversion protein in the malignant cell. It is anticipated that the different forms of ADase noted between patients may be of eventual usefulness in clinical evaluation of the course of the disease, as well as in the development of an effective chemotherapeutic regimen.

Experimental Section

Materials. Xanthine oxidase (crystalline suspension in ammonium sulfate; from buttermilk), nucleoside phosphorylase (crystalline suspension in ammonium sulfate; from calf spleen), bovine serum albumin, 2′-deoxyadenosine, 3′-deoxyadenosine, 6-chloropurine ribonucleoside, imidazole, MTT tetrazolium, and phenazine methosulfate were obtained from Sigma Chem. Co. (St. Louis, Mo.). Methylazoxymethanol acetate, agar (special grade), and sucrose (enzyme grade) were obtained from Schwarz/Mann (Orangeburg, N.Y.), acrylamide and bisacrylamide from Bio-Rad Labs (Richmond, Calif.), and ampholines from LKB Instruments, Inc. (Hicksville, N.Y.). Adenosine was obtained from SBR (Orangeburg, N.Y.) and [8-^{14}C]adenosine (water:ethanol solution, 1:1; 54.6 mCi/mmol) from New England Nuclear (Boston, Mass.). Formycin A was a gift from Meiji Seika Kaisha LTD Pharmaceutical Division (Kawasaki, Japan). *ara*-A was purchased from Parke-Davis & Co. (Ann Arbor, Mich.). APTR was a gift of Dr. Jack Fox and 2,6-diaminopurine ribonucleoside was a gift of Dr. George B. Brown. Molecular weight standards (ovalbumin, chymotrypsinogen, and ribonuclease) as well as all Sephadex gel filtration resins were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Thin-layer cellulose plates were obtained from Eastman Kodak (Rochester, N.Y.). Human erythrocytes were obtained from outdated units of blood supplied by the Memorial Blood Bank. Normal and tumor colon surgical specimens, as well as spleen and colon autopsy samples, were obtained through the Tumor Procurement Service at Memorial Hospital. Diagnostic evaluation of all surgical samples was performed by the Department of Pathology at Memorial Hospital.

Enzyme Assay. ADase activity was most commonly determined spectrophotometrically by modification of the method of Hopkinson and co-workers (1969) in which the inosine produced is converted to uric acid in the presence of commercial nucleoside phosphorylase (0.1 unit/mL) and xanthine oxidase (0.2 unit/mL). The rate of production of uric acid was monitored by continuous recording in a Beckman Acta III recording spectrophotometer using a millimolar extinction coefficient of 12.2 at 293 nm (Kalckar, 1947). The reaction volume of 1.0 mL contained 0.2 mM adenosine, 100 mM potassium phosphate, pH 7.0 at 37 °C. Because of contaminating ADase activity in commercial preparations of nucleoside phosphorylase and xanthine oxidase, it was necessary to substract an appropriate background blank from each determination.

Other substrates of ADase were tested for their relative substrate specificity by spectrophotometric assays employing the following millimolar extinction coefficients and wavelengths: 2′-deoxyadenosine and 3′-deoxyadenosine, 8.33 (265 nm) (Kalckar, 1947); 2,6-diaminopurine ribonucleoside, 5.15 (247 nm) (Simon, 1970); 6-chloropurine ribonucleoside, 5.32 (250 nm) (Baer et al., 1968); APTR, 1.52 (265 nm); formycin A, 6.43 (300 nm); *ara*-A, 8.28 (265 nm). The extinction coefficients for APTR, *ara*-A, and formycin A were determined in this laboratory.

For the determination of the effect of preincubation with specific antibodies on the ADase activity, a radioactivity assay was employed. Incubation with [8-^{14}C]adenosine for 15–45 min at 37 °C was performed under the conditions indicated in the individual experiments. After the reaction was stopped by boiling for 2 min, the radioactive products, inosine and hypoxanthine, were separated from the substrate on thin-layer cellulose plates using water-saturated 1-butanol: concentrated ammonium hydroxide (99:1) as the developing solvent. Both substrate and product spots were cut out and radioassayed by liquid scintillation.

A unit of activity is defined as the amount of enzyme that deaminates 1 μmol of substrate per min under the specified steady-state assay conditions. For determination of specific activity protein was quantitated by the method of Lowry (1951) using bovine serum albumin as standard.

Isoelectric Focusing. Analytical isoelectric focusing was performed in a flat bed of 5% polyacrylamide containing 2% ampholines, pH 4 to 6, and 5% sucrose using the LKB Multiphor 2117. The anode and cathode were composed of 1 M sulfuric acid and 0.50% (w/v) pH 5 to 7 ampholines, respectively. Approximately 0.02 mL of sample exhibiting an activity of 0.1–0.2 unit/mL was soaked into a 5 × 10 mm cellulose acetate strip, which was applied to the surface of the gel. Focusing was allowed to proceed for approximately 2 h to final voltage of about 900 V and a maximum power output of 40 W. Immediately after the termination of the run the gel was overlaid with a thin layer of 1% agar containing adenosine (1.5 mM), MTT tetrazolium (0.1 mg/mL), phenazine methosulfate (0.1 mg/mL), xanthine oxidase (0.0075 unit/mL), and nucleoside phosphorylase (0.005 unit/mL) dissolved in 25 mM potassium phosphate, pH 7.5, essentially as described by Spencer and co-workers (1968). After a 30–60-min incubation at 37 °C in the dark, blue bands corresponding to ADase activity appeared.

Preparative isolectric focusing was performed in the LKB Uniphor 7900 in the pH range of 4 to 6 in a 5% to 50% linear sucrose gradient (approximately 150 mL) containing 1% (w/v) ampholines. The sample to be focused was included in the heavy (50%) sucrose solution. Cathode and anode solutions were composed of 2.9% (v/v) ethanolamine in 64% sucrose and 1% (v/v) sulfuric acid, respectively. Runs were allowed to proceed for approximately 48 h to a final voltage of between 1000 and 1200 V with a maximum power output of 3 W. Fractions of approximately 1.0 mL were collected.

Preparation of Antibodies. Purified preparations of low molecular weight (type C) ADase were injected into rabbits in two injections 2 weeks apart at 0.5–1.0-mg doses in Freund's adjacent solution. After 6 weeks a similar dose was given and blood was drawn 10 days later. The serum was fractionated with ammonium sulfate to 33% and the pellet redissolved in

TABLE I: Specific Activities of Adenosine Deaminase in Normal Human Colon and Colon Tumors.[a]

Patient	Spec. act. (units/mg of protein) Normal colon[b]	Colon tumor
I	0.0024	0.0052
II	0.0016	0.0056
III	0.0065	0.0078
IV	0.0075	0.0115
V	0.0033	0.0084
VI	0.0037	0.0052
VII	0.0050	0.0064
VIII	0.0038	0.0070
IX	0.0043	0.0082
X	0.0163	0.0274
XI	0.0053	0.0049
XII	0.0026	0.0026
XIII	—[c]	0.0105
XIV	—	0.0085
XV	—	0.0066
Mean ± SEM:	0.0052 ± 0.0011	0.0084 ± 0.0014

[a] The preparations on which activities were determined were supernatants from homogenates of fresh surgical specimens. Enzyme assays were performed spectrophotometrically at 293 nm by enzymatic coupling of the inosine produced to uric acid, as described in Materials and Methods. Duplicate activity determinations generally agreed to within 5%. [b] "Normal" refers to morphologically normal areas of tissue adjacent to the tumors. [c] A dash indicates that a specimen of normal colon was not available from this patient for assay.

100 mM potassium phosphate, pH 7.0. The fractionation procedure was repeated and the preparation was then exhaustively dialyzed against 10 mM potassium phosphate, pH 7.0. No serum adenosine deaminase was detectable in this preparation.

Determination of Conversion Activity. The sample to be analyzed for conversion activity was preincubated with a partially purified preparation of human erythrocyte ADase for 25 min at 37 °C in 50 mM imidazole hydrochloride, 100 mM sodium chloride, pH 7.0. The ADase used in this assay was partially freed of contaiminants by a preparative isoelectric focusing run (see above). Samples were than applied to an Ortec Model 4200 vertical electrophoresis apparatus containing a 4.85% acrylamide–0.15% bisacrylamide gel equilibrated with 100 mM Tris–acetate, pH 7.0. The run was terminated when the bromphenol blue marker reached the bottom of the gel, and the gel was stained for ADase activity as described above for the analytical isoelectric focusing procedure.

Preparation of Tissue Homogenates. Fresh human surgical specimens were obtained from the Tumor Procurement Service of Memorial Hospital and immediately homogenized as 50% solutions in a glass homogenizer in 250 mM sucrose, 20 mM imidazole hydrochloride, pH 7.0. Assay of activity as well as gel filtration chromatography were performed as soon as feasible on the clear supernatants obtained by centrifugation at 10 000 *g* for 15 min. Rat tissues were obtained from male CFN rats (Carworth Farms, Rockland County, N.Y.) weighing between 130 and 170 g. They had been fed a diet of Purina rat chow and tap water ad lib. After sacrifice of the animal by decapitation, the appropriate tissues were excised, immediately cooled on dry ice, and homogenized as described above.

Chemical Induction of Tumors. Colon tumors were induced by intravenous injection of methylazoxymethanol acetate (35 mg/kg of body weight) (Laqueur and Spatz, 1968) into 21 day old weanlings and excised after about 3 months.

Molecular Weight Estimation. Apparent molecular weight values were estimated by chromatography on a Sephadex G-75 column (62 × 1.6 cm) equilibrated with 50 mM imidazole hydrochloride, 100 mM sodium chloride, pH 7.0. The standards employed, which were detected by their absorption at 280 nm, were bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease.

Results

ADase Specific Activity in Colon Tumors and Normal Colon. Specific ADase activity for surgical samples of several human colon tumors and the adjacent normal colons is summarized in Table I. The mean specific activity calculated for the normal colon (0.0052 unit/mg of protein) is among the lowest observed for human tissue. This result is of particular interest because of the fact that other sections of the gastrointestinal tract, in particular the duodenum, jejunum, and stomach, generally exhibit high specific activities in man and other species (Brady and O'Donovan, 1965; Ma and Magers, 1975). This activity level does appear to be a real property of the normal colon since the activity of ADase from colons of noncancer patients has also been demonstrated in our laboratory to be low. As also shown in Table I the specific ADase activity from the malignant cells was uniformly higher than from the normal colon with only two exceptions. The tumors for which no adjacent normal tissue were available were also significantly above the mean normal specific activity.

The activities obtained in the rat colon represent an interesting contrast to the corresponding data in man. The mean specific activity of several MAM-induced tumors of the descending colon was 0.114 ± 0.024 unit/mg of protein, which is an order of magnitude higher than the corresponding value obtained in man (0.0082 unit/mg of protein). This value represents a decrease in activity from that of several pooled normal rat descending colons, which was found to be 0.229 unit/mg of protein. The specific activities of the rat tumors were higher than the activities of the entire rat colon, which generally appeared in the range of 0.05–0.08 unit/mg of protein.

Tumor-Specific Variants of ADase. The tissue samples listed in Table I were analyzed for the molecular weight distribution of the ADase variants by chromatography on Sephadex G-75 superfine; the results of a typical experiment are demonstrated in Figure 1 (upper). Greater than 80% of the enzyme present in the normal colonic tissue consistently eluted at or near the void volume of this column. We have therefore classified the normal enzyme as predominantly type A, consistent with the terminology of Ma and co-workers (1968). This result is consistent with previous data which demonstrate that a number of the tissues that display low specific activities are also characterized by a high molecular weight enzyme (Ma and Magers, 1975). In all of the colorectal tumors examined except one, however, the striking result was noted that most of the activity now eluted with a substantially lower apparent molecular weight. The most common result is shown in Figure 1 (upper) where the apparent molecular weight is approximately 35 000, characteristic of the normal human erythrocyte (type C) enzyme (Edwards et al., 1971). An interesting exception was noted in one rectal tumor (patient IV, Table I), which displayed the majority of its activity as the high molecular weight form. This tumor, however, differed from the others listed in that it was not an adenocarcinoma but was epidermal in origin.

The percentage of enzyme present lower in molecular weight than type A varied from patient to patient. This value ranged from 25% to over 95% of the enzyme present as the type C

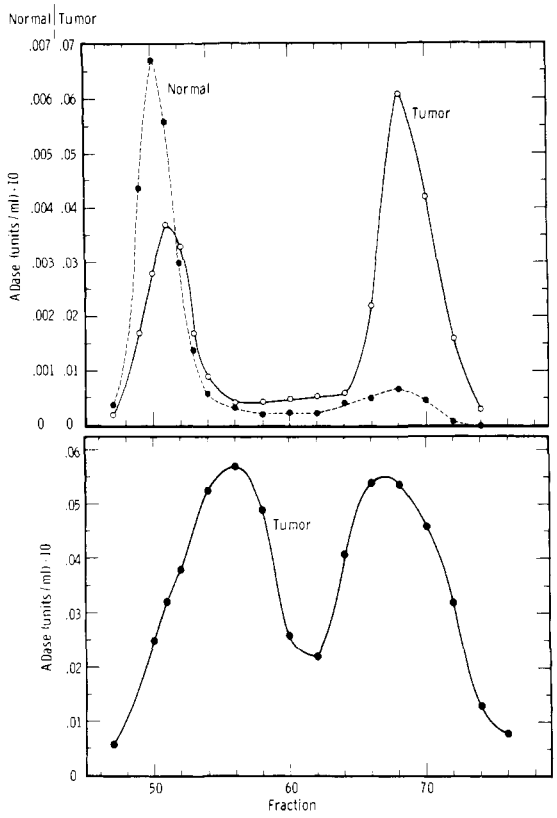

FIGURE 1: Sephadex G-75 superfine chromatography of normal human colon and colon tumor adenosine deaminase. (*Upper*) Supernatant after centrifugation of a normal colon and colon tumor homogenate from the same patient; (*lower*) supernatant after centrifugation of a colon tumor homogenate (patient different from the individual in upper trace). For each of the chromatographies, the Sephadex column (62 × 1.6 cm) was equilibrated at 4 °C with 50 mM imidazole hydrochloride, 100 mM sodium chloride, pH 7.0. Samples were applied in a total volume of 0.6 mL in 15% sucrose in the same buffer and eluted at a flow of approximately 3–4 mL/h. Fractions (0.9 mL) were collected. The void or excluded volume was determined with blue dextran 2000 and is located at approximately tube 49. The recovery of applied activity was typically 75–85%. The preparation of homogenates and spectrophotometric assay of activity by conversion to uric acid are described in Materials and Methods.

form. However, when this percentage was compared with the comparable percentage from the adjacent normal tissue from the *same* individual, the proportion of high molecular weight enzyme was always diminished in the tumor with the one exception noted.

Several of the tumor samples examined displayed an unusual ADase variant which eluted as a discrete peak at a position intermediate between the type A and type C enzymes, corresponding to an apparent molecular weight of 72 000 ± 4000. This species is shown as the first major activity peak in the elution profile of the tumor shown in Figure 1 (lower). This form has not been previously described in human tissues. We have designated this enzyme "tumor type B" since it corresponds approximately in size to the normal type B enzyme (mol wt ~ 100 000) that is common in lower vertebrates but generally absent in higher animals (Ma and Fisher, 1968, 1971, 1972; Ma and Magers, 1975). This unusual variant appears to be distinct from the minor species reported by Van der Weyden and Kelley (1976) in human splenic homogenates that exhibits a molecular weight of 114 000.

The apparent molecular weight values of the tumor variants A, B, and C were estimated by gel filtration chromatography to be greater than 100 000, 68 000–76 000, and 35 000–37 000, respectively. A more precise estimate for the molecular

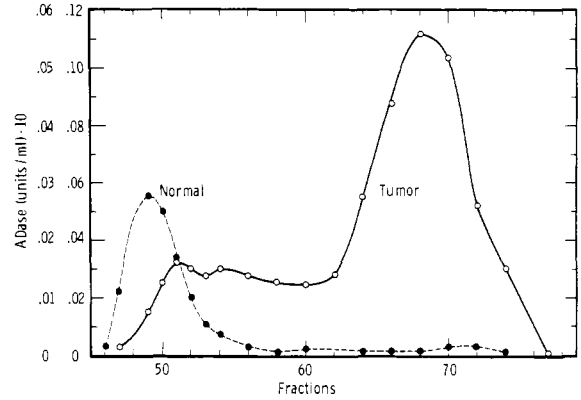

FIGURE 2: Sephadex G-75 superfine chromatography of a normal human kidney and kidney tumor adenosine deaminase. The tumor was diagnosed as a clear cell renal carcinoma. The details of the chromatography are as described in Figure 1.

weight of type A was not attempted from these chromatographies since its elution position was at or near the excluded volume of the column. The migration of this form on electrophoresis in a 7% polyacrylamide gel was virtually identical with that of normal human high molecular weight ADase from either kidney or spleen. This result suggests that the true molecular weight of this form may actually be in excess of 200 000, which is characteristic of the normal human type A enzyme (Edwards et al., 1971). Interestingly the exact elution position of each of the three species varied from patient to patient to a greater extent than anticipated from duplicate runs on the same sample. The range in values obtained for the apparent molecular weight values of types B and C may in fact represent a true biological variation.

Normal human kidney has also been reported to exhibit predominantly type A enzyme (Ma and Magers, 1975). It was therefore of interest to establish whether a similar shift toward lower molecular weight species occurred in a kidney tumor compared with adjacent normal tissue (Figure 2). A striking increase in the proportion of the type C variant, which was barely detectable in the normal tissue, was noted, similar to the results with colon (Figure 1). Although no discrete type B species was observed, a substantial amount of activity was observed to elute as a broad area in the region of 70 000 molecular weight. Also in parallel with the colon data these changes in molecular weight distribution were associated with a specific activity increase from 0.0012 (normal tissue) to 0.0064 (tumor) unit/mg of protein.

Alterations in ADase associated with chemically induced rat colon tumors have also been examined. In distinction to the human data documented above, both normal and tumor-specific rat colon ADase eluted on Sephadex G-75 with the apparent molecular weight of a type C enzyme. No evidence for the presence of higher molecular weight species was noted for either tumor or normal variants. Preparative isoelectric focusing in a sucrose gradient, however, demonstrated the presence of two electrophoretic variants (Figure 3). These species, which exhibit close p*I* values of approximately 4.85 and 4.74, have been termed tumor ADase I and II, respectively. Although the closeness of the two p*I* values did not allow complete resolution, analytical isoelectric focusing experiments indicated that areas on the ascending and descending portions of the two peaks, respectively, were essentially free of the second variant. These purer fractions were employed for further kinetic studies (see later section).

The isoelectric forms of ADase present in rat colon tumor,

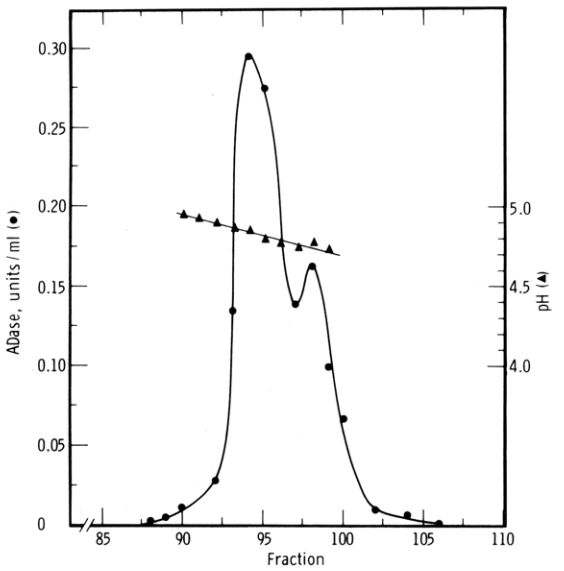

FIGURE 3: Preparative isoelectric focusing of the adenosine deaminase in a chemically induced rat colon tumor. The sample subjected to focusing was the supernatant after centrifugation of a tumor homogenate. Recovery of activity was approximately 80% of the applied units. Details of the induction of the tumor, homogenization, and preparative isoelectric focusing procedures are presented in Materials and Methods.

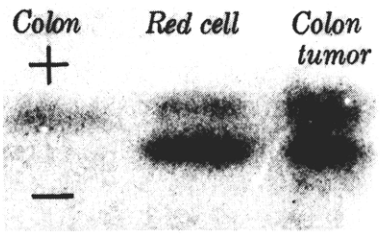

FIGURE 4: Analytical isoelectric focusing in polyacrylamide gel of the adenosine deaminase from a normal rat colon, colon tumor, and erythrocytes. The colon samples applied were the supernatants obtained after centrifugation of homogenates. Erythrocytes were washed several times with physiological saline and lysed by repetitive freezing and thawing; the hemolysate obtained after centrifugation at 10 000 *g* for 15 min was employed for the run. Other details of sample handling, isoelectric focusing, and staining for ADase activity are described in Materials and Methods.

normal rat colon, and rat red cell were compared by analytical isoelectric focusing in a polyacrylamide flat bed (Figure 4). It is clear from this experiment that the major tumor variant ADase I shown in Figure 3 is virtually undetectable in a total colon scrape. Similarly, this form was also absent in areas of normal colon from animals treated with methylazoxymethanol or 1,2-dimethylhydrazine.[2] An erythrocyte hemolysate, however, displays variants similar in isoelectric point to those characteristic of both normal and malignant colon. This similarity in electrophoretic behavior is not proof of the identity of these forms because of possible differences in uncharged areas of the molecule.

An interesting parallel between the human and rat tumor variants is their resemblance to the normal erythrocyte form in certain of their properties. Thus the low molecular weight (type C) enzyme which appears in the human tumor is the only molecular weight range found in the human erythrocyte

[2] Multiple injection of 1,2-dimethylhydrazine (Druckrey, 1967) produced tumor ADase variants identical with those observed after methylazoxymethanol treatment.

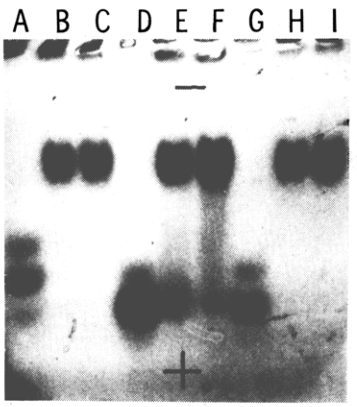

FIGURE 5: Determination of human conversion factor activity by polyacrylamide gel electrophoresis. (A) Low molecular weight human erythrocyte ADase partially purified from the lysates of a 2-1 phenotype by preparative isoelectric focusing; (B and C) same sample as in A plus 0.005 and 0.010 mL, respectively, of a normal kidney homogenate; (D) type C kidney ADase; (E and F) same sample as D plus 0.01 and 0.02 mL, respectively of a normal kidney homogenate; (G) type C colon tumor ADase; (H and I) same samples as in G plus 0.01 and 0.02 mL of normal kidney homogenate. Colon and kidney type C enzyme were obtained by Sephadex G-75 chromatography as described in Figure 1. The normal kidney homogenate indicated here is the supernatant fraction obtained after centrifugation. When this fraction was run alone, only traces of ADase activity were noted on the gel. Electrophoresis was performed in a continuous buffer system of 0.1 M Tris–acetate, pH 8.0. Other details are described in Materials and Methods.

(Edwards et al., 1971). A determination of the amount of red cell contamination by the amount of hemoglobin present (Van Kampen and Zijlstra, 1961) indicated that less than 2% of the total activity in both human and rat tumors (as well as normal tissue) could be attributed to red cell contamination. Thus the variants appear to be true tumor-specific forms.

Effect of Tumor Development on the Presence of Conversion Factor. A so-called conversion factor protein has been demonstrated which converts the low molecular weight human erythrocyte ADase to the high molecular weight species characteristic of the other tissues (Nishihara et al., 1973; Hirschhorn, 1975). Conversely, various procedures have been described that can convert the naturally occurring high molecular weight type A ADase to a 35 000 molecular weight subunit (Akedo et al., 1970, 1972). In addition, the presence of free cytoplasmic conversion protein has been demonstrated in those tissues characterized by a high proportion of type A species (Hirschhorn, 1975). It therefore was of interest to ascertain whether the increased proportion of type C enzyme in tumors was produced by a modification of the ADase molecule itself rendering it incapable of interaction with the conversion factor. The alternative hypothesis was also explored that the conversion factor was absent or biologically inactive in the malignant cell.

An assay for conversion activity was developed based on the difference in migration of the type A and C proteins in a 5% polyacrylamide gel electrophoresis (Figure 5). Upon the addition of the supernatant or a normal colon homogenate to a partially purified erythrocyte ADase preparation, no conversion to a higher molecular weight species was detected. It was therefore concluded that the conversion protein was not free in the cytoplasm of the colon, but was most probably present as a complex with ADase in the high molecular weight form of the enzyme. In distinction, when an extract of the normal kidney was added to red cell enzyme a strong conversion to the high molecular weight species occurred (Figure 5B,C). A kidney tumor was tested for this conversion activity after first

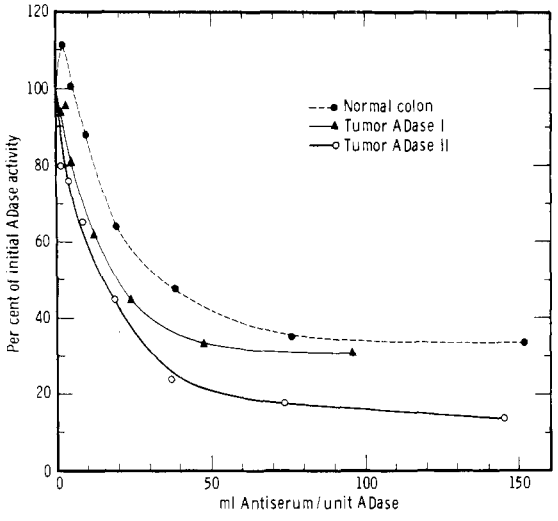

FIGURE 6: Inactivation of normal rat colon and colon tumor adenosine deaminase by antibodies to normal rat enzyme. The enzyme preparation against which antiserum was raised was ADase I partially purified (approximately 300-fold) from rat jejunum. Enzyme and antiserum were preincubated at 37 °C for 15 min and assayed for activity essentially as described in the legend to Table II. The abscissa represents the milliliters of antiserum present per unit of ADase activity in the assay medium.

purifying the conversion factor from interfering low molecular weight enzyme on Sephadex G-75. Fractions in the excluded volume corresponding to the normal elution position of the conversion factor were mixed with the erythrocyte enzyme. An electrophoretic pattern essentially identical with that shown in Figure 5A was obtained indicating an absence of free conversion activity.

Low molecular weight ADase from malignant kidney and colon were incubated with the partially purified conversion factor, and the mixture was subjected to gel electrophoresis (Figure 5D-G). In each case conversion could be produced to a species at approximately the same location as the type A enzyme. This conversion proceeded to virtually total completion under relatively mild incubation conditions. It was therefore concluded that the normal sites enabling a type C enzyme to interact with conversion factor were present in the tumor type C enzyme.

Immunochemical Studies. A comparative study was undertaken to determine whether tumor and normal enzyme could be distinguished by differences in their antigenic determinants. Antibodies were raised in a rabbit to a partially purified preparation of rat jejunal adase I previously characterized by studies conducted in our laboratory (Trotta and Balis, 1977a,b). A preparation of this antiserum was demonstrated to partially inactivate both normal colon and tumor ADase (Figure 6). However, the extent of inhibition as a function of antiserum present in the assay clearly distinguished normal and tumor forms. Interestingly, although the normal colon ADase and tumor ADase II actually exhibit similar isoelectric points (Figure 4), they are readily distinguished by this difference in their sensitivity to the antiserum.

Antibodies to low molecular weight human spleen enzyme were also tested for their reactivity against the human colon tumor types A, B, and C (Table II). Each of the forms was highly sensitive to this antiserum and was inactivated by 90% or greater. However, approximately twice as much antiserum was required to produce this effect in the type B enzyme as in type A or type C. This result is further evidence for a common catalytic subunit shared by both types A and C, which has already been implied by the interconversion of the two forms

TABLE II: The Effect of Antibodies to Human Spleen Adenosine Deaminase on the Activity of Human Colon Adenosine Deaminase.[a]

Antigen	mL of antiserum/ml of assay mix	% of initial act.
Type A ADase	0.01	100
	0.02	16
	0.04	0
Type B ADase	0.01	100
	0.02	75
	0.04	12
Type C ADase	0.01	115
	0.02	0
	0.04	33

[a] Enzyme (adjusted to 0.7 μunit per mL for each preparation) and antiserum were preincubated at 37 °C for 15 min in 25 mM imidazole hydrochloride, 50 mM sodium chloride, pH 7.0. Activity was assayed at 37 °C by incubation with 0.17 mM [8-^{14}C]adenosine in 100 mM potassium phosphate, 10 mM imidazole hydrochloride, 20 mM sodium chloride, pH 7.0, followed by separation of the radioactive products on thin-layer cellulose. Antibodies were raised in a rabbit against a 300-fold purified preparation of low molecular weight spleen ADase (type C). Additional experimental details are described in Materials and Methods.

TABLE III: Relative Substrate Specificities of Adenosine Deaminase from Normal Colon and Colon Tumors.[a]

	Relative substrate specificity				
	Rat			Human	
Substrate	Normal	Tumor I[b]	Tumor II	Normal	Tumor
Adenosine	1.00	1.00	1.00	1.00	1.00
2′-Deoxyadenosine	1.03	1.07	1.11	0.62	0.59
3′-Deoxyadenosine	0.55	0.53	0.55	0.80	0.78
ara-A	0.088	0.097	0.14	0.12	0.13
6-Chloropurine ribonucleoside	0.25	0.27	0.26	0.11	0.12
2,6-Diaminopurine ribonucleoside	0.17	0.34	0.33	0.30	0.25
Formycin A	1.98	1.87	1.88	1.12	1.20
APTR	2.68	2.49	2.46	2.39	2.03

[a] Activity with each of the substrates was determined spectrophotometrically as described in Materials and Methods and expressed relative to the value for adenosine which was set equal to 1.0. All rat preparations were partially purified by preparative isoelectric focusing. The human preparations were supernatants from homogenates that were exhaustively dialyzed against 50 mM imidazole hydrochloride, 100 mM sodium chloride, pH 7.0. Data represent the average of at least two independent determinations. [b] "Tumor I" and "tumor II" refer to the cathodal and anodal forms, respectively, of the two tumor variants shown in Figure 4.

promoted by the protein conversion factor. The sensitivity of normal colon ADase, which is predominantly type A enzyme (Figure 1 (upper)), to this antiserum was not distinguishable from that observed for the type A tumor variant.

Characterization of Selected Kinetic Parameters. A comparative study was undertaken of the relative substrate specificity of the tumor and normal ADase from rat and man (Table III). For the human enzyme the specificities against various substrates were virtually indistinguishable. For the rat both 2,6-diaminopurine ribonucleoside and *ara*-A appeared to be better substrates for tumor enzymes.

Selected K_m values were also determined for these various

TABLE IV: Selected K_m Values for Adenosine Deaminase from Normal Colon and Colon Tumors.[a]

	Substrate	K_m values (mM) Normal colon	Colon tumor
A. Rat ADase	Adenosine	0.045 ± 0.003[b]	0.021 ± 0.004 (I)[c] 0.093 ± 0.006 (II)
	ara-A	0.065 ± 0.012	0.130 ±0.013 (I) 0.150 ± 0.010 (II)
B. Human ADase	Adenosine	0.039 ± 0.002	0.045 ± 0.003

[a] Enzyme activities were determined spectrophotometrically by following uric acid production at 293 nm with adenosine as substrate in a coupled enzymatic assay, or as by monitoring the decrease in absorbance at 265 nm with *ara*-A as substrate; details are described in Materials and Methods. All rat preparations were partially purified by isoelectric focusing in a sucrose gradient over the pH range 4 to 6. The human preparations represented the supernatant fractions from tissue homogenates, which were exhaustively dialyzed against 50 mM imidazole hydrochloride, 100 mM sodium chloride, pH 7.0. The human normal colon preparation was obtained from autopsy material; the human tumor was a surgical specimen. K_m values were determined from Eadie-Hofstee or Lineweaver-Burk kinetic analysis. [b] ±SD. [c] I and II refer to the cathodal and anodal forms, respectively, of the two tumor variants shown in Figure 4.

forms and are tabulated in Table IV. K_m values for adenosine were identical for the human tumor and normal tissue. However, the rat K_m values for adenosine for variants I and II were demonstrated to be distinguishable from the corresponding values for the normal enzyme. Similarly the K_m value for *ara*-A for each of the two tumor forms was about double that for the normal rat enzyme. This result indicates that, at a higher substrate concentration of *ara*-A than actually employed in the relative substrate specificity studies, the difference between normal and tumor would be magnified.

These data can be interpreted as indicating no substantial kinetic difference between the human normal and malignant forms, while some significant differences exist among the rat variants. Further study at other values of pH, ionic strength, temperature, and so forth may as yet reveal more substantial differences.

Discussion

The data presented here document striking changes in the physicochemical characteristics of adenosine deaminase associated with tumor development in the colon. Human adenocarcinomas were characterized by a large proportion of type C (mol wt 35 000) enzyme. The latter form was frequently only barely detectable or a relatively minor component of the adjacent normal tissue, which was characterized by a high molecular weight type A enzyme. The only exception to this trend was noted in a tumor of epidermal origin, which suggests the interesting possibility that these results are an expression of certain cell types. This shift in the molecular weight distribution was accompanied frequently, but not always, by a substantial increase in specific ADase activity. It is notable that this pattern is also generally associated with the distribution of ADase in normal tissues. Thus, those tissues with relatively high specific activity generally have a greater proportion of low molecular weight ADase than those that exhibit lower activities (Ma and Magers, 1975). In spite of the increase in specific activity in the colon tumor, however, this value is still one of the lowest reported for human tissues.

One mechanism which can be considered is an alteration in the synthesis and/or complexing capability of the conversion factor protein. Previous data have suggested that this protein is responsible for the production of the various tissue-specific high molecular weight adenosine deaminases by direct interaction with the low molecular weight form (Nishihara et al., 1973; Hirschhorn, 1975). This interaction apparently results in the incorporation of this factor into the subunit structure of the molecule. Studies presented here indicate that conversion activity is lost in cancerous tissue of the kidney, a result which has also been noted previously by Nishihara and associates (1973) in a cancerous lung. A change in the biosynthesis of the factor or a modification of its structure so that it can no longer interact properly with ADase represents possible explanations for these data. In either case the result would be an increase in specific activity if the inherent activity of the low molecular weight ADase exceeded that exhibited by the converted enzyme. Although one report (Akedo et al., 1972) does conclude that the specific ADase of the type C enzyme is in fact several fold higher than that of the type A variant, data obtained in our own laboratory suggest that the addition of conversion factor to low molecular weight enzyme does not substantially influence enzymic activity. The true function of the conversion factor must be considered as yet unknown.

The absence or modification of conversion factor may be a property of the malignant cell. Alternatively there may be some defect in this cell that produces an increase in the synthesis of the enzyme itself or a decrease in its degradation. Recent data from our laboratory suggest the regulation of ADase activity in normal mammalian cells by one of these mechanisms (Trotta et al., manuscript in press, 1978). We have observed that in response to infusion of the inhibitors 9-*erythro*-(2-hydroxy-3-nonyl)adenine and 9-β-D-arabinofuranosyl-6-hydroxylaminopurine, ADase activity levels in certain normal mouse tissues undergo large increases which are dose dependent. The ADase activity in SV 40 sarcoma cells has also been shown to be capable of a similar response (Trotta and Balis, unpublished observation). Thus if the amount of enzyme protein is stimulated in the malignant colon, the increased specific ADase activity, as well as the loss of free conversion factor, which is presumably complexed with ADase, is readily explained. A similar mechanism may explain the 45–70-fold stimulation reported to be associated with a dominantly transmitted hemolytic anemia (Paglia et al., 1970; Valentine et al., 1977).

Data presented by Hirschhorn and Levytska (1974) provide evidence that some normal cells that are rapidly proliferating may also undergo alterations in ADase similar to those described here for the malignant cell. These authors have demonstrated a decrease in the proportion of high molecular weight type A ADase when peripheral blood lymphocytes were stimulated by mitogens. After 72 h of stimulation of lymphocytes with phytohemagglutinin the low molecular weight ADase became the predominant form. However, the mechanism for this effect may in fact be distinct from that responsible for similar effects in malignant cells since no increase in ADase activity was associated with the mitogen stimulation.

The high proportion of the 72 000 molecular weight variant observed in a number of colon tumors is of special interest since this form has not been previously described in mammalian tissues. The difference in its sensitivity to inhibition by antibodies to normal human enzyme suggests the appearance of new antigenic determinants. Possible mechanisms for its production include: (1) the de-repression or activation of a distinct gene; (2) a modification of a preexisting low molecular weight protein which promotes dimerization; or (3) interaction

of the low molecular weight enzyme with a modified form of conversion factor which may be related to the increased proportion of type C enzyme.

The data obtained in the rat also support the conclusion that specific changes in ADase are associated with tumorigenesis. In distinction to the observations with human colon tumors, however, the rat tumors were characterized by a form of ADase with a higher p*I* value than the normal enzyme, but with substantially the same elution pattern on gel filtration. It is also notable that the specific activity characteristic of the tumors of the colon was significantly less than the activity in normal descending colon, in distinction to the increased activity observed in human tumors. These opposing results suggest that the activity changes per se may actually be incidental to more fundamental changes in function associated with the formation of new variants. Such changes might result, for example, as a consequence of an alteration in the interaction of adenosine deaminase with other cellular components. It may be relevant that evidence does exist for an interaction of ADase with the plasma membrane (Schrader et al., 1972; Mustafa et al., 1972; Agarwal and Parks, 1975).

It is hoped that the data presented here might be useful in the development of a rational approach to the chemotherapy of colon cancer. The fact that forms of ADase seem to be tumor-specific makes plausible the development of corresponding tumor-specific inhibitors. A combination of such an inhibitor with an anti-tumor nucleoside which is normally inactivated by deamination would be expected to enhance greatly the specificity of the drug. Strong precedent already exists for this type of chemotherapeutic approach. Thus, the combination therapy of 9-*erythro*-(2-hydroxy-3-nonyl)adenine with the antimetabolite 9-β-D-arabinofuranosyl-6-aminopurine has been shown to extend the survival time of mice bearing the Ehrlich ascites tumor (Plunkett and Cohen, 1975), while 2′-deoxycoformycin greatly potentiates the cytotoxicity of this drug against L1210 leukemic cells (Cass and Au-Yeung, 1976; Johns and Adamson, 1976). The critical role that adenosine and certain of its metabolites play in the control of the growth and differentiation of certain mammalian cells provides a further basis for this type of approach (Green and Chan, 1973; Ishii and Green, 1973).

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Purine Nucleoside Phosphorylase from Human Erythrocytes: Physicochemical Properties of the Crystalline Enzyme†

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ABSTRACT: The major physicochemical properties of human erythrocytic purine nucleoside phosphorylase (PNPase) have been described. The molecular weight, estimated by ultracentrifugation, molecular sieving and sucrose density gradient centrifugation, ranged from 87 000 to 92 000. Other physical constants of erythrocytic PNPase were: sedimentation coefficient ($s_{20,w}$), 5.4 S obtained by sedimentation analysis and 5.5 S by the sucrose density gradient procedure; Stokes radius, 38 Å; calculated diffusion coefficient ($D_{20,w}$), 5.7×10^{-7} cm^2 s^{-1}; frictional ratio, 1.29; and partial specific volume calculated from amino acid analysis, 0.73 cm^3 g^{-1}. The CD spectra of the human erythrocytic and bovine spleen PNPases were almost identical and indicated a very low α-helical content. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the molecular weight of the PNPase subunit is 30 000 ± 500. These results corroborate earlier reports that the native enzyme is a homologous trimer. Comparative studies with crystalline bovine spleen PNPase confirmed that it is also a trimer but is somewhat smaller than the human erythrocytic enzyme with a molecular weight of about 86 000.

The purification, crystallization, and some properties of human erythrocytic purine nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) have been reported earlier (Kim et al., 1968a,b; Sheen et al., 1968; Agarwal and Parks, 1969, 1971; Agarwal et al., 1975), including a recent review of the literature by Parks and Agarwal (1972). Substrate binding studies (Agarwal and Parks, 1969) and the electrophoretic patterns of rare allelic variants (Edwards et al., 1971) have indicated that the enzyme consists of three catalytically active subunits. A trimeric structure has been demonstrated for the PNPase[1] from bovine spleen (Edwards et al., 1973); Chinese hamster liver, kidney, and V79 tissue culture cells (Milman et al., 1976), and chicken liver (Murakami and Tsushima, 1976), with the last possessing subunits of two different sizes. Hybrids formed from human and mouse liver PNPases suggest that each of these has three electrophoretically identical subunits (Edwards et al., 1971). However, trimers are rare among enzymes (Klotz et al., 1975) and a variety of quaternary structures has been reported for PNPases from other sources. Rabbit liver PNPase is monomeric whereas bovine brain PNPase is dimeric (Lewis and Glantz, 1976a,b). The enzymes from *E. coli* and *S. typhimurium* have six subunits of equal size (Jensen and Nygaard, 1975). The present report offers further evidence for the trimeric structure of human erythrocytic PNPase, describes some of the important physicochemical properties of the enzyme, and compares them with those of the crystalline bovine spleen PNPase. A preliminary report of these studies has appeared earlier (Agarwal et al., 1973).

Materials and Methods

Materials. Peroxidase (horseradish) was purchased from P-L Biochemicals, Milwaukee, Wis., and alcohol dehydrogenase (crystalline, horse liver), creatine kinase (crystalline, rabbit muscle), and cytochrome *c* (horse heart type VI) were obtained from Sigma Chemical Co., St. Louis, Mo. Chymotrypsinogen A, ovalbumin, ribonuclease, blue dextran 2000, and Sephadex were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Purine nucleoside phosphorylase (crystalline, bovine spleen) and lactate dehydrogenase (crystalline, beef heart) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and crystalline human erythrocytic PNPase was prepared according to the method described earlier (Agarwal and Parks, 1969).

Methods. PNPases were assayed by the coupled xanthine oxidase method of Kalckar (1947) as modified by Kim et al. (1968a). Lactate dehydrogenase, alcohol dehydrogenase, and creatine kinase activities were measured spectrophotometrically by the methods of Kornberg (1955), Bonnichsen and Brink (1955), and Tanzer and Gilvarg (1959), respectively. Peroxidase activity was determined by following the rate of decomposition of hydrogen peroxide in the presence of *o*-dianisidine as hydrogen donor essentially as described in the Manual of Worthington Biochemical Corp., Freehold, N.J. (1968). The increase in color development was followed spectrophotometrically at 460 nm. Cytochrome *c* and chymotrypsinogen were determined by following absorbancy at 410 and 280 nm, respectively.

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[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoate); PCMB, *p*-chloromercuribenzoate; PNPase, purine nucleoside phosphorylase; $NaDodSO_4$, sodium dodecyl sulfate.

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