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MULTIPLE FORMS OF HUMAN ADENOSINE DEAMINASE

II. ISOLATION AND PROPERTIES OF A CONVERSION FACTOR FROM HUMAN LUNG

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SUMMARY

1. A conversion factor, which can convert the small form (E_S; 47 000 mol. wt) of human adenosine deaminase (EC 3.5.4.4) to the large form (E_L; 230 000 mol. wt), was purified about 600-fold from normal human lung by $(NH_4)_2SO_4$ fractionation, Sephadex and DEAE-cellulose chromatography, and preparative acrylamide electrophoresis.

2. The purified preparation, heat labile and free from the deaminase activity, exhibited upon disc electrophoresis a single protein band associated with the converting activity and also showed a maximum absorption at 280 nm. Its molecular weight was found to be 139 000 when judged by gel filtration with Sephadex G-200.

3. The factor did not require sulfhydryl compounds nor bivalent metal ions for exerting its activity.

4. Results of quantitative analysis of the rate of formation of E_L from known amounts of conversion factor and E_S, immunochemical characterizations of the two forms of the enzyme in relation to conversion factor by using antiserum against the factor, and polyacrylamide electrophoresis of E_L and the factor in the presence of sodium dodecyl sulfate strongly suggest that E_L is a complex of E_S and conversion factor.

5. No detectable activity of conversion factor was demonstrated in an extract of lung cancer tissue.

INTRODUCTION

As described in our previous reports[1,2], we established the presence of two forms of human adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) which were

Abbreviations: PMS, phenazine methosulfate; PCMS, *p*-chloromercuriphenylsulfonate; MTT tetrazolium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide.

markedly different in their molecular sizes. Determination of the molecular weights using a calibrated Sephadex G-200 column[3] resulted in the values of 230 000 and 47 000 for the large form (E_L) and the small form (E_S) of the enzyme, respectively. Similar values were obtained by sucrose density gradient centrifugation[4] (225 000 and 44 000). It has been revealed with both enzyme preparations purified to essential homogeneity that E_S is considerably less stable than E_L, but that E_S is 4 to 5 times greater in molecular activity (units of activity per mole of enzyme) than E_L. Apart from these properties, however, the two forms were virtually indistinguishable catalytically, and they were also indistinguishable by immunochemical neutralization using antisera against the two enzyme species. Thus no direct evidence indicating the distinct physiological roles of the two forms of this particular enzyme has been obtained, although the fact that cancerous tissues of the lung and the liver contain fairly large amounts of E_S which is hardly detected in the corresponding normal tissues seems to suggest their distinct roles.

Interestingly, an apparent interconversion between E_L and E_S was demonstrated by gel filtration with Sephadex G-200 when E_L was treated with guanidine hydrochloride or when E_S was incubated with normal lung supernatants (see refs. 1 and 2). By the latter finding we assumed the existence of unknown factor(s) capable of stimulating the conversion of E_S to E_L and designated the factor as conversion factor.

The present paper describes the purification and properties of conversion factor as well as the results of studies on the mechanism of action of the factor toward E_S and further on the structural relationship between E_L and E_S.

MATERIALS AND METHODS

Materials

Human tissue specimens were obtained by autopsy and immediately stored at −20 °C until use. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT tetrazolium), phenazine methosulfate (PMS), *p*-chloromercuriphenylsulfonate (PCMS) and trypsin inhibitor from soybean (type I-S) were obtained from Sigma Chemicals Co. Myoglobin (horse skeletal muscle) was purchased from Miles–Seravac, Ltd. Xanthine oxidase (EC 1.2.3.2) (approx. 0.4 unit/mg) and nucleoside phosphorylase (EC 2.4.2.1) (25 units/mg) were from Boehringer Mannheim Biochemicals. Sources of other major compounds used here were described previously[2].

Assay methods

Adenosine deaminase activity and protein concentrations were determined as reported previously[2]. The definition of the enzyme unit and specific activity is also the same[2].

The method used for measuring the activity of conversion factor quantitatively was as follows; 0.25 unit of stomach E_S, which had been purified through the initial four steps of the purification[2] (specific activity; 2.0–8.0), and an appropriate amount of a sample to be tested were mixed in 30 mM phosphate buffer (pH 7.4) in a total volume of 0.5 ml, followed by incubation at room temperature (20–25 °C) for 1 h. The mixture was then loaded on a Sephadex G-200 column (1.6 cm × 24.5 cm) previously equilibrated with 30 mM phosphate buffer (pH 7.4) containing 5 mM 2-mercaptoethanol. The column was washed with the same buffer at a flow rate of 8 ml/h

and 1-ml fractions were collected. Adenosine deaminase activity of each fraction was measured colorimetrically[2]. A satisfactory and reproducible separation of the two forms of the deaminase is attainable under these conditions and more than 90% of the enzyme applied is usually recovered. When a sample to be tested has an appreciable E_L activity, the sample was incubated without adding the E_S sample and subjected to separation with the Sephadex column. The enzyme units recovered in the E_L fraction were then subtracted from those obtained with the mixture of E_S and the sample. The units of activity of conversion factor were calculated according to the following equation;

$$(F_L/F_L + F_S) \cdot 100 = \text{units of conversion factor}$$

where F_L and F_S represent the total enzyme units in the fractions comparable to E_L and E_S, respectively. Specific activity of conversion factor is expressed as units per mg of protein.

A more rapid and convenient semi-quantitative test for the activity can be achieved by thin-layer polyacrylamide gel electrophoresis followed by activity-staining for the deaminase; appropriate amounts of samples to be tested were pre-incubated at room temperature for 1 h with 0.05-unit aliquots of the stomach E_S in a total volume of 0.10 ml at pH 7.4. Then approx. 30-μl aliquots of the mixtures were subjected to the electrophoresis[2] and subsequently activity-staining as described below. The samples having the converting activity produce stained spots in the location of E_L in addition to those in the location of E_S. This method is very useful for testing the activity of a large number of samples in such cases as chromatographic experiments.

Staining of adenosine deaminase activity

The method of staining of adenosine deaminase activity on polyacrylamide gel plates was essentially the same as reported by Spencer *et al.*[5]. The staining mixture consisted of 40 mg of adenosine, 10 mg of MTT tetrazolium, 10 mg of PMS, 20 μl of xanthine oxidase (0.08 unit), 20 μl of nucleoside phosphorylase (0.50 unit) and 1 g of special agar-Noble (Difco Lab.) in 100 ml of 25 mM phosphate buffer (pH 7.0). The mixture lacking the enzymes, MTT and PMS, was heated at 100 °C for about 20 min and cooled down to around 45 °C. The omitted components were then mixed rapidly with the heated mixture, and it was overlaid on the polyacrylamide gel plate. After incubation at 37 °C for certain periods of time, purple-colored spots are visualized at the location of adenosine deaminase.

Preparation of antibody

A conversion factor preparation of the highest purity (0.81 mg protein in 1.3 ml) was emulsified with 2.6 ml of Freund's complete adjuvant (Difco Lab.), followed by intramuscular injection to a male rabbit at several depots. One month later a blood sample (100 ml) was collected by cutting an ear vein. The γ-globulin fraction was prepared by ammonium sulfate precipitation (0–33% saturation). The precipitated protein was dissolved in and dialyzed against 0.9% phosphate-buffered saline (pH 7.4) and was thereafter stored at 0 °C.

RESULTS

Purification of conversion factor from human lung

Preliminary experiments revealed that the activity of conversion factor was lost completely when the lung supernatant was heated at 60 °C for 10 min, but that the activity was not affected by exhaustive dialysis. Since these heat-labile and non-dialyzable features of this factor seemed to suggest that it may be protein-like in nature, we decided to adapt general isolation techniques for protein to the purification of the factor. All procedures were carried out at 0–4 °C.

Step 1. Extraction of the factor. Frozen lung tissues (560 g wet weight) were thawed overnight in the cold room and then washed extensively with cold distilled water to remove blood as thoroughly as possible. The tissue material was minced using an electric meat grinder and the minced tissue was homogenized in 2 vol. of 30 mM phosphate buffer (pH 7.4) using a Waring blendor. The homogenate was centrifuged at 39 000 × *g* for 20 min and the precipitate was discarded.

Step 2. $(NH_4)_2SO_4$ fractionation. To the supernatant fluid (1200 ml), 468 g of crystalline $(NH_4)_2SO_4$ were added in small portions with stirring to give a final saturation of 60%. After the mixture was stirred for an additional 2 h, it was centrifuged at 39 000 × *g* for 20 min. The resulting supernatant fluid (1270 ml) was brought to 80% saturation with $(NH_4)_2SO_4$ by adding 182 g of the salt slowly under stirring. The precipitated protein obtained by spinning was dissolved in and dialyzed against the phosphate buffer. By this procedure more than 90% of E_L present in the crude extract was eliminated from the obtained fraction and then the existence of the conversion factor became more valid.

Step 3. Gel filtration with Sephadex G-100. The dialyzed solution (72 ml) was loaded on a Sephadex G-100 column (4.5 cm × 45 cm) previously equilibrated with the phosphate buffer. The column was eluted with the same buffer at a flow rate of 45 ml/h and 15-ml fractions were collected. The converting activity was tested by the qualitative assay method (see Materials and Methods). Fractions 14–30 were combined and concentrated by adding solid $(NH_4)_2SO_4$ to 80% saturation.

Step 4. Gel filtration with Sephadex G-200. After desalting, the sample (73 ml) from Step 3 was applied to a Sephadex G-200 column (6 cm × 36 cm) which had been equilibrated with the phosphate buffer. The column was eluted with the same buffer at a flow rate of 30 ml/h. Fractions of 15 ml were collected. Fractions 29–43 were pooled and concentrated with $(NH_4)_2SO_4$ (80% saturation), followed by dialysis against 5 mM phosphate buffer (pH 7.4).

Step 5. DEAE-cellulose chromatography. The dialyzed solution (18 ml) was applied to a DEAE-cellulose column (3 cm × 25 cm) previously equilibrated with 5 mM phosphate buffer (pH 7.4). The column was first washed with the same buffer at a flow rate of 60 ml/h and 10-ml fractions were collected. Elution was made by a linear gradient of 5–100 mM phosphate buffer (pH 7.4) in a total volume of 600 ml. Conversion factor emerged from the column at a phosphate concentration of about 30 mM. Fractions 44–70 were combined and concentrated with $(NH_4)_2SO_4$ (0–80% saturation). The precipitate obtained was redissolved in and dialyzed against 30 mM phosphate buffer (pH 7.4).

Step 6. Second gel filtration with Sephadex G-200. The dialyzed solution (7.9 ml) was again passed through a Sephadex G-200 column (3 cm × 38 cm) which had been

equilibrated with the buffer described above. The column was washed with the same buffer and 5-ml fractions were collected. Flow rate was maintained at 20 ml/h. Fractions 12–20 were combined and concentrated to 4.1 ml by ultrafiltration using Amicon Diaflo membrane PM-10.

Step 7. Second DEAE-cellulose chromatography. The sample of Step 6 previously dialyzed against 5 mM phosphate buffer (pH 7.4) was applied to a DEAE-cellulose column (1.6 cm × 24 cm) which was then washed successively with 200 ml each of 5 mM, 20 mM, 30 mM and 40 mM phosphate buffer (pH 7.4). Fractions of 5 ml were collected. More than 80% of the originally applied activity was found in the fractions eluted with 30 mM buffer but the fractions eluted with 40 mM buffer also contained an appreciable activity of the factor, so that these fractions were combined and concentrated by ultrafiltration to 2.5 ml.

Step 8. Preparative acrylamide gel electrophoresis. The concentrated sample was subjected to the electrophoresis according to the same method as described previously[2]. The eluates from the macerated gel segments associated with the activity were concentrated by ultrafiltration and finally dialyzed against 30 mM phosphate buffer (pH 7.4). The purified preparation was water-clear and was free from adenosine deaminase activity. The converting activity is so stable that no significant loss of activity was noted when stored at 0 °C for one month. The results obtained in the purification are summarized in Table I.

TABLE I

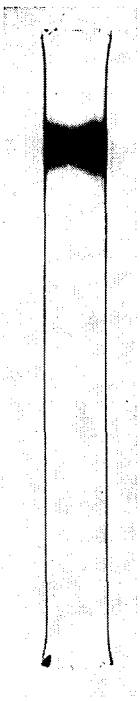
SUMMARY OF PURIFICATION OF CONVERSION FACTOR FROM HUMAN LUNG

Purification step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
1. Crude extract	14628	10240	0.7	100
2. $(NH_4)_2SO_4$ 60–80%	4986	8476	1.7	82.8
3. Sephadex G-100	2837	8227	2.9	80.3
4. Sephadex G-200	353	6707	19	65.5
5. DEAE-cellulose	56	6664	119	65.1
6. Sephadex G-200	34	6562	193	64.1
7. DEAE-cellulose	11	4158	378	40.6
8. Polyacrylamide electrophoresis	3.6	1476	410	14.4

Properties of purified conversion factor

The degree of purity of the final preparation of conversion factor was tested by disc polyacrylamide gel electrophoresis according to Davis[6]. A single stainable protein band was observed as illustrated in Fig. 1. Thin-layer acrylamide gel electrophoresis[2] also exhibited a single protein band which was ascertained to be associated with the converting activity when it was tested by the qualitative assay after eluting the protein from the gel.

The ultraviolet absorption spectrum of the purified sample showed a sharp and symmetrical peak having a maximum absorption at 280 nm (Fig. 2). The 280/260 ratio was calculated to be 1.64, indicating the nucleic acid content of the preparation to be less than 0.25% according to Layne[8]. Although carbohydrate and lipid con-



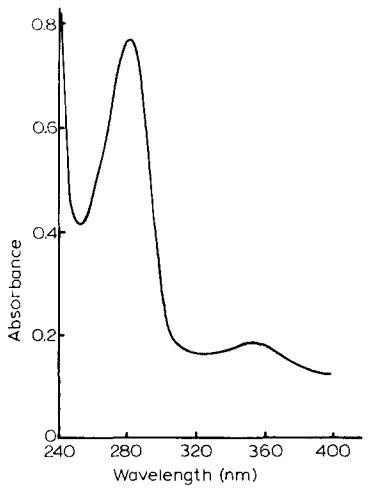


Fig. 1. Disc gel electrophoresis of purified conversion factor. Electrophoresis was carried out with 100 μg of the most purified sample of the factor using 7.5% polyacrylamide gel in Tris–glycine buffer (pH 8.9) for 1.5 h at 3 mA per tube. Protein was stained with 0.05% Coomassie blue[7]. Direction of migration is to the bottom.

Fig. 2. Ultraviolet absorption spectrum of purified conversion factor. Cary recording spectrophotometer, Model 14 was used.

tents have not yet been determined, due mainly to lack of enough sample for this purpose, evidence so far obtained is in support of the conversion factor being a protein.

According to the method of Andrews[3], the molecular size of conversion factor was studied with a calibrated Sephadex G-200 column (1.6 cm × 24.5 cm) equilibrated with 30 mM phosphate buffer (pH 7.4). The column was washed with the same buffer at a flow rate of 5 ml/h and 1-ml fractions were collected. Ovalbumin, bovine serum albumin and human γ-globulin were used as standards, and elution volumes of these standard proteins and conversion factor were measured by the absorbance at 280 nm. By plotting the elution volume of each standard protein against the log of its molecular weight, the molecular weight of the conversion factor was calculated to be 139 000. Any other methods for determining the molecular weight more accurately have not yet been carried out.

The conversion factor does not apparently require thiol compounds nor bivalent metal ions for its activity; preincubation of a sample of the factor (Step 7 in Table I) with 1 mM PCMS did not significantly affect the activity. Likewise, such an added thiol compound as 2-mercaptoethanol at a concentration of 5 mM had little effect. Exhaustive dialysis of the sample against 1 mM EDTA-containing buffer caused no

inactivation of the factor. Similarly, $MgCl_2$ and $MnCl_2$ (1 mM each) showed neither inhibitory nor stimulatory effect on the activity. ATP (0.25 mM), AMP (0.5 mM), adenosine (0.1 mM), inosine (0.1 mM) and hypoxanthine (0.1 mM) had also no effect.

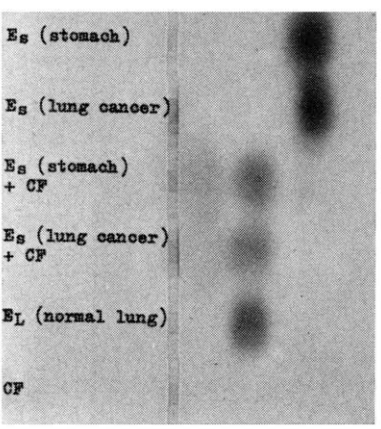
The conversion factor appears to have no organ-specificity with respect to its substrate, E_S; conversion factor purified from lung, as well as from liver, was able to convert the E_S preparations either from stomach or from lung cancer tissues.

Identification of the reaction product

The product formed by incubating E_S from the stomach with the conversion factor shows the same elution profile on Sephadex G-200 chromatography as that of the native lung adenosine deaminase (E_L) in so far as judged by the deaminase activity[2]. Upon thin-layer polyacrylamide electrophoresis (pH 8.4) the reaction product also showed the same migration as that of the native lung E_L (Fig. 3). In addition, no apparent differences in such catalytic properties as optimal pH, substrate specificity, metal and thiol requirements and K_m values were found between the native E_L and the reaction product. Thus all the data obtained so far in this regard support the identity of the product with the native E_L.

Time course of the conversion reaction

Two different amounts (17 and 51 units) of the purified conversion factor were incubated at room temperature for various periods of time with 0.70-unit aliquots of the E_S sample and the amounts of E_L formed were estimated by Sephadex G-200 chromatography. In both cases, the formation of the product reached a maximum



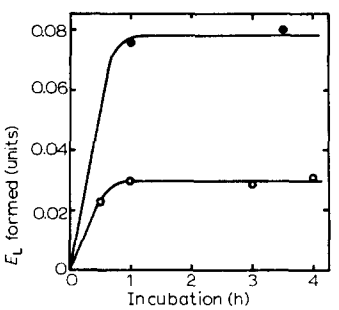


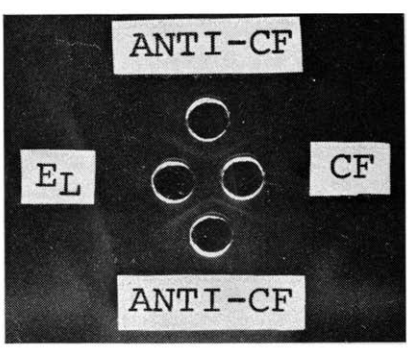
Fig. 3. Electrophoretic comparison of the enzyme species formed *in vitro* from the small forms of adenosine deaminase (E_S) in the presence of conversion factor with the native large form of the enzyme (E_L). 0.01-unit aliquots of E_S preparations partially purified either from stomach or from lung cancer tissue were incubated for 1 h with 13.4 units of conversion factor in a total volume of 0.10 ml and about 20-μl aliquots of the mixtures were subjected to 5% polyacrylamide gel electrophoresis in Tris–borate buffer (pH 8.4, $I = 0.05$) at 20 mA for 2 h. The E_S samples (0.002 unit) and conversion factor (1.9 units) were separately applied to the gel as controls. After electrophoresis adenosine deaminase activity was stained as described in Materials and Methods.

Fig. 4. Time course of the conversion reaction in the presence of two different amounts of conversion factor. ○—○, with 17 units of conversion factor; ●—●, with 51 units of conversion factor. Details are given in the text.

in 60 min, as shown in Fig. 4. However, the final amount of the product was found to be proportional to the added amount of the conversion factor.

Immunochemical characterization

In order to clarify the mechanism of action of conversion factor, immunochemical investigations were performed by preparing the immune γ-globulin against the factor as described in Materials and Methods. Antibody activity was first tested by immunodiffusion[9] in 1% agarose in veronal–acetate buffer (pH 8.6, $I = 0.05$), using a purest sample of the factor as antigen. One major and two minor precipitin lines were then observed. Immunodiffusion between the immunoglobulin and human serum revealed two precipitin lines which were comparable to the minor precipitin lines observed in the reaction between the conversion factor and its antibody, suggesting that the purified sample of the factor contained at least two minor antigenic substances derived from the serum. Accordingly, the immune γ-globulin was absorbed with 0.2 vol. of the serum. By this treatment one of the minor lines disappeared and another line became faint without affecting the intensity of the major line. Fig. 5 demonstrates interactions of the absorbed antibody with conversion factor and also



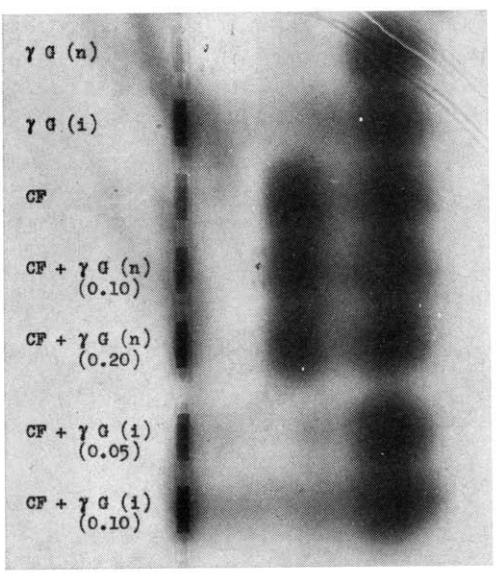


Fig. 5. Immunodiffusion analysis of conversion factor and the large form of adenosine deaminase (E_L) with antibody against conversion factor. An E_L sample (0.86 mg/ml, specific activity 2.22) and a purified sample of conversion factor (0.38 mg/ml, specific activity 384) were used. The immunoglobulin against conversion factor (13.73 mg/ml) was previously absorbed with human serum as described in the text.

Fig. 6. Precipitation of conversion factor with antiserum against the factor. 57 units of purified conversion factor were incubated at 37 °C for 1 h with 1.39 or 2.78 mg of immune γ-globulin against the factor (γG(i)) or with 1.24 or 2.48 mg of non-immune γ-globulin (γG(n)), followed by centrifugation at 2000 rev./min for 20 min to remove the precipitates. The supernatant fluids of 0.08-ml aliquots were then mixed and incubated at room temperature for 1 h with 0.06-unit aliquots of E_S from stomach. Subsequently the mixtures were applied to polyacrylamide gel electrophoresis followed by staining of the deaminase activity.

with E_L in an Ouchterlony double-diffusion agarose plate[9], where a continuous precipitin line is clearly observed. However, an additional minor line is also seen for both interactions.

To examine whether the proteins precipitated by the antibody involved the conversion factor or E_L, the immunoglobulin was first incubated at 37 °C for 1 h with the purified preparation of the factor, followed by centrifuging at 2000 rev./min for 20 min. The resulting supernatant fluid was tested for the converting activity by the qualitative assay method. Non-immune γ-globulin fraction prepared from unimmunized rabbit serum by the same procedure as used for preparing the immune γ-globulin served as control. As seen in Fig. 6, preincubation of the conversion factor with the immunoglobulin caused an almost complete loss of the activity in the supernatant, in contrast, preincubation with the non-immune γ-globulin did not affect the activity. Thus, this indicates that the immunoglobulin contains a precipitin against the conversion factor. Similar experiments were carried out for testing cross-reactivity of the antibody to E_L and E_S. After preincubations of various amounts of the immunoglobulin with 0.040-unit aliquots of E_L or with 0.021-unit aliquots of E_S, the mixtures were centrifuged to separate supernatants and precipitates, and adenosine deaminase activity of the two fractions was measured. As shown in Fig. 7, E_L

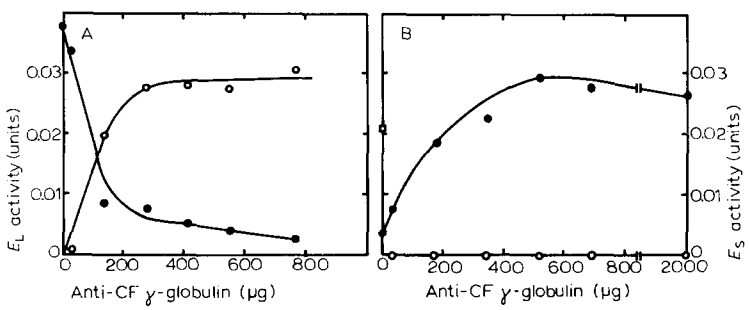


Fig. 7. Precipitation of the two forms of adenosine deaminase with antiserum against conversion factor. Various amounts, as indicated, of immune γ-globulin against conversion factor were incubated at 37 °C for 1 h with 0.04 unit of E_L from lung (A) or with 0.021 unit of E_S from stomach (B). The mixtures were centrifuged at 2000 rev./min for 20 min. Adenosine deaminase activity was measured with the resulting supernatant fluids and the precipitates previously dissolved in 0.30-ml portions of 0.9% phosphate-buffered saline (pH 7.4). ●—●, supernatants; ○—○, precipitates; □, the original activity before the incubation.

was precipitated in the presence of the immunoglobulin. The E_L activity lost from the supernatants was recovered in the precipitates with high yields (more than 80%). On the other hand, E_S activity remained in the supernatant fluids although a very small amount of precipitate was produced. The non-immune γ-globulin precipitated neither E_L nor E_S. The reason why E_S activity is apparently activated in the presence of the immune (or non-immune) γ-globulin has not yet been clarified. By these results it is indicated that conversion factor and E_L share a common antigenic determinant which differs from the catalytic site of E_L.

Dissociation of the large form of adenosine deaminase with sodium dodecyl sulfate

In order to examine the subunit structure of E_L, a purified lung E_L sample

Fig. 8. Disc gel electrophoretic patterns of purified large adenosine deaminase (E_L). 170 μg of an E_L sample purified from lung (specific activity 2.22) were subjected to disc polyacrylamide gel electrophoresis: (A) ,in Tris–glycine buffer (pH 8.9) at 3 mA per tube for 1.5 h; (B), in β-alanine–acetate buffer (pH 4.5) at 8 mA per tube for 2 h. Direction of migration is to the bottom.

Fig. 9. Sodium dodecyl sulfate–polyacrylamide electrophoresis of purified samples of the large form of adenosine deaminase (E_L) and conversion factor. Such standard proteins (50 μg each) as bovine serum albumin (A), ovalbumin (B), trypsin inhibitor (C), and myoglobin (D) were incubated at 37 °C for 1.5 h in 0.01 M sodium phosphate buffer (pH 7.0) containing 2.5% each of sodium dodecyl sulfate and 2-mercaptoethanol. The same E_L sample (75 μg) as used in Fig. 9 (E), a partially purified conversion factor (Step 6 in Table I; 23 μg) (G), and the most highly purified conversion factor (Step 8 in Table I; 15 μg) (H) were also treated in the same manner as the standard proteins except that the incubation was prolonged to 3 h. The same E_L sample (75 μg) (F) was incubated at 37 °C for 10 min in the phosphate buffer containing 1% each of sodium dodecyl sulfate and 2-mercaptoethanol. All the proteins thus treated were subjected to 5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol[11]. Protein bands were visualized with 0.05% Coomassie blue[7].

which had exhibited a single protein band by disc polyacrylamide electrophoresis at pH 8.9 (ref. 6) and also at pH 4.3 (ref. 10) (Fig. 8), was incubated at 37 °C for 3 h in 0.01 M sodium phosphate buffer (pH 7.0) containing 2.5% each of sodium dodecyl sulfate and 2-mercaptoethanol. Thereafter, the treated sample was subjected to thin-layer polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, essentially according to Weber and Osborn[11]. In six different experiments, two stainable protein bands were reproducibly observed, as shown in Fig. 9. When bovine serum albumin (68 000 mol. wt), ovalbumin (43 000), trypsin inhibitor (21 500) and myoglobin (17 200) served as standards, molecular weights of the two proteins were

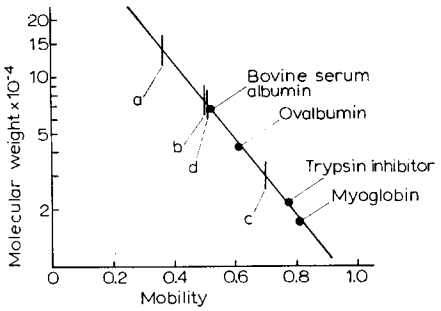


Fig. 10. Determination of molecular weights of the subunits of the large form of adenosine deaminase (E_L) and conversion factor by sodium dodecyl sulfate–polyacrylamide electrophoresis. The electrophoretic mobility of each standard protein was plotted against the log of its molecular weight[11]. Each mobility of the subunits a, b and c originating from E_L, and that of the subunit d originating from the conversion factor (see Fig. 9) were as indicated.

estimated to be 73 000 ± 2000 and 30 000 ± 1000 (mean ± S.D.), respectively (Fig. 10). The same treatment of a purified sample of conversion factor resulted in a single stained band which showed a very similar mobility to that of the larger species in the treatment of E_L (Fig. 9). When the E_L sample was treated with SDS under much milder conditions (1% in sodium dodecyl sulfate and 2-mercaptoethanol, 10 min), an additional protein band was observed. Its molecular weight was estimated to be approx. 135 000, which corresponds well to the molecular weight of native conversion factor. Similar treatment of E_S has not been performed because of lack of a pure sample of the enzyme.

Conversion factor in lung cancer tissue

The fact that E_S occurs in cancerous lung and liver tissues led us to examine the activity of conversion factor in the cancer tissues. From a case of lung cancer, tissue specimens of cancerous and normal parts of the lung were obtained by autopsy. The tissues were homogenized with 2 vol. of 30 mM phosphate buffer (pH 7.4) followed by centrifugation at 35 000 × *g* for 30 min. The precipitates were discarded. Since the extracts contained considerably strong activity of E_L which would interfere with

TABLE II

DETECTION OF THE ACTIVITY OF CONVERSION FACTOR IN EXTRACTS OF NORMAL AND CANCEROUS LUNG TISSUES

Detailed experimental procedures are described in the text.

Tissue	*Adenosine deaminase activity (units/mg protein)*			*Conversion factor activity (units/mg protein)*
	Total	E_L*	E_S**	
Normal	0.006	0.006	0	1.4
Cancer	0.015	0.001	0.014	N.S.***

* The large form of human adenosine deaminase.
** The small form of human adenosine deaminase.
*** Not significant.

the estimation of the converting activity of the extracts, they were first dialyzed for 24 h against 30 mM phosphate buffer (pH 7.4) containing 1 mM PCMS and subsequently dialyzed exhaustively against the buffer containing no PCMS to remove unreacted PCMS. Adenosine deaminase contained in the extracts was inactivated completely by this procedure which had no effect on the activity of conversion factor. Thus treated extracts were then tested for the converting activity according to the quantitative assay method. The results obtained are shown in Table II. The extract from the normal part of the lung had substantial activity, but the extract from the cancerous part showed no detectable activity of conversion factor.

DISCUSSION

The data presented here have substantiated the existence of conversion factor which converts the smaller adenosine deaminase (E_S) to the larger enzyme species (E_L) of human tissues. The factor has been successfully purified from the lung tissue extract through 8 steps of purification procedures generally employed for isolating proteins. The final purified preparation has exhibited upon disc gel electrophoresis a single stainable protein band associated with the converting activity. The activity was completely destroyed by heating at 60 °C for 10 min or 80 °C for 2 min. By these results together with the ultraviolet absorption spectrum of the purified sample it is reasonably concluded that the conversion factor is a protein, although its chemical composition has to be investigated further.

Although no direct evidence of the identity between the native E_L and the E_L formed from E_S *in vitro* in the presence of conversion factor has been obtained, both enzymes are found to be identical with respect to their molecular sizes, electrophoretic mobilities, and general enzymic properties. In assuming the identity between them, we can suppose two possible modes of action of conversion factor toward E_S: (1) Conversion factor acts as a catalyst of polymerization of E_S to yield E_L, where a monomer–tetramer relationship is assumed the most reasonable for the structures of E_L and E_S on the basis of their molecular weights. (2) Conversion factor can be bound directly with E_S to produce E_L.

Results of the quantitative analysis of the rate of formation of E_L in the presence of known amounts of E_S and conversion factor seem to eliminate the first possibility, because in a catalytic reaction the final equilibrium level should not be changed by varying the amount of catalyst to be added. Immunochemical characterizations of E_L, E_S and conversion factor by using the immunoglobulin against the factor revealed the cross-reactivity between E_L and conversion factor, supporting the second assumption that E_L is a complex of E_S and the factor. On the other hand, the data from sodium dodecyl sulfate electrophoresis of the purified lung E_L indicate that the enzyme comprises at least two polypeptide chains of different sizes. The molecular weight of the larger peptide chain (73 000) appears to be comparable to a half of that of conversion factor (139 000), and the same sodium dodecyl sulfate treatment of the preparation of the factor resulted in producing a peptide chain of similar size. Furthermore, another peptide which was well consistent in molecular size with the conversion factor was visualized by the treatment under much milder conditions (1% in sodium dodecyl sulfate and 2-mercaptoethanol, 10 min). In view of these facts, it seems reasonable that conversion factor is a subunit of E_L and also that

conversion factor is a dimer of the larger polypeptide chain. Although it is not possible at present to say that the smaller peptide chain (30 000 mol. wt.) arose from E_S, all the findings accumulated so far, together with the results of guanidine treatment of E_L where an apparent interconversion between E_L and E_S was demonstrated upon gel filtration, strongly support the mechanism as proposed below for the action of conversion factor;

$$E_S + CF \rightleftharpoons 2E_S \cdot CF = E_L \quad (CF: \text{conversion factor})$$

It seems unlikely that conversion factor could bind other protein(s) besides E_S, since the molecular size of the factor apparently did not vary during the purification and in addition a conversion factor sample exerted its activity to similar extents upon different E_S preparations at various stages of the purification from stomach. But this problem remains to be examined further in association with the following problem as discussed below.

The physiological significance of this conversion factor is still obscure. On the basis of the molecular structures of human adenosine deaminases as proposed above and by the observed differences in catalytic properties between the two forms of this particular enzyme[2], conversion factor is thought to exert inhibitory and at the same time stabilizing effects on E_S when the two proteins become bound with each other, and hence the conversion factor can be interpreted as a regulatory protein for adenosine deaminase. An analogous structure is presented for protein kinases[12–15], which undergo a reversible dissociation when bound with cyclic AMP. However, any reversible dissociation of human adenosine deaminases has not yet been ascertained under physiological conditions despite the fact that E_L can be dissociated reversibly into E_S by the treatment with guanidine HCl. Furthermore, in view of the fact that the detectable amounts of conversion factor in lung and liver tissues are considerable, there could be some other physiological role(s) of the conversion factor besides the regulation of the enzymic activity. To see the possible roles, we have tested a purified sample of the factor for several enzymic activities related somehow to the metabolism of adenosine; *i.e.* activities of AMP deaminase, 5′-nucleotidase, adenosine kinase, nucleoside phosphorylase and glutamine synthetase, as well as an activity of transamination between adenosine and α-ketoglutarate, but none of these activities was substantially demonstrated.

Interestingly, any significant activity of conversion factor was not detected in a lung cancer tissue extract. This finding is consistent with our previous observation that the cancer tissue contains a considerable amount of E_S which is hardly detected in the corresponding normal tissue and suggests the possibility that the lung tissue may be deprived of the capacity to elaborate the conversion factor or may acquire some unknown mechanism to inactivate the factor during the carcinogenesis. This characteristic alteration of the conversion factor arising during carcinogenesis might imply some crucial role of this protein played in the pathogenic process and may also suggest an alternative approach to clarify the unknown physiological role of this factor.

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REFERENCES

1 Akedo, H., Nishihara, H., Shinkai, K. and Komatsu, K. (1970) *Biochim. Biophys. Acta* 212, 189–191
2 Akedo, H., Nishihara, H., Shinkai, K., Komatsu, K. and Ishikawa, S. (1972) *Biochim. Biophys. Acta* 276, 257–271
3 Andrews, P. (1965) *Biochem. J.* 96, 595–606
4 Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372–1379
5 Spencer, N., Hopkinson, D. A. and Harris, H. (1968) *Ann. Hum. Genet., Lond.* 32, 9–14
6 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
7 Chrambach, A., Reisfeld, R. A., Wyckoff, M. and Zaccari, J. (1967) *Anal. Biochem.* 20, 150–154
8 Layne, E. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 3, pp. 451–454, Academic Press, New York
9 Ouchterlony, O. (1948) *Acta Pathol. Microbiol. Scand.* 26, 507–515
10 Reisfeld, R. A., Lewis, U. J. and Wiliams, D. E. (1962) *Nature* 195, 281–283
11 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
12 Kumon, A., Yamamura, H. and Nishizuka, Y. (1970) *Biochem. Biophys. Res. Commun.* 41, 1290–1297
13 Tao, M., Salas, M. L. and Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 408–414
14 Gill, G. N. and Garren, L. D. (1970) *Biochem. Biophys. Res. Commun.* 39, 335–343
15 Reimann, E. M., Brostrom, C. O., Corbin, J. D., King, C. A. and Krebs, E. G. (1971) *Biochem. Biophys. Res. Commun.* 42, 187–194