CHROMATOGRAPHIC PURIFICATION OF CYSTEINYL-GLYCINASE*

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INTRODUCTION

Cysteinylglycinase from pig kidney has been reported to be a protein-free RNA, and it has been suggested that RNA may act in protein synthesis through this enzymic activity[2,3]. Because of the remarkably interesting nature of these statements, an attempt was made to purify this dipeptidase further.

In the experiments, CGase prepared from pig kidney was further purified by chromatography on Ca phosphate, Dowex-2 and DEAE-cellulose columns. Comparison of the chromatographic behaviour of CGase activity and RNA on these materials, together with some properties of the purified material obtained, does not support the theory that this enzyme is of a non-protein nature.

MATERIAL AND METHODS

Material. Na β-naphthoquinone-4-sulfonate (Eastman), glutathione (Eastman), histidine (Hoffmann La Roche), trypsin (Merck), chymotrypsin (Worthington Biochemical Co.), crystalline pancreatic RNase (Sigma), tris(hydroxymethyl)aminomethane (Sigma), and Na *p*-chloromercuribenzoate (Sigma) were used. Trypsin was tested for CGase (see below), phosphomono- and diesterase[4] and RNase***[5] activities: the preparation used was found free from these activities, at least under the conditions in which its effect on CGase was tested. RNase was also found free from CGase activity.

For dialysis, Visking (Chicago, Ill.) cellophane bags were used.

Analytical methods. Phosphorus: method of SCHAFFER *et al.*[6]. Total N: micro-Kjeldahl method of HILLER *et al.*[7]. Protein N; LOWRY's modification of FOLIN's method[8], the standard curve being made with Pro-Sol Standard protein (Standard Scientific Supply Co., New York 12, N.Y.). Pentose: BROWN's method[9]. Orcinol (Eastman) was used without further purification. As a standard, a 0.1 m*M* solution of adenosine-5'-phosphate (Schwartz) was used, the concentration being determined by its optical density at 260 mμ. Cl^-: mercurimetric method[10].

Assay of the activity. The CGase activity was determined essentially by the method described by OLSON AND BINKLEY[1]; the volumes were reduced to one tenth, the final volume of the incubation mixture being 1 ml; Mn^{++} was used as activator. A unit of CGase activity is here defined as the quantity of enzyme able to hydrolyse 10% (= 50 μg) of the substrate in 10 minutes at 37° C §.

As a substrate, a partial hydrolysate of glutathione was used[1]. Its composition was checked by paper electrophoresis.

* The following abbreviations are used: CGase: Cysteinyl-glycinase; I-B-CGase: CGase prepared from pig kidney according to BINKLEY's first method[1]; II-B-CGase: CGase prepared from pig kidney according to BINKLEY's second method[2]; RNA: ribonucleic acid; RNase: pancreatic ribonuclease; THAM: tris(hydroxymethyl)aminomethane.

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*** Highly polymerized RNA prepared by ALLEN's method, was used as substrate.

§ A comparison of the data reported here with those reported by BINKLEY on CGase units basis is somewhat difficult. In fact, owing to some misprint, the two definitions given by him[1,2] are contradictory. It was thus thought more advisable to define it again.

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At pH 10.1 it is possible to separate some of the glutathione breakdown products. Whatman No. 1 paper strips (34 cm long and 10 cm wide) in 0.0125 *M* borax-NaOH buffer pH 10.1 are used, and in 2 hours (at 440 V, 8.10 mA) the products travel towards the cathode as follows:

Cysteine	11.5 cm	Cysteinylglycine	10 cm
Glycine	10 cm	Glutathione	11.5 cm
Glutamic acid	12.5 cm		

This pH value was shown to be the only one that permitted a separation of cysteinylglycine from cysteine. A separation of cysteinylglycine from glycine could not be achieved at any pH tested.

No non-hydrolysed glutathione or free cysteine were detectable in the partial hydrolysate of glutathione that was used as a substrate.

Owing to the extensive oxidation of the SH groups that occurs at pH 10.1, the use of electrophoresis for purification of cysteinylglycine is precluded.

In some cases, the electrophoretic separation of the incubation mixture has proved useful in showing the appearance of the spot of free cysteine. In this case, of course, the use of ninhydrin-positive buffers (such as histidine) was avoided.

The effect of the buffers used in the chromatographic procedures on the CGase activity was tested. As might be expected, phosphate buffers, metaphosphate, etc. (as well as other anions interfering with the metal activator) were found to be inhibitors; they were removed by dialysis. After two day's dialysis no loss in the activity was detectable. THAM-HCl buffer had no effect on the CGase activity test, at least at the concentrations used for chromatography on Dowex-2 or DEAE-cellulose columns.

Preparation of cysteinyl-glycinase. CGase was prepared from pig kidney by either the first (I-B-CGase)[1] or the second (II-B-CGase)[2] of BINKLEY's methods. Both methods involve alcohol fractionation and treatment with chloroform-octanol (9:1) mixture. At this stage, in fact, CGase activity is not sensitive to the latter treatment, and it can be recovered quantitatively in the aqueous phase.

In the case of II-B-CGase, the last step (adsorption on Dowex-1, and elution with 0.5 *M* NaCl) was not performed.

As solutions of the enzyme preparations were not found to lose any activity when kept in the cold in the presence of chloroform over a period of some weeks, lyophilisation was usually avoided. The experiments that will be described in the following, except that on calcium phosphate, were carried out with the original non-lyophilized solutions of enzyme.

The average proteolytic coefficient (C_1)* (on total N basis) of I-B-CGase was about 5.5 and of II-B-CGase about 85.

Chromatographic procedures. Preparation of Ca phosphate (hydroxyapatite) and chromatographic procedures performed on it were carried out according to TISELIUS *et al.*[11]. Potassium phosphate buffers at pH 6.8 were used. Attempts to elute the CGase from Ca phosphate with anions such as acetate or sulfate, which should not interfere with the metal activator, were unsuccessful. Phosphate buffer was removed by dialysis before determination of activity and protein and pentose content.

Chromatographic purification of CGase on Dowex-2 X-8[Cl^-] (mesh 200–400) was performed as described by BOMAN AND WESTLUND[12].

Because the use of a cationic buffer in protein chromatography on anion exchangers permits better pH control throughout the whole experiment[13], THAM-HCl buffer was used in chromatographic experiments on Dowex-2 and on DEAE-cellulose. Unless otherwise stated, the molarity of THAM-HCl buffer refers to the concentration of THAM. When necessary, most or all of the THAM was removed either by dialysis or by shaking with Dowex-50 in Na^+ form. Under these conditions (pH 7.3) no loss of CGase activity occurred.

DEAE-cellulose was prepared following PORATH's modification[14] of the PETERSON AND SOBER method[15].

The fraction collectors used operated on a time basis.

All the experiments were carried out at + 4° C.

RESULTS

On Ca phosphate, Dowex-2 and DEAE-cellulose columns, the chromatographic behaviour of CGase is closer to that of proteins than to that of RNA. Some typical experiments will be described in detail.

* The proteolytic coefficients were calculated from determinations giving an activity < than 50%, and a first-order reaction was assumed.

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Chromatography of CGase on Ca phosphate

In the experiment reported in Fig. 1, 10 mg of lyophilized CGase dissolved in 1 ml of 0.01 *M* K phosphate buffer pH 6.8, were applied to a column (1.0 × 5.0 cm) of Ca phosphate (hydroxyapatite), which had been previously equilibrated overnight against the same buffer. The elution was performed with stepwise increase of the concentration of phosphate buffer as indicated by the arrows in Fig. 1. The flow rate was 2 ml/hour. Fractions were collected every 90 minutes.

From Fig. 1, it can be seen that most of the proteins are adsorbed in 0.01 *M* phosphate buffer (tube 1) and most of them are eluted at a concentration of buffer lower than 0.20 *M*. Most of the RNA, however, is eluted at higher phosphate buffer concentration (1 *M*).

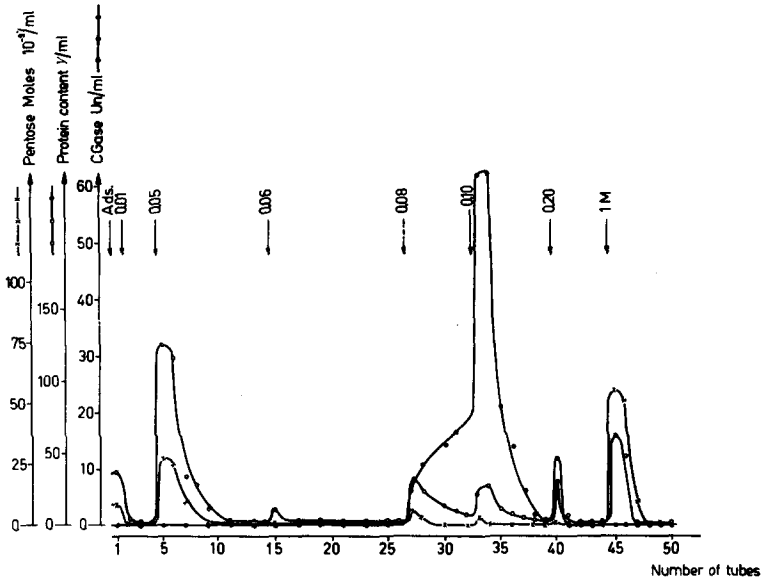

Fig. 1. Chromatography of CGase on Ca phosphate. The sample (I-B-CGase) was applied, dissolved in 0.01 *M* potassium phosphate buffer pH 6.8, to a column (1 × 5 cm) of Ca phosphate that had been equilibrated against the same buffer: elution was carried out with phosphate buffer at pH 6.8 whose molarities (as well as the moment at which they were applied to the top of the column) are indicated by the numbers and the arrows in the upper part of the figure. CGase activity: ●——●——●; protein content: ○——○——○ pentose content: ×——×——×. For other details, see RESULTS.

As to the CGase activity, elution is effected by 0.08–0.10 *M* K phosphate buffer, pH 6.8* (tubes 27–37), *i.e.*, at a concentration where most of the RNA is still adsorbed on the column. Only a small quantity of orcinol-positive material is eluted together with the activity.

When a slow increase in eluting buffer concentrations is used (as in the experiment reported here), the CGase begins to be eluted at 0.08 *M* phosphate buffer (with an $R_F < 1$), and $R_F \simeq 1$ is obtained with a slightly higher concentration, 0.10 *M*. The small peak of activity which comes out with 0.20 *M* phosphate is certainly due to the tailing of the preceding zone, which is taken up by the following eluent. Pictures

* See footnote on page 405.

like these are often met in stepwise elution chromatography, and they cannot be taken as an indication of heterogeneity of a substance[16,11].

The recovery of the activity in this experiment was 82%. The purification was about 7-fold when calculated from the Folin-Lowry reaction, or 12-fold when calculated from the orcinol reaction (tube 33–34).

Chromatography of CGase on Dowex-2

Fig. 2 shows an experiment with a column of 287 ml (2.7 × 50.0 cm) of Dowex-2 X–8 [Cl^-], mesh 200–400. After equilibration against 0.04 *M* THAM-HCl buffer, pH 7.3, 250 ml of I-B-CGase in 0.04 *M* THAM-HCl buffer was applied to the column (64 units/ml, total units applied: 16,000; total N: 184 μg/ml, total N applied: 46 mg). The elution was performed by stepwise increase of the buffer concentration. The arrows in the figure indicate the start at the top of column of the respective THAM-HCl buffer concentrations. The water pressure of the column was adjusted to give a flow

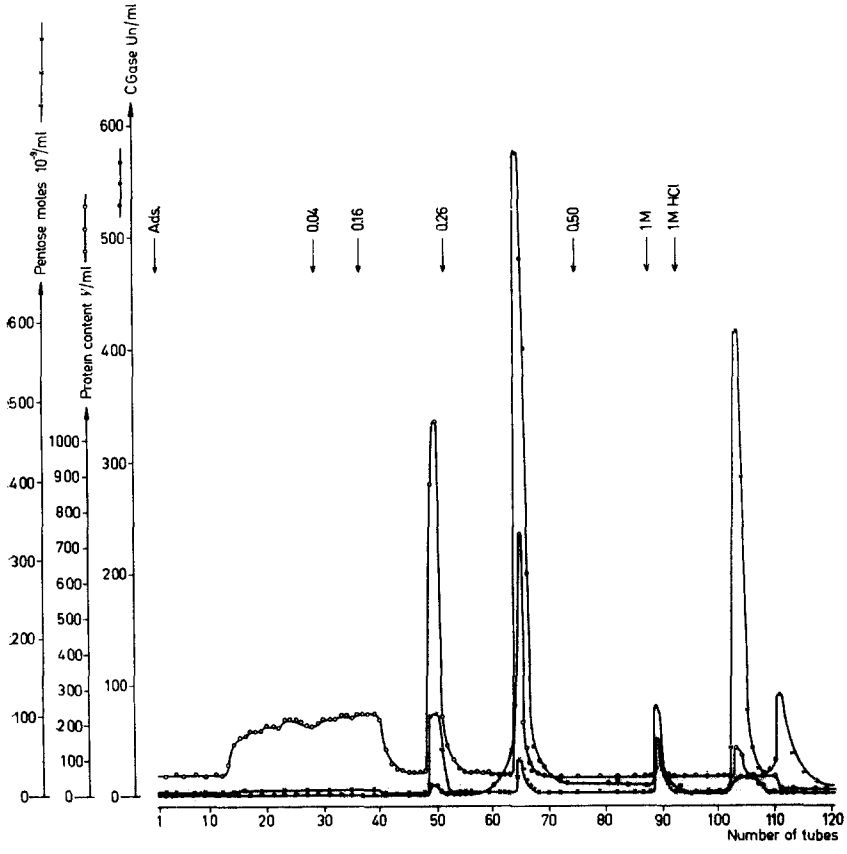

Fig. 2. Chromatography of CGase on Dowex-2. The sample (I-B-CGase in 250 ml of 0.04 *M* THAM-HCl buffer, pH 7.3) was applied to a Dowex-2 [Cl^-] column (2.7 × 50.0 cm), which had been equilibrated against the same buffer. The elution was carried out with THAM-HCl buffers, pH 7.3, whose molarities (as well as the moment at which they were applied to the top of the column) are indicated by the numbers and the arrows in the upper part of the figure. The dead volume of the column corresponded to about 12 fractions. CGase activity: ●——●——●; protein content: O——O——O; pentose content: ×——×——×. For other details, see RESULTS.

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rate of about 20 ml per hour. Fractions of approximately 10 ml each were collected every 30 minutes.

As can be seen from Fig. 2, more than half of the protein material is not adsorbed at 0.04 *M* THAM-HCl, but passes straight through the column as a first zone (tubes 13–43). Most of the adsorbed proteins are eluted between 0.16 and 0.50 *M* THAM-HCl buffer. As to the orcinol-positive material, it is almost completely adsorbed at 0.04 *M* THAM-HCl; a small amount of it is eluted between 0.16 and 0.50 *M* THAM, and most of it by 1 *M* THAM-HCl buffer (tubes 102–108).

The CGase is almost completely eluted by 0.26 *M* THAM-HCl buffer, pH 7.3 (tubes 63–70), (Cl^- concentration: 0.23–0.24 *N*); this THAM-HCl buffer concentration is within the range of concentrations at which most of proteins of the sample are eluted, and fairly far from those at which the bulk of ribonucleic acid is eluted. Only a small amount of orcinol-positive material is eluted together with the activity.

The appearance of some minor peaks of CGase activity should not be taken as an indication of the possibility that CGase is bound to more than one substance. The same phenomenon has also been observed, in fact, with homogeneous proteins (such as human serum albumin) on Dowex-2, and was found to depend upon the quantity of protein applied and the capacity of the column used[12].

In the experiment reported in Fig. 2, the activity was concentrated about 8.5-fold (in fraction 65); its recovery was about 80%; its purification was 13-fold (Folin-Lowry reaction) or 31-fold (orcinol reaction) (fraction 65).

In some experiments, the fraction eluted from Ca phosphate with 0.10 *M* phosphate buffer was made 0.04 *M* in THAM-HCl buffer, pH 7.3, and applied to a Dowex-2 column. Under these conditions also, the enzyme was quantitatively adsorbed and could be eluted at the same THAM-HCl buffer concentration as usual; the overall recovery of the activity was, however, very small.

Chromatography of CGase on DEAE-cellulose

CGase can be quantitatively adsorbed on DEAE-cellulose [Cl^-] in 0.04 *M* THAM-HCl buffer, pH 7.3, and can be eluted with 0.20 *M* THAM-HCl buffer at the same pH (Cl^- concentration: about 0.18 *N*)*.

1. In the experiment reported in Fig. 3A, 100 ml of II-B-CGase in 0.04 *M* THAM buffer (120 units/ml; total units applied, 12,000; total N, 14.3 μg/ml; protein, 40 μg/ml; pentose, 34 moles $\times 10^{-9}$/ml; see Fig. 3, column at the left) were passed through a DEAE-cellulose column (1 × 15 cm) which had been previously equilibrated against the same buffer (tubes 6–64). After washing with 0.04 *M* THAM buffer elution was accomplished with 0.40 *M* (tubes 70–75) and 1 *M* THAM buffer (tubes 76–79); the arrows in the figure indicate the start at the top of the column of the respective THAM-HCl (pH 7.3) concentrations. Flow rate: 0.2 ml per min. Fractions (of about 2 ml) were collected every 10 minutes. In this experiment, the activity was concentrated about 15.5-fold; it was purified 3-fold (Folin-Lowry reaction) or 10-fold (orcinol reaction) (tubes 70–71).

2. For rechromatography, fractions 70–74 were pooled, the Cl^- concentration was determined, enough 1 *M* THAM-HCl buffer, pH 7.3, was added to bring the Cl^- concentration to 0.24 *N* (*i.e.* approximately the same as the Cl^- concentration of

* It should be pointed out that these figures are to be considered as approximate, since they are subject to small variations in different batches of the adsorbing material.

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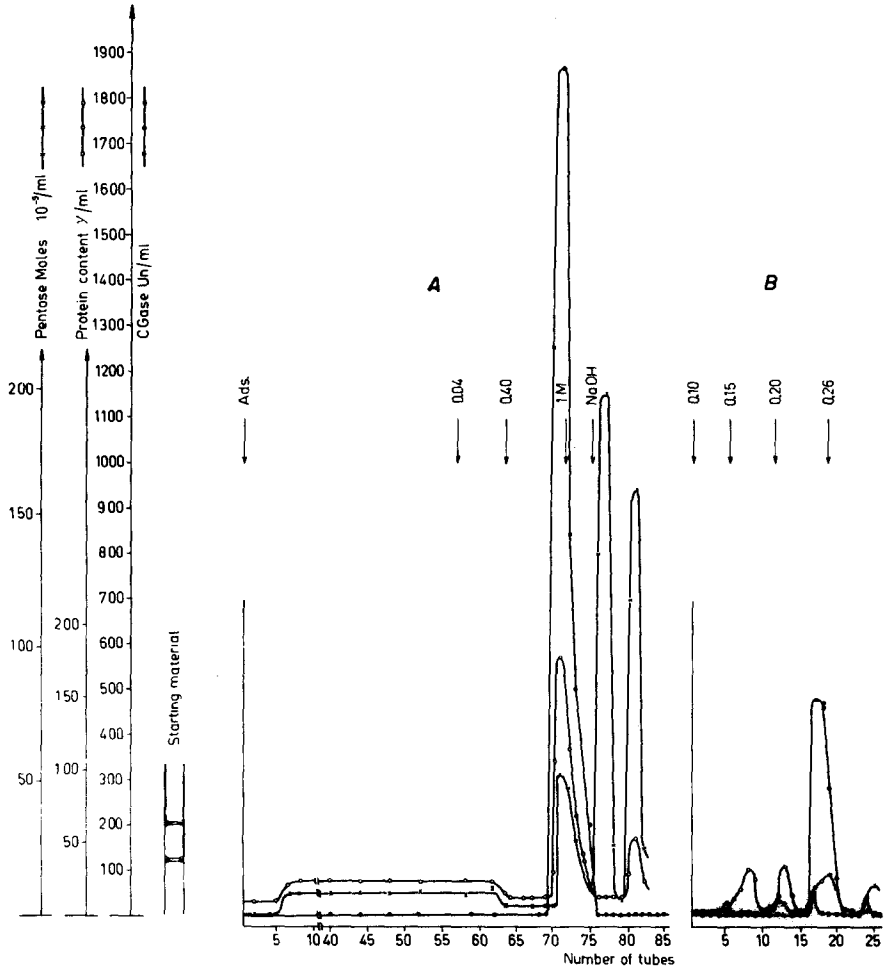

Fig. 3. Chromatography of CGase on DEAE-cellulose. In the experiment reported in Fig. 3*A* the sample (II-B-CGase in 100 ml 0.04 *M* THAM-HCl buffer, pH 7.3) was applied to a column (1 × 15 cm) of DEAE-cellulose [Cl^-] which had been previously equilibrated against the same buffer. Elution was carried out with THAM-HCl buffers, whose molarities (as well as the moment at which they were applied to the top of the column) are indicated by the numbers and the arrows in the upper part of the figure. The last washing was performed with 1% NaOH. The dead volume of the column corresponded to about 5 fractions. CGase activity: ●——●——●; protein content: ○——○——○; pentose content: ×——×——×. 3*B*: Re-rechromatography of a part of the CGase on the same column. Symbols, as for 3A. For other details, see RESULTS.

0.26 *M* THAM-HCl buffer, at pH 7.3) and applied to the same DEAE-cellulose column, after washing with 1% NaOH and equilibration against 0.26 *M* THAM-HCl buffer. In this step, 90% of the activity was recovered unadsorbed and only slightly diluted; a further 3-fold (Folin-Lowry reaction), or 7-fold (orcinol reaction) purification was obtained.

3. The unadsorbed fraction was diluted to make the Cl^- concentration equal to that of 0.04 *M* THAM-HCl buffer, pH 7.3, and again applied to the same column, which had been previously washed with 1% NaOH, and equilibrated against 0.04 *M*

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THAM buffer. Elution was carried out by stepwise increase of the buffer concentration, and this third part of the experiment is shown in Fig. 3B. Here again, the arrows indicate the start of each eluent at the top of the column, and the respective THAM-HCl buffer (pH 7.3) concentrations are indicated. The recovery of the activity in this step was about 80%. In tube 18, a further approx. 1.2-fold purification (Folin-Lowry reaction) was achieved; no pentose could be detected in this fraction.

The purification of CGase throughout these three steps on DEAE-cellulose column was about 10-fold (Folin-Lowry reaction) or 23-fold (total N).

It is evident from these experiments on DEAE-cellulose (Fig. 3) that here (as well as on Ca phosphate and Dowex-2) CGase follows more closely the proteins than the orcinol-positive material. From Fig. 3A, one can observe, in fact, that some proteins are not adsorbed in 0.04 *M* THAM buffer, but pass through the column as a first zone (tubes 6–64); most of those that are adsorbed are eluted with 0.40 *M* THAM buffer (tubes 70–75). The greatest portion of the pentose-containing material is eluted instead by 1 *M* THAM buffer (tubes 76–79), or comes out with the final washing with 1% NaOH (tubes 80–83).

CGase activity is eluted with the first eluent, together with most of the proteins and a small amount of orcinol-positive material. In the following rechromatographic experiments (2 and 3) the ratio between the activity and pentose content increased more than the ratio between the activity and the protein content. The proteolytic coefficient (C_1) of this preparation was about 2000.

In other preparations, a lower C_1 was obtained at this stage (1,000–1,500); in these cases, an additional chromatography on Dowex-2 gave a product with $C_1 \simeq 2{,}000$.

SOME DATA ON THE PURIFIED CYSTEINYL-GLYCINASE

The purified cysteinyl-glycinase* preparations obtained were free from glycyl-glycinase and leucyl-glycinase activities, at pH 7.3. A test on filter paper described elsewhere[17] was used.

When gradient elution chromatography of the "purified" CGase was carried out, a single protein component coincident with the activity peak was obtained. Fig. 4 shows such an experiment on a Dowex-2 column. A purified CGase solution in 0.04 *M* THAM-HCl buffer, pH 7.3 (2 ml; 150 Units/ml), was applied to a Dowex-2 [Cl^-] column, (1.07 × 10 cm), which had been previously equilibrated against the same buffer. After a first washing with 0.04 *M* THAM buffer, and a second one with 0.14 *M* THAM buffer (first arrow in Fig. 4) a gradient elution was carried out from 0.14 to 0.35 *M* THAM buffer (the closed mixing chamber[18] contained at the beginning 20 ml of 0.14 *M* THAM, and the reservoir 0.35 *M* THAM buffer). The volume of every fraction was about 1.65 ml. The flow rate was about 0.1 ml per min. The whole experiment was carried out in the cold. The recovery of the activity was about 90%.

As can be seen, a single peak of activity and of protein was obtained and the proteolytic coefficient (C_1) was practically constant in the fractions.

Similar behaviour was observed in gradient elution chromatography on Ca phosphate.

* Here and in the following, CGase preparations obtained by repeated chromatographic runs on DEAE-cellulose (at times followed by chromatography on Dowex-2), and having a proteolytic coefficient (C_1) of about 2000, are referred to as "purified CGase".

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Paper electrophoresis of CGase was carried out at room temperature, in a humid-chamber horizontal electrophoresis apparatus. Whatman No. 54 paper strips (7 cm wide and 42 cm long), and 0.04 *M* THAM-HCl buffer, pH 8.2, + 0.0005 *M* $MnCl_2$ were used. With 400 V, (1.5 mA), during 4.5 hours, the CGase travelled about 9 cm towards the anode. The activity was detected by the paper test[17]. A single spot of CGase activity was always found, at every stage of the chromatographic purification.

As reported above, the proteolytic coefficients (C_1) of the preparations obtained (with or without additional chromatography on Dowex-2) were about 2000, and this

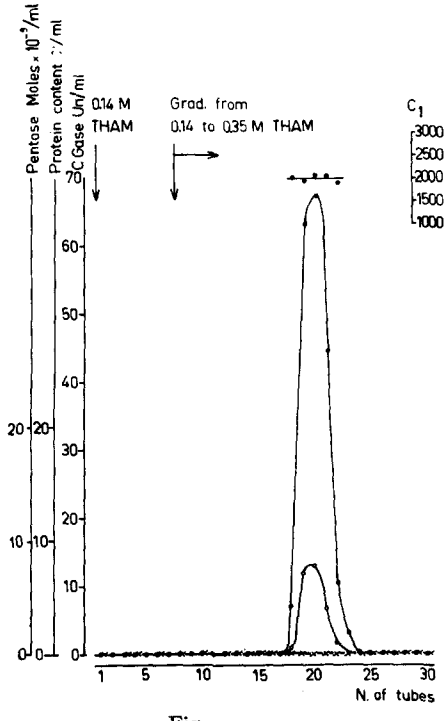

Fig. 4.

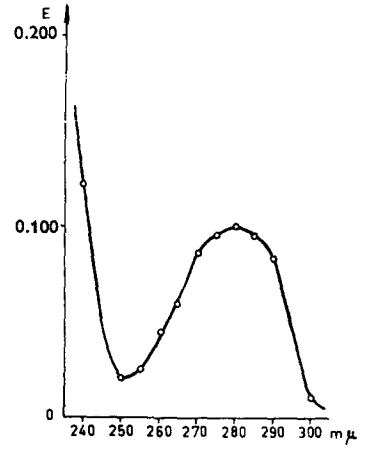

Fig. 5.

Fig. 4. Gradient elution chromatography of the "purified CGase" on Dowex-2. A sample of "purified CGase" (300 units) in 2 ml of 0.04 *M* THAM-HCl buffer, pH 7.3, was applied to a column of Dowex-2 [Cl^-], (1.07 cm × 10 cm), which had been previously equilibrated against the same buffer. After a washing with 0.04 *M* THAM buffer 0.14 *M* THAM was applied (first arrow) at the top of the column; then (second arrow) a gradient elution was carried out from 0.14 to 0.35 *M* THAM (for details see text). The proteolytic coefficient (C_1) is reported in the upper part of the figure. CGase activity: ●——●——●; protein content: O——O—O; pentose content: ×——×——×.

Fig. 5. UV spectrum of purified CGase at pH 7.3 (see text).

figure was fairly constant in the different preparations. It should be pointed out that this C_1 is higher than that of the purest of BINKLEY's preparations (from Dowex fractionation)[19], and it is about 23–25 times higher than that of the II-B-CGase that was used as a starting material for the chromatographic purification.

All preparations of CGase, at any stage of purification, were Folin-Lowry positive. The final preparations from DEAE-cellulose chromatography were negative to both tests for pentose and P. The lower sensitivity limits of these tests (as applied for example in fraction 18, Fig. 3B) were: 1 μg of pentose per ml, and 0.1 μg of P per ml.

The purified CGase did not pass through the dialysis membrane, during 2 days dialysis, and the whole activity was recovered inside the dialysis bag. The somewhat

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different behaviour reported for II-B-CGase[2] may be due to the different membrane used.

Fig. 5 shows the UV spectrum at pH 7.3 of a purified preparation of CGase. Most of the THAM was removed with Dowex-50 [Na^+]; the readings were taken against THAM-HCl buffer, which had been treated in the same way. Other preparations of CGase had the same spectrum in 0.04 *M* THAM HCl buffer, pH 7.3.

Neither I-B-CGase nor II-B-CGase is chloroform-sensitive[1,2]. After chromatographic purification, however, it is strongly chloroform-sensitive: when treated with a chloroform-octanol mixture (9:1) at room temperature, a solution containing 100 CGase units per ml in 0.40 *M* THAM-HCl buffer, pH 7.3, failed to show any CGase activity.

In the presence of 6 *M* urea the CGase is inactive. This effect of urea is completely (or almost completely) reversible. Table I gives the results of an experiment demonstrating this.

From the experiment reported below, it is evident that CGase is not active in the presence of 6 *M* urea (tube 1), and that the effect of urea is reversible (*cf.* tubes 2 and 3).

TABLE I

EFFECT OF UREA ON CGASE

A solution of purified CGase in 0.2 *M* THAM-HCl buffer, pH 7.3, was incubated for 16 hours at room temperature, in the presence of 6 *M* urea (final concentration). A parallel blank (tube 3) was run, in which water was substituted for urea. Tubes 1–5 were made up as shown.

Tube No.	*THAM buffer* 0.5 *M*, *pH* 7.26 *µl*	*Urea* 8 *M* *µl*	*Water* *µl*	$MnSO_4$ 0.005 *M* *µl*	*CGase in* 6 *M urea* *µl*	*CGase in water* *µl*	*% hydrolysis* of substrate*
1	50	750	—	100	50	—	0
2	50	—	750	100	50	—	20
3	50	—	750	100	—	50	22
4	50	750	50	100	—	—	0
5	50	—	800	100	—	—	0

* After preincubation of tubes 1–5 for 30 min at 40° C, the substrate was added (partial hydrolysate of glutathione, 1 mg per tube in 0.1 ml) and the incubation carried out for 15 min at 40° C.

Purified CGase was incubated (either with or without Mn^{++}) with trypsin, or chymotrypsin or RNase, either in THAM or in veronal buffer, pH 7.3. Under these conditions, no effect was detectable, either on the activity, the electrophoretic mobility, or the dialysability of the enzyme.

When CGase was incubated with trypsin or chymotrypsin in the presence of 6 *M* urea, a different result was obtained.

Table II gives the data of such an experiment.

From Table II it is evident that, when trypsin or chymotrypsin are incubated with the CGase in presence of 6 *M* urea, the CGase activity is reduced. This is particularly evident for chymotrypsin, but it is also true for trypsin. It should be emphasized that care has been taken to overcome (at least to a certain extent) the denaturing effect that urea has on both trypsin and chymotrypsin[20]. This was accomplished by taking advantage of the fact that the effect of urea is not instantaneous, and by

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TABLE II

EFFECT OF CHYMOTRYPSIN AND TRYPSIN ON CGASE IN THE PRESENCE OF 6 *M* UREA

A solution of purified CGase (147 units/ml) in 0.25 *M* THAM buffer, pH 7.3, was diluted 1:4 with 8 *M* urea (final concn. 6 *M*). After standing at room temperature (14 hours), trypsin or chymotrypsin were dissolved in the CGase solution in 6 *M* urea (final concentration of trypsin or chymotrypsin: 10 mg/ml), and incubated at 37° C for 1 hour. (A sample of CGase in 6 *M* urea was similary treated without any trypsin or chymotrypsin.) Then, 100 and 50 μl samples were taken to test the activity.

	Percent hydrolysis after 15 min at 37°C	
	100 μl sample	*50 μl sample*
Trypsin-incubated CGase	30	21
Chymotrypsin-incubated CGase	16.5	15
Control CGase	38	26

adding large amounts of trypsin or chymotrypsin immediately before the incubation. The lack of proportion between the 100 μl and the 50 μl samples, is possibly due to the fact that the samples contained 6 *M* urea, which was diluted in the mixture used for the enzymic test. Chymotrypsin and trypsin themselves have no CGase activity.

An experiment was carried out to determine whether a part of the activity became dialysable after chymotrypsin or trypsin incubation in the presence of 6 *M* urea. When samples treated as in Table II were dialyzed against 0.04 *M* THAM-HCl buffer, pH 7.3, in the cold for 24 hours, no activity could be detected outside the cellophane bag.

A lower electrophoretic mobility was shown by the trypsin- and by the chymotrypsin-treated CGase, when the incubation was carried out in the presence of urea. An experiment was carried out exactly as in Table II, except that the CGase + trypsin (or chymotrypsin) + 6 *M* urea were incubated at room temperature for 24 hours instead of 1 hour. The electrophoresis was carried out in 0.04 *M* THAM-HCl buffer, pH 8.2, + 0.0005 *M* $MnCl_2$. After 5 hour's run, at room temperature, (400 V), the control CGase travelled 11 cm towards the anode, while the trypsin- and the chymotrypsin-treated CGase travelled 8.5 cm. A tailing could be noticed, probably owing to the fact that all the samples were applied to the paper in 6 *M* urea.

The effect of Na *p*-chloromercuri-benzoate on CGase was also tested. 0.1 ml of a $2 \cdot 10^{-3}$ *M* solution of Na *p*-ClHg-benzoate in 0.04 *M* THAM-HCl buffer, pH 7.3, was added to 0.3 ml of purified CGase in 0.04 *M* THAM buffer, pH 7.3; the incubation lasted for 24 hours, at room temperature. A sample of CGase was added to THAM buffer, and used as a blank. The determination of the activity gave the following results: (incubation at 37° C for 30 min):

25 μl of Na *p*-ClHg-benzoate-treated CGase: % hydrolysis, 46
25 μl of control CGase: % hydrolysis, 63.

The activity is therefore lower in Na *p*-ClHg-benzoate-treated CGase.

It is uncertain whether this difference is due to a binding of Na *p*-ClHg-benzoate with the enzyme (partially reversed by the cysteinylglycine used as substrate) or to a binding of *p*-ClHg-benzoate with the cysteinyl-glycine, leading to a product provided with inhibitory action.

The Na *p*-ClHg-benzoate-treated CGase has the same electrophoretic mobility at pH 8.2 as the non-treated CGase.

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DISCUSSION

On all three materials used, the chromatographic behaviour of the bulk of the proteins is remarkably different from that of the bulk of RNA; that is, the affinity of the latter for the adsorbing material is higher than that of the protein. This is true for Ca phosphate (hydroxyapatite), Dowex-2 and DEAE-cellulose. In this connection, chromatography of these materials might be suggested as a useful tool in separating proteins from nucleic acids, especially in the later steps of enzyme preparations.

CGase is eluted at buffer concentrations that are well within the limits between which most of the protein material present in the preparation is eluted. This is true on all adsorbents tested (Ca phosphate, Dowex-2 and DEAE cellulose).

The small amount of pentose-containing material that is eluted together with the activity peak is probably due to mutual displacement phenomena. Pictures like these are often met with in chromatographic experiments on the adsorbing materials used here[11,12]. This interpretation is supported by rechromatographic experiments (see Fig. 3B): the ratio CGase activity/pentose content was increased progressively by repeated rechromatographic runs, indicating that the CGase activity is more likely to be associated with a protein, than with a RNA. In Fig. 3B it can be seen that in the final rechromatographic experiment of this preparation, a fraction of CGase was obtained (tube 18), which was orcinol-negative.

The chromatographic behaviour of CGase on Ca phosphate, DEAE-cellulose and Dowex-2 would then agree more with a protein nature of CGase than with an RNA nature. Some properties of the purified CGase are listed below.

1. CGase has a UV spectrum with a maximum at about 280 mμ, and a minimum at 250 mμ (Fig. 5). The same spectrum could also be given by an RNA containing a large amount of cytosine, which might suggest that CGase is such a substance. This possibility is unlikely, however, since CGase would then be very sensitive to RNase.

2. Folin-Lowry positive, orcinol-negative and phosphorus-negative preparations were obtained. The negative data cannot exclude, of course, the presence of a nucleotide coenzyme; but they render rather unlikely the possibility that traces of RNA (possibly still present) might be responsible for the activity: in that case the proteolytic coefficient (C_1) would have to be not less than $45 \cdot 10^6$ (as in the preparation reported in Fig. 3B). It may be recalled that the proteolytic coefficients reported for other peptidases are of a different order of magnitude: SMITH's preparation of leucine-aminopeptidase[21], for example, has a $C_1 \simeq 80$, or $\simeq 100$ (after electrophoretic purification).

3. Both the activity (see Table II) and the electrophoretic mobility of CGase (at pH 8.2) are affected by the incubation of the dipeptidase with trypsin or chymotrypsin *in the presence of urea* whilst the incubation of CGase with trypsin or chymotrypsin, in the absence of urea, fails to affect either the activity or the electrophoretic mobility. These results suggest that CGase is a protein, which can be attacked by chymotrypsin and trypsin only in its unfolded state.

4. The purified CGase is strongly sensitive to the chloroform treatment, while the original preparations (obtained with either the 1st or the 2nd BINKLEY method[1,2]) are quite insensitive to it. The simplest explanation for this difference is that in I-B-CGase and II-B-CGase the enzyme might be in a sort of combination with some other tissue component (possibly RNA), protecting it from the denaturating action of chloroform, and this combination may be split during chromatographic procedures.

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5. The purified CGase (as well as the starting material) is not RNase-sensitive.

6. Hydrogen bonds are present in the CGase molecule, as shown by the complete lack of activity of the enzyme in 6 *M* urea, suggesting that it can only be active in its folded state. The action of urea is reversible (see Table I).

7. The partial inhibition that is determined by the Na *p*-chloromercuribezoate cannot be given an unequivocal explanation, since at least two possibilities exist: the Na *p*-chloromercuri-benzoate forms with the substrate (cysteinyl-glycine) a compound having an inhibiting action, or the Na *p*-chloromercuri-benzoate does cause a complete inactivation of the CGase during the pre-incubation period, but this action is partially reversed by the cysteinyl-glycine itself, during the enzymic assay.

8. The CGase activity is not dialysable through cellophane bags. No dialysable activators could be detected.

Some of these data (*viz.* Nos. 1 to 4) and the chromatographic behaviour of CGase, do not support the suggestion that this enzyme is a protein-free non-RNase sensitive RNA*. On the contrary, they indicate that the cysteinyl-glycinase is, or is bound to, a single non-dialysable protein**.

It should also be emphasized that these purified preparations, dealt with here, represent a further purification of II-B-CGase, which was used as a starting material. A further 10-fold (Folin-Lowry reaction) or 23-fold (total N) purification was obtained. The final preparations had a proteolytic coefficient (C_1) of about 2,000, which is higher than the highest one reported by BINKLEY[19].

The possibility that more than one CGase is present in BINKLEY's preparations, one RNA, and others non-RNA in nature, is unlikely. In fact, the CGase activity behaved as a single substance in all chromatographic experiments, while the recoveries of CGase activity were almost quantitative (from 80 to 95%). In addition, only one spot of CGase activity could be detected in all electrophoretic experiments on paper, during the steps of the chromatographic purification.

These "purified CGases" also fulfil certain criteria of purity: final $C_1 \simeq 2{,}000$ was also obtained by slightly different procedures, (alternate chromatography on DEAE-cellulose and Dowex-2 columns, for example); the preparation is chromato-

* It may be recalled here that, if a non-RNase sensitive RNA were responsible for protein synthesis, the inhibiting effect of RNase on protein synthesis in onion roots and amoebae[22], would be difficult to explain.

** When this work was finished, I had the opportunity of discussing some of BINKLEY's unpublished results, which he was kind enough to communicate. With a different chromatographic procedure, he was able to isolate five different CGases, composed of a "peptide" (and therefore Folin-Lowry positive) and a "nucleotide" part. This seems to rule out the possibility, previously reported[2], that a "protein-free" RNA might be responsible for the CGase activity.

As the C_1 of these CGases is between 1000 and 2000, it would seem that a lower purification has been reached than that reported in the present paper; in no case was there a higher purification.

One cannot, of course, rely simply on the negative tests for pentose or P (the amount of material available was fairly small) to deny the possible presence of a firmly bound nucleotide coenzyme. The disagreement between these results seems therefore to be reduced to the size of the "peptide" bound, or not bound, to a nucleotide coenzyme: in no case was I able to recover any CGase activity outside the dialysis membranes, whilst BINKLEY's CGases[2] are reported to be slowly dialysable. The possibility exists, however, that during the extensive digestion or the chromatographic procedure (which are carried out in the new, unpublished, BINKLEY method) some degradation might occur, leading to a product of relatively low molecular weight, still retaining the original activity: a partial degradation of some enzymes, not followed by a disappearance of the activity, has already been reported by several authors (*e.g.*, HILL AND SMITH[23] have degraded papain with their leucine-aminopeptidase). But it should be pointed out that this hypothesis is merely given here as one of the possible explanations.

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graphically pure (see Fig. 4); it is free from other dipeptidase activities, such as glycyl-glycinase and leucyl-glycinase. It is understood, however, that fulfillment of these criteria is not sufficient to allow one to assert that a "pure" enzyme has been obtained.

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SUMMARY

In view of the importance of the statement that cysteinyl-glycinase is a protein-free RNA, and that RNA acts in protein synthesis through this dipeptidase activity, a further purification of this enzyme was carried out. By chromatographic procedures (on Ca phosphate, Dowex-2, DEAE-cellulose) a further 25-fold purification (on total N basis) was obtained; the final proteolytic coefficient (C_1) was about 2000. The chromatographic behaviour of the enzyme, as well as the characteristics of the purified material obtained, do not support the hypothesis that cysteinyl-glycinase is of an RNA nature; they indicate, instead, that it is (or is bound to) a single non-dialysable protein.

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