Biochem. J. (1978) **172**, 301–309
Printed in Great Britain

Calcium-Dependent Golgi-Vesicle Fusion and Cathepsin B in the Conversion of Proalbumin into Albumin in Rat Liver

By PAUL S. QUINN* and JACOB D. JUDAH†
Department of Experimental Pathology, University College Hospital Medical School, London WC1E 6JJ, U.K.

(*Received* 6 *September* 1977)

1. An enzyme from rat liver that converts proalbumin into albumin is described. Partial purification, inhibitor studies and the conditions for maximum activity suggest that the enzyme is cathepsin B. 2. A membrane-bound enzyme, located mainly in lysosomes, also converts proalbumin into albumin. This appears to be a membrane-bound form of cathepsin B. 3. Isolated Golgi vesicles, incubated under conditions suitable for cathepsin B, convert endogenous proalbumin into albumin. 4. This conversion in Golgi vesicles has an absolute requirement for Ca^{2+} at micromolar concentrations. Mg^{2+} does not affect or substitute for Ca^{2+}. Both the proalbumin and the albumin formed from it are intravesicular. 5. Converting activity is enhanced by pretreatment with the known chemical fusogen, poly(ethyleneglycol). 6. Vesicles preincubated at pH above 7 in the presence of dithiothreitol show a marked fall in converting activity. This can be partially restored by incubation with native vesicles. These results suggest that vesicle fusion is a requirement for conversion of proalbumin into albumin.

Previous work has shown that proalbumin is the immediate biosynthetic precursor of serum albumin. Proalbumin differs from albumin in having an *N*-terminal extension Arg-Gly-Val-Phe-Arg-Arg- (Geller *et al.*, 1972; Russell & Geller, 1973; Urban *et al.*, 1974; Quinn *et al.*, 1975; Russell & Geller, 1975). Proalbumin may be converted into albumin by limited tryptic hydrolysis (Judah *et al.*, 1973). Experiments *in vivo* failed to identify any newly formed albumin in liver organelles (Geller *et al.*, 1972; Dorling *et al.*, 1975). In the present work we have identified a converting enzyme from rat liver and provide evidence for its site of action in the liver cell.

Materials and Methods

The preparation of serum albumin, electrofocusing on pH gradients, determination of protein, chromatography of albumin and proalbumin on DEAE-cellulose with linear salt gradients and the determination of radioactivity were as described by Judah *et al.* (1973). Chromatography of acid-soluble peptides on SP (sulphopropyl)-Sephadex C-25 was as described by Russell & Geller (1975). The preparation of labelled and unlabelled proalbumin was as described by Quinn *et al.* (1975). Smooth endoplasmic reticulum was prepared as described by Geller *et al.* (1972). Plasma membranes were prepared as described by Dorling & le Page (1973). The preparation of Ca^{2+} buffers with EGTA was as described by Portzehl *et al.* (1964). The pH was adjusted as required by the addition of 1M-Tris base, and the concentration of free Ca^{2+} (down to 1μM) was monitored with a Ca^{2+} electrode (type IS 560; Philips Industries, Eindhoven, The Netherlands). Allowance was made for the presence of EGTA (1mM) in the Golgi preparations (see below) in adjusting the Ca^{2+} concentrations in the experiments.

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK').

* Present address: Department of Biochemistry, University of Chicago, Chicago, IL, 60637, U.S.A.

† To whom reprint requests should be addressed.

Preparation of 'large granules'

This was a modification of the method of de Duve *et al.* (1955). Rat liver (30g) was homogenized in 150ml of 250mM-sucrose containing 50mM-K_2HPO_4/KH_2PO_4 buffer, pH6.2, and 1mM-EDTA, pH6.2, with Tris base. The homogenate was centrifuged ($3000g_{av.}$, 10min) and the supernatant centrifuged again ($30000g_{av.}$, 10min). The pellet was resuspended in the homogenization medium and recentrifuged ($30000g_{av.}$, 10min). All operations were at 2°C in the SS 34 rotor of a Sorvall RC2B centrifuge (Dupont Instruments, Hitchin, Herts., U.K.). The final pellet was suspended in 10ml of 100mM-phosphate buffer, pH6.2.

For the preparation of the soluble extract, the suspension was frozen and thawed four times and the membranes were removed by centrifugation ($100000g_{av.}$, 60min) in the no.65 rotor of a Beckman L265B centrifuge (Beckman RIIC Ltd., Glenrothes,

Scotland, U.K.). For the preparation of the 'large-granule' membranes, the 'large granules' were suspended in 50ml of 10mM-phosphate buffer, pH6.2. The membranes were sedimented ($30000g_{av.}$, 10min), washed with 4×50ml and finally suspended in 10ml of the same buffer. Both soluble and membrane fractions were kept for no more than 72h at 2°C.

Assay of cathepsin B was by the method of Barrett (1972) with α-benzoyl-DL-arginine *p*-nitroanilide as substrate.

Assay of conversion of proalbumin into albumin by large-granule enzymes

This assay was used to measure the conversion of exogenously added proalbumin into albumin by liver extracts or by purified enzymes such as trypsin or cathepsin B. It is based on the presence of 26 arginine residues in proalbumin, of which three are in the *N*-terminal peptide. On conversion, the *N*-terminal peptide is released, and since it is acid-soluble it may easily be separated from the protein and its radioactivity determined, when it is appropriately labelled. The assay system, in a final volume of 50μl, contained proalbumin (2mg/ml), [^{14}C]arginine-labelled proalbumin (specific radioactivity 750000d.p.m./mg; 200μg/ml), crystalline bovine serum albumin (40mg/ml), 40mM-Mes (adjusted to pH6.0 with KOH) and enzyme preparation. The addition was 260μg of protein/ml of reaction mixture for the soluble extract, and 500μg of protein/ml for the membrane preparation. For full activity, the enzyme preparations were reduced before assay with 2mM-dithiothreitol in 50mM-Mes, pH6.0, for 10min at 37°C. This was done to a concentrated enzyme preparation, portions of which could then be assayed. Incubation was for varied times at 37°C and was ended by addition of 50μl 17.5% (w/v) trichloroacetic acid. The mixture was then centrifuged for 2min (Eppendorf 3200 micro-centrifuge, in the tubes of which the assay can conveniently be done) and the acid-soluble radioactivity determined in a portion of the supernatant. This radioactivity is assumed to be derived entirely from the *N*-terminal peptide of proalbumin, as none is found if instead of [^{14}C]arginine-labelled proalbumin, [^{14}C]arginine-labelled albumin or [^{14}C]-leucine-labelled proalbumin are used as substrates. The release of [^{14}C]arginine correlated well with the formation of albumin as estimated after purification on DEAE-cellulose (see above).

Chromatography of the enzyme in the soluble fraction on Sephadex G-75 or DEAE-cellulose was done as described by Davidson & Poole (1975).

Preparation of Golgi vesicles and cisternae

The preparation was a modification of the methods of Leelavathi *et al.* (1970) and of Ehrenreich *et al.* (1973). Livers from female rats (approx. 200g body wt.) were homogenized in 6vol. of 250mM-sucrose containing 1mM-EGTA (adjusted to pH7.4 with Tris base) in an all-glass Dounce homogenizer (Blaessig Glass Co., Rochester, NY, U.S.A.), kept cold in crushed ice. All operations were done at approx. 2°C thereafter. The homogenate was centrifuged ($700g_{av.}$, 10min) and 8ml portions of the supernatant were layered over 5ml of 1.3M-sucrose containing 1mM-EGTA and centrifuged ($200000g_{av.}$ for 60min) in a SW41Ti swinging-bucket rotor in a Beckman L2 65B centrifuge. The membranes at the interface were collected (about 8ml of suspension). To the suspension was added about 12ml of 2M-sucrose, containing 1mM-EGTA. Portions (5ml) of this suspension were overlaid with (successively) 1.1M-sucrose (3ml), 0.85M-sucrose (3ml) and 0.25M-sucrose (approx. 2ml). All the sucrose solutions contained 1mM-EGTA. The tubes were centrifuged (SW41Ti rotor) at $200000g_{av.}$ for 120min. The Golgi-vesicle fraction was collected from the interface between 0.25M-sucrose and 0.85M-sucrose layers. The Golgi cisternae (contaminated with vesicles and by smooth endoplasmic reticulum as seen by electron microscopy) were found at the interface between 0.85M- and 1.1M-sucrose. The composition of the vesicle fraction was judged by both electron microscopy and the use of enzyme markers. It closely resembled a mixture of the GF1 and GF2 fractions described by Ehrenreich *et al.* (1973). For some experiments (see the Results section) the vesicles were taken from the gradient and used without further treatment. They are called 'unpelleted vesicles' in the text. They were recovered in a sucrose concentration of approx. 0.7M. In other experiments, the vesicle suspension was diluted (approx. 4-fold) with 250mM-sucrose containing 1mM-EGTA, sedimented ($100000g_{av.}$ for 20min) and the pellets were resuspended (as desired) for the experiment. These are called 'pelleted vesicles'.

Preparation of vesicles containing radioactive proalbumin

The rats were anaesthetized by an intraperitoneal injection of ethyl carbamate (125mg/100g body wt.) and then 300μCi of L-[2,3-^{3}H]valine was injected into a tail vein. The rats were killed 15min later by cervical dislocation and preparations made as described above. The vesicles contained 200000–350000d.p.m. of ^{3}H in (anti-albumin)-precipitable protein, of which approx. 70% was found in proalbumin (Dorling *et al.*, 1975).

Determination of radioactive albumin within Golgi vesicles

The extent of conversion of proalbumin into albumin within Golgi vesicles could not be measured by the assay method described above. Instead, the accumulation of radioactive albumin was determined. After incubation, the 1ml reaction mixtures (see the

Results section for details) were treated with 1M-Tris/HCl, pH7.8 (200μl) and sodium deoxycholate (10%, w/v; 50μl). Then rat serum albumin (750μg) and 5ml of 150mM-NaCl were added and the albumin fractions were precipitated with anti-(rat serum albumin) (prepared by Wellcome Reagents Ltd., Beckenham, Kent, U.K.) and left overnight at 2°C. The antigens were recovered as described by Quinn *et al.* (1975) and albumin was separated from proalbumin by chromatography on DEAE-cellulose (Dorling *et al.*, 1975).

Preincubation of vesicles with poly(ethylene glycol)

An 80% (w/v) solution of poly(ethylene glycol) (mol.wt.6000; obtained from BDH Chemicals, Poole, Dorset, U.K.) was freshly made up in 20mM-Mes, pH6.0, containing 1mM-EGTA. To maintain its fluidity, it was kept at 37°C until used. Unpelleted Golgi vesicles were mixed with an equal volume of this poly(ethylene glycol) solution, whereupon the temperature of the mixture was approx. 20°C. After 60s, the vesicle suspension was cooled in crushed ice, diluted approx. 3-fold with ice-cold 70mM-sucrose/1mM-EGTA and centrifuged (100000$g_{av.}$ for 20min, no. 65 Beckman rotor). The supernatant was carefully removed (with a Pasteur pipette) and the pellet suspended in 2ml of 70mM-sucrose/1mM-EGTA. Control vesicles were mixed with an equal volume of 70mM-sucrose/1mM-EGTA/20mM-Mes, pH6.0 (which was warmed to 37°C before use) and were processed in exactly the same way and at the same time as the vesicles pretreated with poly(ethylene glycol).

Materials

Leupeptin and pepstatin were gifts from Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, U.K. Diaflo XM300 membranes were obtained from Amicon, High Wycombe, Bucks., U.K. L-[U-^{14}C]Arginine and L-[2,3-^{3}H]valine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Leupeptin was entrapped in liposomes for use with the Golgi-vesicle preparation. Phosphatidylcholine (20μmol), cholesterol (20μmol), stearylamine (20μmol) and leupeptin (10μM) were sonicated together in 2ml of 5mM-potassium phosphate, pH7.0 in a M.S.E. sonic disintegrator at full power (150W) and at 22°C. About 30% of the leupeptin was entrapped, but no attempt was made to separate the free inhibitor from that contained in liposomes.

Results

Conversion of exogenous proalbumin by soluble enzymes

The soluble fraction of large granules (see the Materials and Methods section) converted proalbumin into albumin. The conversion has a pH optimum at pH6.0, activity was negligible at pH7.0 and only 20% of maximum at pH5.0. The system was saturated with substrate at 1mg of proalbumin/ml, and K_m was approx. 400μg/ml.

Activity is linear with time and enzyme concentration provided that no more than 30% of the substrate is consumed.

Identity of the soluble converting enzyme

Conversion of proalbumin was inhibited by leupeptin and Tos-Lys-CH_2Cl, but not by pepstatin. The activity was destroyed if the extract was first reduced with 1mM-dithiothreitol at pH6.0 (10min at 37°C) and the pH then raised to pH7.0 or above, and the mixture incubated at 37°C for 15min (see Table 1). These findings suggested that cathepsin B or a similar enzyme was responsible for the converting activity in the large-granule extract. This idea was supported by the results of chromatography of the soluble fraction on Sephadex G-75 (Davidson & Poole, 1975) which indicated a mol.wt. of 25000 for the converting enzyme, which is that expected for cathepsin B (Otto, 1971). As a result of gel filtration, the specific activity of the converting enzyme rose 6-fold from 20mg of proalbumin converted/h per mg of protein. The enzyme fraction obtained from the Sephadex column was then chromatographed on DEAE-cellulose (Davidson & Poole, 1975) and eluted with a further 5-fold increase in specific activity. The converting activity co-chromatographed with cathepsin B activity (Barrett, 1972). It was found that a purified

Table 1. *Effect of proteinase inhibitors on the conversion of exogenous proalbumin into albumin*

The enzymes were prepared and assayed as described in the Materials and Methods section, with [^{14}C]-arginine-labelled proalbumin as substrate. Incubations were for 5min at 37°C. The soluble extract or membranes added was 200μg of protein. Values are means, with the ranges given in parentheses, for the number of experiments shown. The activity of the soluble enzyme varied from 15 to 25mg of proalbumin split/h per mg of protein and that of the membrane-bound enzyme from 5 to 10mg of proalbumin split/h per mg of protein.

Inhibitor	Concn. (μM)	% inhibition: Soluble enzyme	% inhibition: Membrane-bound enzyme	No. of expts.
Leupeptin	3	—	70 (65–75)	5
	10	90 (75–100)	85 (80–90)	5
Pepstatin	10	nil	nil	2
Tos-lys-CH_2Cl	10	50 (40–60)	50 (40–60)	5

sample of bovine cathepsin B (kindly supplied by Dr. A. J. Barrett) converted proalbumin into albumin.

Membrane-bound converting enzyme

When the frozen and thawed suspension was centrifuged (100000$g_{av.}$, 60min) most of the converting activity was found in the supernatant. Some activity was found associated with the membranes, but the activity varied considerably from one preparation to another. The large-granule fraction was therefore subjected to osmotic shock (suspension in 5mM-potassium phosphate buffer, pH6.2) rather than to freeze-thawing. The same amount of converting activity was released into the supernatant as after freezing and thawing, but that associated with the membranes was 25–30% of the total. This activity could not be removed from the membranes by extensive washing. However, freezing and thawing of these membranes led to destruction of 70% of their activity. Sonication (10s, at 0°C under N_2) or treatment with 0.05% (w/v) deoxycholate also resulted in considerable loss of activity.

Table 1 shows that leupeptin and Tos-Lys-CH_2Cl inhibit the membrane-bound enzyme which converts proalbumin into albumin. The enzyme requires the presence of a thiol for full activity and is inactivated by exposure of the reduced form to a pH above 7 for 15min at 37°C. The pH optimum for conversion was about pH6.0. We concluded that it was probably a membrane-bound form of cathepsin B.

The activity found in the large-granule membranes after hypo-osmotic shock resides mainly in the lysosomes. This was shown by isolating lysosomes from the large-granule fraction of livers from rats pretreated with Triton WR1339 (Beaufay, 1972). Over 70% of the total membrane-associated activity was found in the membranes of the Triton-laden lysosomes ('tritosomes'). The specific activity of the crude large-granule membranes initially obtained in this experiment was 5.3mg of proalbumin cleaved/h per mg of membrane protein, whereas the subsequently purified tritosomal membranes had a specific activity of 250mg of proalbumin cleaved/h per mg of membrane protein.

Identification of the products of conversion

When release of [^{14}C]arginine was used as a measure of conversion of proalbumin, the formation of albumin as measured after purification by chromatography on DEAE-cellulose or isoelectric focusing on pH gradients agreed very well. If the release of [^{3}H]valine was followed, less albumin was formed than expected. This was so whether the source of converting enzyme was crystalline trypsin, purified bovine cathepsin B, the soluble extract or the membrane fraction from the large granules. It was concluded that some breakdown product of proalbumin other than albumin was being formed. This can be seen as peak III in Fig. 2 in the paper of Judah *et al.* (1973), which illustrates the action of trypsin on proalbumin. We suspect that proalbumin is being cleaved to yield an arginyl-albumin.

We examined the acid-soluble products of conversion by chromatography on SP-Sephadex C-25 (Russell & Geller, 1975). Since the elution from this anionic exchanger is with a reversed pH gradient, the more basic peptides come off later. Tryptic hydrolysis yielded the intact *N*-terminal hexapeptide, which came off at pH5.4 as reported by Russell & Geller (1975), but the liver enzyme preparations only yielded three unidentified peptides, which were eluted at pH3.1, pH3.9 and pH4.4, labelled with both [^{3}H]valine and [^{14}C]arginine. This suggests that they are less basic than the hexapeptide, presumably through the loss of arginine residues.

Conversion of proalbumin into albumin within isolated Golgi vesicles

It was concluded from the experiments reported above that cathepsin B might be responsible for the conversion of proalbumin in the liver, even though this is mainly a lysosomal enzyme. It was calculated that the activity of the easily soluble and membrane-bound forms was enough to convert approx. 1000mg of proalbumin/h per total liver at 37°C. This compares with a rate of secretion of albumin of 6mg/h per liver. It was concluded that this large excess of activity made it pointless to seek the intracellular site of conversion by determining the intracellular distribution of the converting enzyme. It also seemed most unlikely that the lysosomes could play a part in the secretion of serum albumin. It was therefore decided to attack the problem by isolating subcellular organelles from rats dosed with labelled amino acids and studying the conversion of the endogenous proalbumin without prior disruption of the vesicles. The conditions for incubation were dictated by the properties of cathepsin B (i.e. provision of thiol, incubation at pH6.0) and the Golgi vesicles were chosen for study because of their importance in secretory processes.

In preliminary experiments, it was found that Golgi vesicles isolated from labelled rats (see the Materials and Methods section) contained 60–70% of the label in proalbumin. When incubated (as described in the Materials and Methods section) at pH6.0 and with 1mM-dithiothreitol, some of the proalbumin was converted, but the results were irregular. Variation of pH from 5.0 to 7.0 failed to improve the rate of conversion. It occurred to us that the substrate (proalbumin) and the converting enzyme might exist in separate vesicles which would have to fuse to form a secretory vesicle in which conversion would take place. Electron-microscopic evidence has been obtained that Ca^{2+} can induce fusions between Golgi vesicles (Gratzl & Dahl, 1976) and it was accordingly tested in our system.

Effect of Ca^{2+}, pH and sucrose concentration

In experiments with unpelleted vesicles (see the Materials and Methods section) a rapid conversion of proalbumin into albumin was found at pH5.5 with 100μM-Ca^{2+}. At pH6.0 in the same system, conversion was slow to start, but accelerated, as shown in Fig. 1.

As the unpelleted vesicles were in hyperosmotic sucrose, we tested pelleted vesicles (see the Materials and Methods section) suspended in 70mM-sucrose containing 1mM-EGTA. Fig. 2 shows the conversion of proalbumin found at pH5.5 and pH6.0 in the presence of 100μM-Ca^{2+}. It is clear that the lag period at pH6.0 has disappeared, and that at pH5.5 the rate of conversion was relatively slower.

In other experiments (results not shown), the 'pelleted vesicles' were suspended in 700mM-sucrose/1mM-EGTA, and conversion was examined at pH5.5 and pH6.0. As expected, the initial rate of conversion was far greater at pH5.5 than at pH6.0, but the rate at the latter pH increased with time and the extent of conversion was far greater at the end of the experiment (60min). Little or no conversion was observed at pH5.0 or pH7.0, and none in the absence of Ca^{2+}.

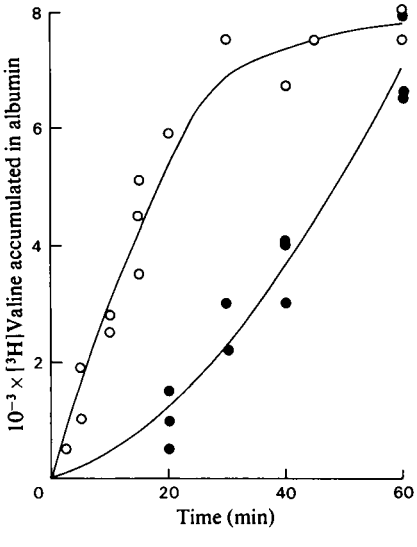

Fig. 1. *Conversion of proalbumin contained within Golgi vesicles suspended in hyperosmotic sucrose*
The unpelleted vesicles were in 1ml of the gradient solution (see the Materials and Methods section). They were incubated at pH5.5 with 50mM-acetate (○) or at pH6.0 with 50mM-Mes (●) together with 1mM-dithiothreitol and 100μM-Ca^{2+} at 30°C. The points shown were obtained in three separate experiments. The contents of the vesicles were released and analysed as described in the Materials and Methods section.

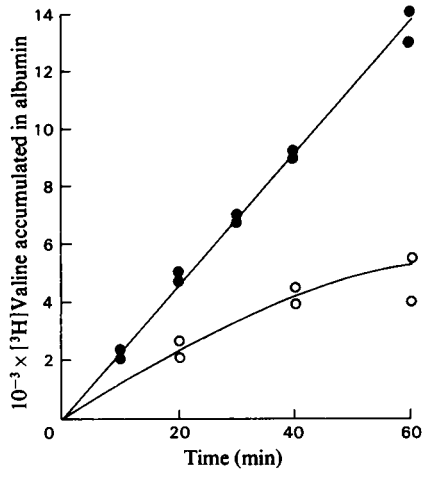

Fig. 2. *Conversion of proalbumin contained within Golgi vesicles suspended in 70mM-sucrose*
The pelleted vesicles were suspended in 70mM-sucrose/1mM-EGTA and incubated at pH5.5 (○) or pH6.0 (●) exactly as described in Fig. 1.

Ca^{2+} requirement for conversion

The effect of a range of Ca^{2+} concentrations on conversion of proalbumin was tested at pH5.5 (by using 'unpelleted vesicles') and at pH6.0 (by using 'pelleted vesicles' suspended in 70mM-sucrose/1mM-EGTA). At pH5.5 30μM-Ca^{2+} gave 60% of the maximum rate, which was reached at 100μM-Ca^{2+}, whereas at pH6.0 60% of the maximum rate was reached at 1μM-Ca^{2+} and full activity at 10μM-Ca^{2+}.

Nature of the Ca^{2+} effect

It is clear that the sucrose concentration in which the vesicles are placed exerts a considerable influence on their ability to convert proalbumin into albumin. Miller & Racker (1976) have shown that the osmotic gradient of sucrose across the membranes of sarcoplasmic-reticulum vesicles determines the rate of the Ca^{2+}-dependent fusion of these vesicles with artificial bilayers. We therefore decided to look for evidence that fusion was necessary for conversion of proalbumin into albumin in Golgi vesicles. This was done in two ways, as shown below.

Combination of inactivated with active vesicles

We observed that incubation of Golgi vesicles for 10–20min at 30°C, at pH7.2 in the presence of 1mM-dithiothreitol, decreased their ability to convert proalbumin into albumin by 50–80%, when they were subsequently incubated at pH6.0 with Ca^{2+}. We reasoned that if vesicles containing labelled proalbumin were thus inactivated and then mixed with

unlabelled, active vesicles, an increase in the conversion of proalbumin into albumin would be evidence for a fusion. The experiments in Table 2 show that the combination of active unlabelled vesicles with labelled inactive vesicles does indeed lead to increased conversion of proalbumin. The experiments were done with 'unpelleted vesicles' so as to minimize handling, at pH5.5 and pH6.0. The conversion was shown to be intravesicular by the methods described below.

Effect of the fusogen poly(ethylene glycol) on conversion

Poly(ethylene glycol) has been shown to promote fusion in several systems (Ahkong *et al.*, 1975; Fodor & Alföldi, 1976; Schaeffer *et al.*, 1976; Lucy, 1977). When tested in our system (as described in the Materials and Methods section), poly(ethylene glycol) (mol.wt.6000) induced a striking increase in the initial rate of conversion of proalbumin, as shown in Table 3.

The Ca^{2+} requirement of the poly(ethylene glycol)-treated vesicles is the same as that of the controls. The fact that poly(ethylene glycol) is effective after a brief exposure of the particles is reminiscent of the findings of Schaeffer *et al.* (1976) with the fusion of bacterial protoplasts. If poly(ethylene glycol) is allowed to remain in contact with the vesicles during incubation at a concentration of 40% (w/v), the conversion of proalbumin is abolished. It was calculated that as a result of our washing procedure, less than 6mg of poly(ethylene glycol)/ml was likely to remain in the vesicle preparations, but at this concentration in the reaction mixture it had no effect on the conversion of proalbumin.

We conclude that these experiments provide good, if not final, evidence of vesicle fusion as an essential step in the conversion of proalbumin into albumin.

Table 2. *Evidence for fusion between inactivated and native Golgi vesicles*

Five independent experiments were done with unpelleted vesicles derived from [^{3}H]valine-labelled rat liver. The vesicles were inactivated as described in the text. In Expts. 1 and 2, the incubations were at pH6.0, with 50mM-Mes, for 30min at 30°C. In the remainder, the pH was 5.5, with 50mM-sodium acetate, and incubation was for 15min at 30°C. All the systems contained 1mM-dithiothreitol and 100μM-Ca^{2+}. The vesicles were added in 1ml of the gradient solution (see the Materials and Methods section).

	[^{3}H]Valine (c.p.m.) accumulated in newly formed albumin in	
Expt. no.	Inactivated labelled vesicles	Combination of inactivated labelled and native unlabelled vesicles
1	8850	17500
2	6300	15000
3	200	9350
4	9500	13400
5	10500	16600

Table 3. *Effect of poly(ethylene glycol) on the conversion of proalbumin contained within Golgi vesicles*

Preincubation with poly(ethylene glycol) was done as described in the Materials and Methods section. The Golgi vesicles were suspended in 70mM-sucrose/1mM-EGTA and incubated for 10min at 30°C in the presence of 50mM-Mes, pH6.0, 1mM-dithiothreitol and 100μM-Ca^{2+}. The reaction was stopped and the vesicle contents were analysed as described in the Materials and Methods section.

	[^{3}H]Valine accumulated in newly formed albumin (c.p.m.)	
Expt. no.	Control	Poly(ethylene glycol) treated
1	2500	7800
2	2000	3300
3	2500	8000
4	2025	4560
5	3500	6500
6	4500	5000

Proof of intravesicular conversion

At the end of incubation, approx. 80% of the (anti-albumin)-precipitable radioactivity was retained in the vesicles. This was shown by layering the reaction mixtures (1ml) over 0.85M-sucrose and centrifuging in a swinging-bucket rotor at 200000$g_{av.}$ for 60min. Other samples were filtered through Amicon XM300 membranes. The soluble fractions so obtained contained albumin and proalbumin in much the same proportions as those found in the vesicles. In a typical experiment, the vesicles contained 7500c.p.m. of ^{3}H in the albumin in the absence of Ca^{2+}, and 13200c.p.m. of ^{3}H in its presence. In the supernatants was found albumin containing 1600 c.p.m. of ^{3}H in the absence of Ca^{2+} and 1900c.p.m. in its presence. Similar observations were made in four other experiments. These methods were used because centrifugation at 100000$g_{av.}$ for 30min in angle rotors resulted in the release of 50–100% of the albumin fractions to the supernatant.

Products of conversion in Golgi vesicles

Fig. 3 shows the formation of albumin and the disappearance of proalbumin in Golgi vesicles in the presence or absence of Ca^{2+}. It is noteworthy that the 'intermediate' form of albumin (referred to above) is absent. Fig. 4 shows the two acid-soluble peptides that appear during conversion. These are eluted at

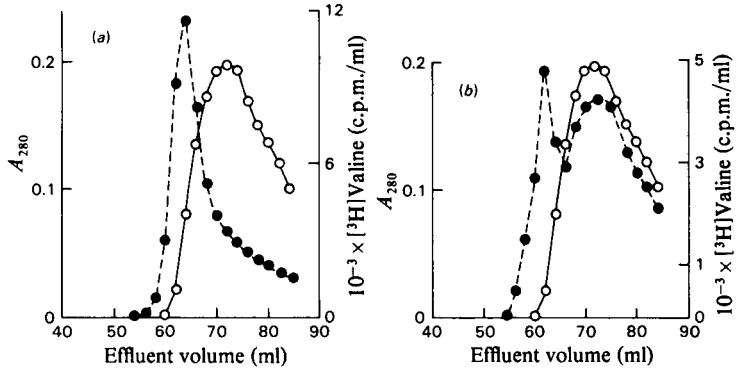

Fig. 3. *Conversion of proalbumin into albumin within Golgi vesicles as shown by DEAE-cellulose chromatography*
The unpelleted Golgi vesicles were incubated at pH 5.5 for 30 min at 30°C without Ca^{2+} (Fig. 3*a*) or with 100 μM-Ca^{2+} (Fig. 3*b*). All other details were as described in Fig. 1. Chromatography on DEAE-cellulose was as described in the Materials and Methods section, with 10 mg of rat serum albumin added as carrier. A_{280} (representing the concentration of albumin added as carrier), ○; ^{3}H, ●.

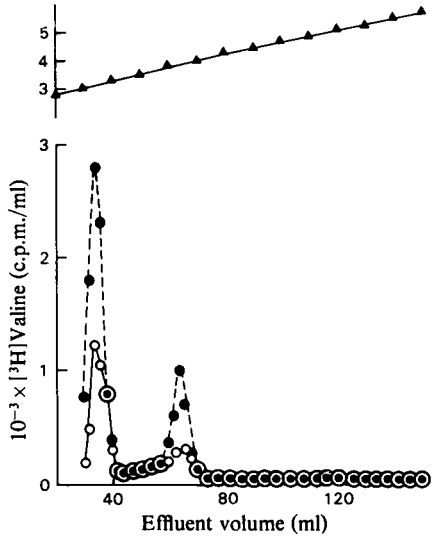

Fig. 4. *Chromatography on SP-Sephadex C-25 of acid-soluble products of conversion within Golgi vesicles*
The experiment was done with duplicate samples of the Golgi vesicles shown in Fig. 3. At the end of incubation, Triton X-100 (final concn. 1%, v/v), bovine serum albumin (1 mg) and 1.25 ml of trichloroacetic acid (17.5%, w/v) were added. The mixtures were centrifuged (3000*g*, 20 min) to remove the precipitate and the supernatants extracted with 3 × 3 ml of diethyl ether. Then they were subjected to chromatography with a reversed pH gradient as described by Russell & Geller (1975). The Figure shows the distribution of radioactivity in the eluate: without Ca^{2+}, ○; with 100 μM-Ca^{2+}, ●; pH, ▲.

pH 3.1 and pH 3.9 and correspond to two of the three peptides released by the large-granule enzyme. No hexapeptide appears.

Table 4. *Effect of proteinase inhibitors on conversion of proalbumin contained within Golgi vesicles*
The Golgi vesicles were derived from rats labelled with [^{3}H]valine and the newly formed albumin was determined as described in the Materials and Methods section. For the incubation, at 30°C for 15 min, the vesicles were suspended in 1 ml of 250 mM-sucrose containing 1 mM-EGTA, 50 mM-Mes, pH 6.0, 1 mM-dithiothreitol and 100 μM-Ca^{2+}. Leupeptin was added partly entrapped in multilamellar vesicles as described in the Materials and Methods section. Inactivation at pH 7.2 was done by incubation for 15 min at 30°C in 5 mM-Tris/HCl, pH 7.2, in the presence of 1 mM-dithiothreitol. The pH was then adjusted to 6.0 by addition of 50 mM-Mes, pH 6.0. [^{3}H]Valine (c.p.m.) accumulated in newly formed albumin ranged from 4500–7000 c.p.m. (between 10–20% of the radioactivity present in proalbumin). Values given are means with the ranges in parentheses, for the numbers of experiments given.

Inhibitor	Concn. (μM)	% inhibition of accumulation of [^{3}H]valine (c.p.m.) in albumin	No. of expts.
Leupeptin	10	80	1
Tos-Lys-CH_2Cl	20	50 (40–60)	5
	50	70	1
	100	100	1
Incubation at pH 7.2 with dithiothreitol	1000	80 (75–85)	6

Effect of proteinase inhibitors

Table 4 shows that the Ca^{2+}-stimulated conversion of proalbumin into albumin is markedly inhibited by leupeptin (10 μM, partly entrapped in liposomes) and Tos-Lys-CH_2Cl (20 μM–100 μM). The converting activity is destroyed by exposure to pH 7.2 for 30 min at

30°C, after preliminary treatment with 1mM-dithiothreitol. These data support the conclusion that cathepsin B (or a similar enzyme) is responsible for conversion.

Lack of effect of Mg^{2+} and bovine serum albumin

Mg^{2+} (up to 1mM) did not substitute for or affect the activity of Ca^{2+} in our system. It has been reported that bovine serum albumin enhances the fusion of liposomes in the presence of Ca^{2+} (Papahadjopoulos *et al.*, 1976). Bovine serum albumin has no effect in our system.

Lack of effect of colchicine

Colchicine *in vivo* inhibits the secretion of serum albumin, and also the conversion of proalbumin into albumin (Dorling *et al.*, 1975). At 20μM, colchicine had no effect on the conversion in our system, whereas vesicles separated from rats pretreated with colchicine (2.5mg/kg for 2h) functioned like those from control rats.

Converting activity in other subcellular fractions

Smooth endoplasmic reticulum prepared as described by Geller *et al.* (1972) contained ample amounts of labelled proalbumin, but failed to convert any into albumin when tested at pH5.5 or pH6.0 in the presence of 100μM-Ca^{2+}. The amount of radioactive proalbumin found in the Golgi cisternal fraction was only 15% of that found in the Golgi vesicles under the conditions of our preparation (see the Materials and Methods section). On incubation at pH5.5 in the presence of 100μM-Ca^{2+}, about 20% of the proalbumin in the Golgi cisternal fraction was converted into albumin, compared with 50% in the Golgi vesicles. Much of this conversion is probably due to contaminating vesicles (as judged by electron microscopy). Plasma-membrane preparations contained so little radioactive albumin fractions that they were not investigated further.

Discussion

The easily solubilized enzyme from the large-granule fraction of rat liver which will convert proalbumin into albumin has the properties of cathepsin B. The membrane-bound enzyme is very possibly identical, since it is inhibited by leupeptin and Tos-Lys-CH_2Cl and inactivated by exposure to pH over 7 in the presence of thiol. This applies also to the enzyme that converts proalbumin into albumin in isolated Golgi vesicles.

Smith & van Frank (1975) remark that cathepsin B has been seen to be associated with immature secretory vesicles and infer that lysosomal proteinases might well have functions other than degradation, particularly in the conversion of proinsulin into insulin. The most effective synthetic substrates for cathepsin B contain double basic residues at the point of cleavage, e.g. *N*-benzyloxycarbonyl-Ala-Arg-Arg 4-methoxy-β-naphthylamide. This was originally designed as a substrate for the proinsulin-converting system, and suggested the possibility that cathepsin B or a very similar enzyme catalysed the conversion of proalbumin. In the same way, it is entirely possible that the same system could convert pro-parathyrin, since the *N*-terminal extension of the pro-parathyrin is strikingly similar to that of proalbumin, namely Lys-Ser-Val-Lys-Lys-Arg- (Hamilton *et al.*, 1974). However, it has been reported that pro-parathyrin may be converted into parathyrin by a proteinase from parathyroid gland which is active in the pH range 7–9 (MacGregor *et al.*, 1976). This observation weakens the case for the attractive idea that a single class of proteinases is concerned in the conversion of the pro-proteins known to undergo intracellular modification before secretion, and which may contain the same or similar amino acid residues about their cleavage points. Despite this, we consider that the hypothesis of the existence of a general mechanism for conversion should not be discarded yet.

The effect of Ca^{2+} in our system is quite possibly 2-fold. There is evidence (by no means conclusive) that Ca^{2+} may activate cathepsin B (Szego *et al.*, 1976), and by inference have the same effect on the conversion of proalbumin within Golgi vesicles (see Tables 2 and 4). We provide quite strong evidence for the induction of fusion between two different types of vesicles in the presence of low concentrations of Ca^{2+}. Such a mechanism provides an excellent explanation for the observed inhibition of conversion of proalbumin (Dorling *et al.*, 1975) and of pro-parathyrin (Kemper *et al.*, 1975) when secretion *in vivo* is blocked by anti-mitotic agents. The implication is that Golgi-vesicle fusion, directed by the microtubule system, is necessary for conversion in intact cells, and that the organelle that is produced by the fusion is a secretory vesicle. In consequence, one could infer that it is only the latter secretory vesicle that can recognise the cytoplasmic surface of the plasma membrane and so take part in exocytosis. The hypothesis would also account for the failure to detect proalbumin in the plasma (P. S. Quinn & J. D. Judah, unpublished work; Ikehara *et al.*, 1976) and for the small amounts of pro-parathyrin and of proinsulin found in the blood.

Ikehara *et al.* (1976) have reported that Golgi vesicles are the site of conversion of proalbumin into albumin. This follows from the observation that albumin is the predominant labelled species in Golgi vesicles isolated from rats treated with ethanol (Ehrenreich *et al.*, 1973). In vesicles isolated from colchicine-poisoned rats, proalbumin predominates. Edwardes *et al.* (1976) have suggested that proalbumin is converted into albumin in the smooth endoplasmic reticulum and the Golgi apparatus.

Their suggestion is based on the finding that after a dose of [^{14}C]leucine the ratio of radioactive proalbumin to albumin falls steadily during the progress from the rough endoplasmic reticulum to the smooth endoplasmic reticulum and the Golgi apparatus. This essentially confirms the data of Geller *et al.* (1972), who, however, using a double-label technique, were unable to show that the albumin in these subcellular fractions was newly formed.

Since cathepsin B is found mainly in lysosomes, it may generally be believed that it is only concerned in a general degradative breakdown of proteins. Smith & van Frank (1975) and Novikoff (1976) have pointed out that enzymes may have one function in a given intracellular location and a different one elsewhere, so it seems quite possible that the conversion of proalbumin by cathepsin B is an example of such a difference.

No-one has yet provided a satisfactory explanation for the existence of the 'pro-proteins'. Perhaps the variety of functions excludes any generalization. Elsewhere, we have suggested that cleavage products of the conversion might function as controls on the translation system (Dorling *et al.*, 1975), but these are mere speculations.

We are indebted to the Cancer Research Campaign, the Vandervell Foundation and the Smith, Kline and French Foundation for grants. We thank Dr. R. T. Dean, Clinical Research Centre, Northwick Park, London N.W. for his help and advice.

References

Ahkong, Q. F., Fisher, D., Tampion, W. & Lucy, J. A. (1975) *Nature (London)* **253**, 194–195

Barrett, A. J. (1972) in *Lysosomes, a Laboratory Handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland, Amsterdam

Beaufay, H. (1972) in *Lysosomes, a Laboratory Handbook* (Dingle, J. T., ed.), pp. 1–45, North-Holland, Amsterdam

Davidson, E. & Poole, B. (1975) *Biochim. Biophys. Acta* **397**, 437–442

de Duve, C., Pressman, B. C., Gianetto, R., Wattieux, R. & Applemans, F. (1955) *Biochem. J.* **60**, 604–617

Dorling, P. R. & le Page, R. N. (1973) *Biochim. Biophys. Acta* **318**, 33–44

Dorling, P. R., Quinn, P. S. & Judah, J. D. (1975) *Biochem. J.* **152**, 341–348

Edwardes, K., Fleischer, B., Dryburgh, H., Fleischer, S. & Schreiber, G. (1976) *Biochem. Biophys. Res. Commun.* **72**, 310–318

Ehrenreich, J. H., Bergeron, J. M. M., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 45–72

Fodor, K. & Alföldi, L. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2147–2150

Geller, D. M., Judah, J. D. & Nicholls, M. R. (1972) *Biochem. J.* **127**, 865–874

Gratzl, M. & Dahl, G. (1976) *FEBS Lett.* **62**, 142–145

Hamilton, J. W., Niall, H. D., Jacobs, J. W., Kentmann, H. T., Potts, J. T. & Cohn, D. V. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 653–656

Ikehara, Y., Oda, K. & Kato, K. (1976) *Biochem. Biophys. Res. Commun.* **72**, 319–326

Judah, J. D., Gamble, M. & Steadman, J. H. (1973) *Biochem. J.* **134**, 1083–1091

Kemper, B., Habener, J. F., Rich, A. & Potts, J. T. (1975) *Endocrinology* **96**, 303–312

Leelavathi, P. E., Estes, L. W., Feingold, D. S. & Lombardi, B. (1970) *Biochim. Biophys. Acta* **211**, 124–138

Lucy, J. A. (1977) *Trends Biochem. Sci.* **2**, 17–20

MacGregor, R. R., Chu, L. L. H. & Cohn, D. V. (1976) *J. Biol. Chem.* **251**, 6711–6716

Miller, C. & Racker, E. (1976) *J. Membr. Biol.* **30**, 283–300

Novikoff, A. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2781–2787

Otto, K. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 1–28, North-Holland, Amsterdam

Papahadjopoulos, D., Hui, S., Vail, W. J. & Poste, G. (1976) *Biochim. Biophys. Acta* **448**, 245–264

Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591

Quinn P. S., Gamble, M. & Judah, J. D. (1975) *Biochem. J.* **146**, 389–393

Russell, J. H. & Geller, D. M. (1973) *Biochem. Biophys. Res. Commun.* **55**, 239–245

Russell, J. H. & Geller, D. M. (1975) *J. Biol. Chem.* **250**, 3409–3413

Schaeffer, P., Cami, B. & Hotchkiss, R. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2151–2155

Smith, R. E. & van Frank, R. M. (1975) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 4, pp. 193–249, North-Holland, Amsterdam

Szego, C. M., Seeler, B. J. & Smith, R. E. (1976) *Eur. J. Biochem.* **69**, 463–474

Urban, J., Inglis, A. S., Edwardes, K. & Schreiber, G. (1974) *Biochem. Biophys. Res. Commun.* **61**, 444–451