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CONVERSION OF THE FOURTH COMPLEMENT COMPONENT STUDIED BY CROSSED IMMUNOELECTROPHORESIS

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SUMMARY

C4 in EDTA plasma and partially purified C4 give a β_2 peak on crossed immunoelectrophoresis. During electrophoresis C4 in serum is converted to a product of fast β_1 mobility, usually accompanied by a slow β_2 peak. Conversion in serum is inhibited by EDTA. Storage of serum at room temperature results in a gradual increase of the slow β_2 peak. Storage of EDTA plasma changes the configuration of the native β_2 peak. $C\bar{1}s$, trypsin, chymotrypsin, plasmin or thrombin added to partially purified C4 is capable of producing a fast β_1 C4 protein peak. $C\bar{1}s$, trypsin and chymotrypsin give this conversion product also when added to EDTA serum. $C\bar{1}s$, trypsin and chymotrypsin also give rise to a slow β_2 and an inter α C4 conversion product in serum, probably consisting of complex formations between C4 and other serum proteins. Enzyme inhibitors known to interfere with $C\bar{1}$ inhibit the conversion of C4 in serum on agarose electrophoresis. The results suggest that such conversion is caused by an activation of C1 during electrophoresis.

INTRODUCTION

Susceptibility to the action of proteolytic enzymes occurring in serum is a feature common at least to the second, fourth, third and fifth complement components (Budzko & Müller-Eberhard, 1970). This may help to explain the lability of these complement factors in serum and in partially purified preparations of the corresponding proteins. Changes in electrophoretic mobility of the C4 protein associated with loss of haemolytic activity after treatment with $C\bar{1}s$ and trypsin have been described by Müller-Eberhard & Lepow (1965), and by Budzko & Müller-Eberhard (1970). Crossed immunoelectrophoresis has been applied to the study of the structural polymorphism of C4 by Rosenfeld, Ruddy & Austen (1969). These authors demonstrated a partial conversion of C4 occurring on agarose electrophoresis of serum and that conversion was inhibited in the presence of 0·005 M EDTA. They also examined the effect of $C\bar{1}s$ on partially purified C4.

In a previous study (Laurell, Sjöholm & Johnson, 1970) dealing with the quantitation

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of C4 by the electroimmunoassay unexplained differences between serum and plasma C4 values were found, especially with respect to the effect of storage. The present investigation was designed to analyse these observations qualitatively by crossed immunoelectrophoresis. The influence of various proteolytic enzymes and enzyme inhibitors on the electrophoretic behaviour of the C4 protein was also studied.

MATERIALS AND METHODS

Normal plasma and serum. Blood from healthy donors was drawn into tubes containing EDTA to a final concentration of 0·01 M and into tubes containing no anticoagulant and yielded EDTA plasma and serum from each individual. Centrifugation was carried out at room temperature. All samples were frozen at −80°C within 2 hr.

Storage. Aliquots of plasma and serum from fifteen healthy individuals were left at room temperature for 1, 3 and 5 days before being frozen. In addition samples were frozen within 2 hr. A preservative solution containing 50 μg of streptomycin and 10^5 IU of penicillin per ml was added to the samples to a final concentration of 1:100.

Freezing and thawing. Aliquots of plasma and serum from four healthy adults were frozen at −80°C 1, 2 and 3 times. Frozen samples were thawed by keeping them at room temperature for 1 hr. One aliquot was not frozen, but kept at +4°C until the next day when analysis was performed.

Hageman plasma and serum. Fresh frozen EDTA plasma and serum from an individual deficient of Hageman factor (coagulation factor XII) was obtained from the Coagulation Laboratory, Karolinska sjukhuset, Stockholm, Sweden.

Partially purified C4

C4 (preparation A) was prepared from euglobulin by chromatography on DEAE cellulose essentially according to a procedure described previously (Laurell *et al.*, 1970). To eliminate C1 at an early stage of the purification procedure the blood used as starting material was allowed to flow directly into a siliconized bottle containing a solution of carrageenin. Carrageenin treatment was carried out according to Borsos, Rapp & Crisler (1965). The plasma obtained after centrifugation was retreated twice with carrageenin. EDTA was then added to a final concentration of 0·01 M. Euglobulin was prepared according to Lepow *et al.* (1963). Chromatography was performed in the presence of 0·001 M EDTA. Fractions were dialysed against barbital buffer, pH 7·4, containing 0·001 M EDTA. Calcium and magnesium were then added to the fractions and tests for C4 haemolytic activity with an R4 reagent were performed. Active fractions were pooled and concentrated ten-fold in a model 50 Diaflo Ultrafiltration cell.

C4 (preparation B) was prepared from pseudoglobulin by chromatography on DEAE cellulose. The method described by Vroon *et al.* (1970) was used.

The C4 preparations were haemolytically active and on crossed immunoelectrophoresis they migrated like native C4. No C1s protein was found in the C4 preparations with immunochemical techniques. Preparation A had a protein concentration of 250 μg/ml, preparation B 650 μg/ml. The C4 content was 70 and 100 μg/ml, respectively, as compared to a commercial standard (human serum, stabilized, Behringwerke AG, Marburg, Germany).

*R*1 was prepared according to Fjellström (1962).

*R*3 and *R*4 were prepared according to Kabat & Mayer (1961).

Antiserum to C4 was prepared as described earlier (Laurell *et al.*, 1970).

Antiserum to C1*s* was obtained by immunization of rabbits with purified $C\bar{1}s$ in Freund's complete adjuvant.

Proteolytic enzymes

Purified $C\bar{1}s$ was prepared according to Haines & Lepow (1964). The preparation had an activity of 132 units/ml, as determined by pH-stat titration (Laurell *et al.*, 1965).

Human plasmin (Kabi 1873, DtC 38, KABI, Stockholm, Sweden) was prepared without acidification during the purification process and was glycerol activated at pH 7·4. The preparation was highly purified, also by chromatography on lysine-Sepharose. It contained 15 CTA unit/ml as defined by Johnson *et al.* (1969). Estimation of the proteolytic activity was kindly performed by Dr U. Hedner (Hedner & Nilsson, 1971) at the Coagulation Laboratory, Allmänna sjukhuset, Malmö, Sweden.

Trypsin (2 × crystallized, Bovine pancreas, Type III, Sigma, St. Louis 18, Missouri, U.S.A.) was dissolved in 0·0025 N hydrochloric acid at 10 mg/ml.

Chymotrypsin (2 × crystallized, Bovine pancreas, 9·35 NF/mg, batch 40406, Novo Industri A/S, Copenhagen, Denmark) was handled in the same way as trypsin.

Bovine thrombin (Topical Thrombin, 5000 NIH unit. Parke, Davis & Co., Detroit, Michigan, U.S.A.) diluted in 0·15 M sodium chloride was used. The enzyme was added to the test samples in siliconized tubes.

Enzyme inhibitors

Ethylene diamine tetraacetate. Various dilutions of Na_3EDTA, $Na_3MgEDTA$, K_2Mg-EDTA and Na_2Ca EDTA were added to serum before electrophoresis. Unless specified EDTA denotes Na_3EDTA.

Heparin (10000 IU/ml, Vitrum, Stockholm, Sweden), *Sp* 54 (100 mg/ml, Firma Bene-Chemie, Munich, Germany) and *Liquoid* (F. Hoffmann—La Roche & Co AG, Basle, Switzerland) in serial dilutions were added to serum and in some experiments also to the gel media.

Carrageenin (Sigma, St. Louis, Missouri, U.S.A.) treatment of serum was performed according to Borsos *et al.* (1965). Plasma was treated likewise in the presence of 0·01 M EDTA.

Epsilon amino caproic acid (*EACA*) Epsikapron® 0·4 g/ml, KABI, Stockholm, Sweden) was added both to serum and to the gel.

$C\bar{1}$ *inactivator.* A concentrate of $C\bar{1}$ inactivator (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) was further purified by gel filtration on Sephadex G-200 and by preparative agarose electrophoresis (Laurell & Mårtensson, 1971). The final preparation contained 36 unit/ml of $C\bar{1}$ inactivator (Levy & Lepow, 1959; Laurell *et al.*, 1965). No α_1-antitrypsin, α_2-macroglobulin or α_2-antithrombin (III) was detected in the preparation.

Trasylol (500000 KIE, Bayer AG, Leverkusen, Germany) dissolved in barbital buffer was used in serum and in the gel medium. Fresh solutions were prepared for each experiment.

Soybean trypsin inhibitor (*SBTI*) (Worthington Biochemical Sales Co., Freehold, New Jersey, U.S.A.) in barbital buffer was used.

Crossed immunoelectrophoresis was performed according to the method described by

Laurell (1965). Barbital buffer, 0·075 M, pH 8·6, containing no calcium was used. Unless otherwise stated this buffer was also used as a diluent. The electrophoretic separation step was usually carried out for 2 hr. The electrophoretic apparatus was cooled by water at about 5°C. The plates were stained with Coomassie Brilliant Blue R 250 5 (Mann Res. Lab., N.Y., U.S.A.).

RESULTS

Normal sera and EDTA plasma were analysed for C4 protein by crossed immunoelectrophoresis. EDTA plasma produced a single, sharply outlined C4 protein peak slightly

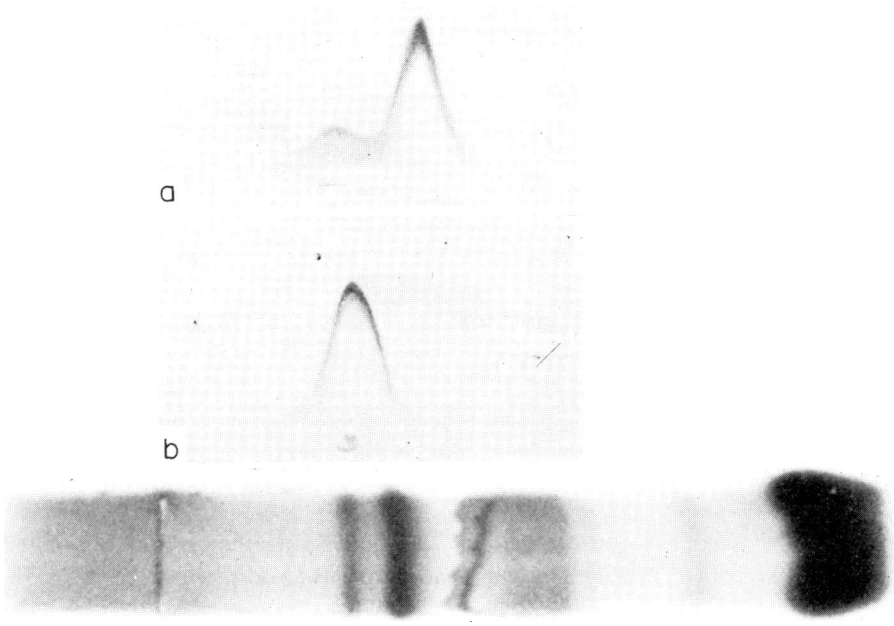

FIG. 1. Crossed immunoelectrophoretic pattern of (a) C4 protein of fresh serum and (b) C4 protein of the corresponding EDTA plasma. The agarose electrophoretic pattern of normal serum is given for reference.

anodal to the β_2 band (Fig. 1). Microheterogeneity of the C4 protein was noted in plasma samples from several individuals. In the light of the results presented by Rosenfeld *et al.* (1969) the pattern seen on analysis of fresh EDTA plasma in the present work was regarded as representing native C4.

Serum invariably gave a C4 protein peak in the β_1 region, usually accompanied by a second peak of β_2 mobility. The former was situated anodally to the β_1 band and is referred to as the fast β_1 peak. The position of the more slowly migrating component was less reproducible from time to time. It corresponded either to the β_2 band or was found in a slightly more cathodal position. In the present work it is referred to as the slow β_2 peak. Typically,

the precipitate of the β_1 C4 protein peak was sharply outlined whereas the slow β_2 peak was stained also inside its outline (Fig. 1).

Storage

Aliquots of serum and plasma were analysed after storage at room temperature for 1, 3 and 5 days (Fig. 2). Fresh serum gave a fast β_1 peak with sparse formation of the slow β_2 component. Storage resulted in a gradual increase of the slow β_2 peak. After 5 days' storage a third peak appeared in the γ region. The alteration of the C4 protein with storage of EDTA plasma manifested itself largely as a characteristic change in the configuration of the β_2 C4 protein peak of fresh plasma. Thus, already after 1 day of storage of plasma

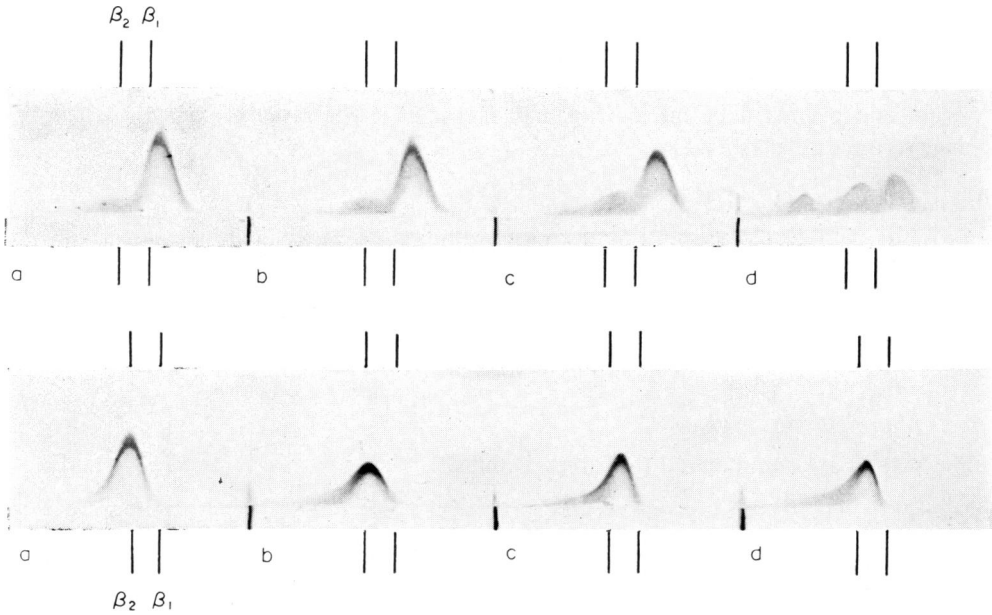

FIG. 2. Crossed immunoelectrophoretic pattern of C4 of serum (above) and EDTA plasma (below). (a) Fresh specimens, (b) storage 1 day, (c) storage 3 days, (d) storage 5 days. Storage at room temperature. Vertical lines indicate the positions of the β_1 and β_2 bands of the corresponding agarose electrophoresis.

the β_2 C4 protein peak developed a cathodal slope. A decrease in the height of the C4 protein peak was also noted. Differences in time of the evolution of the patterns described were observed with samples from different individuals.

Analysis of the C4 protein was also carried out with stored serum and plasma samples after the addition of EDTA to serum and calcium to plasma before electrophoresis. Addition of EDTA to stored serum resulted in a sharply outlined β_2 peak, such as is produced by fresh EDTA plasma, even after storage of serum at room temperature for 5 days. After 5 days' storage, however, serum always gave an additional peak in the γ region. The fast β_1 peak and the slow β_2 peak seen on analysis of fresh or stored serum without EDTA never appeared when EDTA was added before electrophoresis. When plasma stored for 1 day at room temperature was recalcified a minor β_1 C4 protein peak appeared in addition to

the pattern seen in Fig. 2b (below). No changes from the pattern shown in Fig. 2c and d (below) were seen when calcium was added to EDTA plasma stored for 3 or 5 days.

Freezing and thawing

Freezing and thawing of plasma did not influence the C4 protein pattern. For serum a slight increase in the slow β_2 peak was observed. It was concluded that freezing and thawing up to three times does not appreciably affect the C4 protein pattern.

Dilution in barbital buffer

Dilution of fresh serum in barbital buffer without Ca^{++} enhanced the formation of the slow β_2 component and at the same time reduced the β_1 peak. No conversion occurred after dilution of serum in barbital buffer containing EDTA at 0·01 M.

Hageman serum and plasma

Serum and plasma deficient in Hageman factor gave the same C4 protein patterns as normal serum and plasma.

R1 and R3 reagents

R1 prepared by the precipitation of C1 with dilute acetic acid at pH 5·5 gave a single C4 protein peak in the same position as when plasma or EDTA treated serum was analysed. Serum treated with zymosan, yielding an R3 reagent, was also examined. This reagent showed the conversion pattern characteristic of serum. Addition of EDTA to R3 reagents resulted in a single peak corresponding to the plasma C4 protein pattern.

Effects of proteolytic enzymes

The results are summarized in Tables 1 and 2.

$C\bar{1}s$

When $C\bar{1}s$ at final concentrations ranging from 5 unit/ml to 30 unit/ml was added to serum, EDTA serum or R1 reagents immediately prior to electrophoresis, it resulted in a C4 protein pattern consisting of two or three peaks. A major slow β_2 peak was always seen, usually accompanied by a minor peak in the inter α region. Often a fast β_1 peak was seen. Incubation at 37°C for 5 min prior to electrophoresis abolished the two minor peaks leaving only the slow β_2 peak (Fig. 3). Similar amounts of $C\bar{1}s$ added to partially purified C4 never gave rise to a slow β_2 peak. $C\bar{1}s$ treatment of C4 preparation A resulted in a fast β_1 peak. C4 preparation B showed the fast β_1 peak together with an inter α C4 peak after treatment with $C\bar{1}s$.

Plasmin

Plasmin at a final concentration of 5 CTA unit/ml added to serum before electrophoresis produced a single β_1 C4 conversion product. Plasmin at 2·5–1 CTA unit/ml gave partial conversion of C4 to this β_1 product on analysis of some sera, but not of all. Plasmin added to EDTA serum gave a β_1 peak at 5 CTA unit/ml, but smaller doses never influenced C4 mobility. Plasmin added to partially purified C4 at 5–0·5 CTA unit/ml gave rise to C4 conversion patterns similar to those produced by $C\bar{1}s$. Following plasmin treatment preparation A was thus seen to produce a fast β_1 peak and preparation B an additional inter α peak.

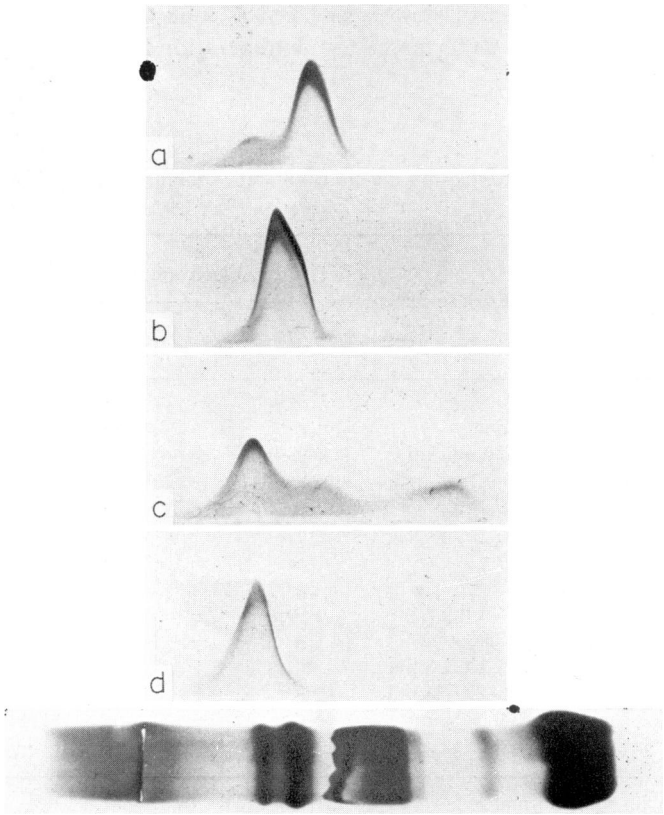

FIG. 3. Effect of $C\bar{1}s$ (13 unit/ml) on the C4 protein in EDTA serum analysed by crossed immunoelectrophoresis. (a) Serum, (b) EDTA serum, (c) EDTA serum+$C\bar{1}s$ added immediately before agarose electrophoresis, (d) EDTA serum+C1s. Mixture incubated for 5 min at 37°C before electrophoresis. Agarose electrophoresis of normal serum shown for reference.

Thrombin

Thrombin added to serum at 100 NIH unit/ml enhanced conversion of the C4 protein. The size of the slow β_2 peak was increased and the fast β_1 peak was correspondingly diminished. Thrombin added to EDTA serum did not influence the mobility of the C4 protein. Thrombin gave rise to a fast β_1 C4 protein peak when added to preparation B. A minor component of γ mobility was seen when preparation A was treated with thrombin.

Trypsin and chymotrypsin

Trypsin added to serum to a final concentration of 2 mg/ml prior to electrophoresis resulted in the appearance of a single C4 protein peak in the posterior α_2 region. Chymotrypsin added to serum at the same concentration gave rise to a fast β_1 C4 protein peak. At doses between 1 mg/ml and 0·2 mg/ml trypsin or chymotrypsin produced C4 conversion patterns similar to those seen when $C\bar{1}s$ was added to serum (Fig. 3). The same results were obtained with EDTA-serum. Trypsin and chymotrypsin added to partially purified C4 at 1 mg/ml

produced a conversion product of slow α_2 and fast β_1 mobility, respectively. Addition of trypsin or chymotrypsin at 0·2–0·005 mg/ml to C4 preparation B gave a fast β_1 peak together with an inter α component.

TABLE 1. Influence of proteolytic enzymes on the electrophoretic mobility of the C4 protein in serum and in EDTA serum

Test sample	Enzyme per ml sample	Electrophoretic position of C4					
		Slow β_2	β_2	β_1	Fast β_1	Slow α_2	Inter α
Serum	None	+	−	−	+++	−	−
EDTA serum	None	−	+++	−	−	−	−
$\overline{C1}$s + serum /EDTA serum	5–30 unit/ml	++	−	−	+	−	+
Trypsin + serum/EDTA serum	2 mg	−	−	−	−	+++	−
Trypsin + serum/EDTA serum	0·2–1 mg	++	−	−	+	−	+
Chymotrypsin + serum/EDTA serum	2 mg	−	−	−	+++	−	−
Chymotrypsin + serum/EDTA serum	0·2–1 mg	++	−	−	+	−	+
Plasmin + serum/EDTA serum	5 CTA unit	−	−	+++	−	−	−
Plasmin + serum*	2·5–1 CTA unit	−	++	++	−	−	−
Plasmin + EDTA serum	2·5–1 CTA unit	−	+++	−	−	−	−
Thrombin + serum	100 NIH unit	++	−	−	++	−	−
Thrombin + EDTA serum	100 NIH unit	−	+++	−	−	−	−

* Only with some normal sera.
The approximate size of the C4 protein peaks is denoted by positive signs.

TABLE 2. Influence of proteolytic enzymes on the electrophoretic mobility of partially purified C4 derived from euglobulin (A) or pseudoglobulin (B)

Test sample	Enzyme per ml sample	Electrophoretic position of C4				
		γ	β_2	Fast β_1	Slow α_2	Inter α
Partially purified C4 (A or B)	None	−	+++	−	−	−
$\overline{C1}$s + A	5–30 unit	−	+	++	−	−
$\overline{C1}$s + B	5–30 unit	−	−	++	−	+
Trypsin + A or B	1 mg	−	−	−	+++	−
Trypsin + B	0·05–0·2 mg	−	−	++	−	+
Chymotrypsin + A or B	1 mg	−	−	+++	−	−
Chymotrypsin + B	0·05–0·2 mg	−	−	++	−	+
Plasmin + A	3 CTA unit	−	−	+++	−	−
Plasmin + B	5–2·5 CTA unit	−	−	++	−	+
Thrombin + A	100–50 NIH unit	+	++	−	−	−
Thrombin + B	100–50 NIH unit	−	+	++	−	−

The approximate size of the C4 protein peaks is denoted by positive signs.

Effect of enzyme inhibitors

The results are summarized in Table 3.

TABLE 3. Influence of enzyme inhibitors on the conversion of C4 in serum on agarose electrophoresis

Inhibitor	Inhibition	Doses used	
		In serum	In serum and gel medium
Na_3EDTA	Yes	0·005 M	–
MgEDTA	Yes	0·1–0·05 M	–
CaEDTA	No	0·1 M	–
Heparin	Yes	1000 IU/ml	10 IU/ml
Sp 54	Yes	1 mg/ml	0·01 mg/ml
Liquoid	Yes	0·1 mg/ml	0·001 mg/ml
Carrageenin	Yes	—	—
EACA	Yes	—	0·5 M
$C\bar{1}$ inactivator	Yes	—	2·2–5·4 unit/ml
SBTI	No	6·25 mg/ml	1·25 mg/ml
Trasylol	(Yes)	–	Possibly partial inhibition at 50–250 KI unit/ml

Ethylene diamine tetraacetate

In repeated experiments the typical plasma C4 protein pattern appeared when EDTA was added to serum to a final concentration of 0·005 M prior to electrophoresis. At lower concentrations conversion to a serum pattern occurred with a continuous increase of the slow β_2 component parallel to decreasing concentrations of EDTA (Table 4). When MgEDTA was added to serum the dose relationships were not so clear-cut. Complete blocking of conversion usually occurred at 0·1 or 0·05 M of $K_2MgEDTA$ or $Na_2MgEDTA$. At 0·02 M or less the serum type of C4 conversion occurred. Variable patterns were seen at intermediate concentrations. $Na_2CaEDTA$ did not inhibit the conversion of the C4 protein even at 0·1 M.

TABLE 4. C4 protein pattern of serum in the presence of various concentrations of EDTA

EDTA	β_2 peak	β_1 peak	Slow β_2 peak
0·005	+++	–	–
0·004 M	–	+++	–
0·003 M	–	++	(+)
0·002 M	–	++	+
No EDTA	–	++	+

The approximate size of the C4 protein peaks is denoted by positive signs.

Heparin

Heparin added to serum prior to electrophoresis blocked conversion of the C4 protein at about 1000 IU/ml. At lower concentrations changes occurred in the configuration of the C4 protein peak. 1 IU/ml did not influence conversion (Fig. 4). When heparin was added both to serum and to the gel C4 conversion was inhibited at about 10 IU/ml.

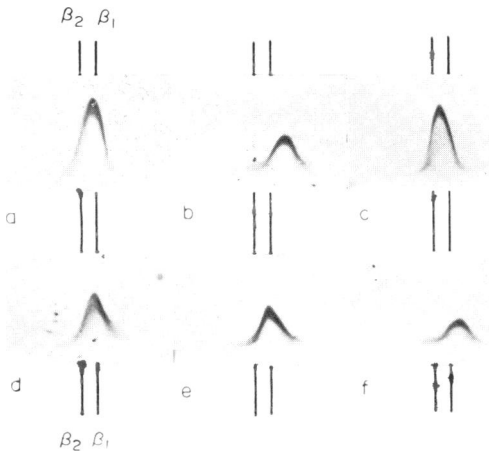

FIG. 4. Crossed immunoelectrophoretic pattern of C4. Heparin added to serum prior to electrophoresis. (a) EDTA plasma, (b) serum, (c) serum+heparin 1000 IU/ml, (d) serum+heparin 100 IU/ml, (e) serum+heparin 10 IU/ml, (f) serum+heparin 1 IU/ml. Vertical lines denote the positions of the β_1 and β_2 bands of the corresponding agarose electrophoresis.

Sp 54 at 1 mg/ml effectively blocked the conversion of the C4 protein when added to serum before electrophoresis. Intermediate patterns were produced at lower concentrations and at 0·125 mg/ml Sp 54 no longer influenced C4 conversion. C4 conversion was completely blocked at 0·010 mg/ml when Sp 54 was also included in the gel medium.

Liquoid

At 0·1 mg/ml liquoid blocked the conversion of the C4 protein after additon to serum before electrophoresis. At 0·05 mg/ml formation of the slow β_2 component of the ordinary conversion pattern was suppressed, while no effect was seen at 0·025 mg/ml. Liquoid in a concentration of 0·001 mg/ml in the gel and in serum produced the pattern of native C4 protein.

Carrageenin

No conversion of the C4 protein occurred on electrophoresis of Carrageenin-treated serum or plasma. Recalcification of Carrageenin-treated plasma did not result in C4 conversion.

Epsilon aminocaproic acid

At a final concentration of 0·5 M in the gel and in the sera tested EACA was capable of inhibiting C4 conversion completely. Concentrations of 0·125 and 0·250 M diminished the slow β_2 peak of the C4 protein conversion pattern of serum. EACA at 0·05 M did not

influence C4 conversion. The specificity of 0·5–0·25 M of EACA in the present system was questionable, as it was found that also comparable amounts of sodium chloride influenced C4 conversion.

$C\bar{1}$ inactivator

No conversion of the C4 protein was seen when $C\bar{1}$ inactivator was incorporated in the gel and added to serum at 5·4 unit/ml. Partial inhibition was observed at 2·2 unit/ml (Fig. 5).

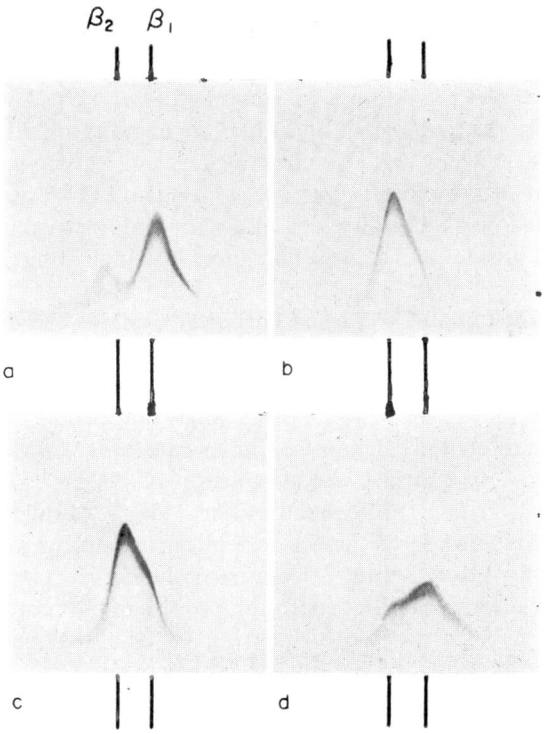

FIG. 5. Crossed immunoelectrophoresis of C4. Influence on C4 conversion of $C\bar{1}$ inactivator included in the gel medium. (a) Serum, (b) EDTA plasma, (c) serum in the presence of $C\bar{1}$ inactivator at 5·4 unit/ml, (d) serum in the presence of $C\bar{1}$ inactivator at 2·2 unit/ml. Vertical lines indicate the positions of the β_1 and β_2 bands.

Soybean trypsin inhibitor

The addition of SBTI to serum prior to electrophoresis in doses as high as 6·25 mg/ml did not influence conversion of the C4 protein. When SBTI was added to serum and to the gel at 1·25 mg/ml a conversion pattern consisting of three C4 protein peaks developed. The major component was found in the β_1 region. Of the two minor peaks one was situated in the β_2 region and the other in the α_2 region.

Trasylol

Unless included in the gel medium Trasylol did not change the electrophoretic behaviour of C4 in serum. In the presence of Trasylol at 50–250 KIU/ml a β_1 C4 protein peak was

seen together with a clearly reduced slow β_2 peak as compared with the conversion pattern of serum analysed in parallel without Trasylol. With increasing concentrations of Trasylol in the gel the C4 protein migrated much slower. Inspection of the initial electrophoresis strip revealed a corresponding cathodal shift of the β_2 band which compromised the interpretation.

DISCUSSION

Analysis of the C4 protein by crossed immunoelectrophoresis showed that undiluted serum gave a major C4 protein peak in the fast β_1 region, while the C4 protein in EDTA plasma migrated slower, giving a peak in the β_2 region.

The present work verifies the findings of Rosenfeld *et al.* (1969) as to the conversion of C4 in serum on agarose electrophoresis and the blocking effect of EDTA. That no conversion is seen with an R1 reagent was also confirmed.

Dilution of serum in electrophoresis buffer containing no EDTA promoted the conversion of C4 to a product of slow β_2 mobility seen also with undiluted serum (Fig. 1a). The slow β_2 peak was stained inside the contourline, possibly indicating a complex formation (Laurell, 1972).

The inhibition by EDTA of C4 conversion partly explains the differences between C4 values in serum and plasma on quantitation of C4 by the electroimmunoassay (Laurell *et al.*, 1970). With reference to the present findings, enhanced conversion of the C4 protein in serum during electrophoresis to a more slowly migrating product seems to explain why fresh and stored serum diluted in calcium-free buffer gave lower C4 values than fresh plasma diluted in EDTA-buffer. In contrast, the sharp drop of plasma C4 values in the electroimmuno assay (Laurell *et al.*, 1970) already after 1 day's storage at room temperature was apparently due to alteration of the C4 protein during storage of plasma (Fig. 2).

It is not known why C4 in serum is converted during electrophoresis. One possible explanation is that the electrophoretic separation process might remove naturally occurring inhibitors of serum enzymes capable of cleaving C4 and that such enzymes might be activated during electrophoresis. In the fibrin agar electrophoretic system plasmin activity is generated on electrophoresis of normal human serum (Heimburger & Schwick, 1962).

$C\bar{1}s$, trypsin, chymotrypsin, plasmin and thrombin changed the electrophoretic mobility of the C4 protein. When added to partially purified C4 all the enzymes produced a C4 protein peak corresponding roughly to the fast β_1 peak seen on analysis of normal serum.

With the exception of thrombin the enzymes gave rise to an additional inter α peak after addition to C4 prepared from pseudoglobulin. Investigation of the possibility of this peak representing a complex formed by C4 and a rapidly migrating protein is in progress.

Plasmin did not give rise to a C4 conversion pattern similar to that of normal serum (Table 1). It therefore seems less likely that plasmin is responsible for the conversion of C4 seen on analysis of normal serum.

All the enzymes tested except plasmin produced a slow β_2 C4 peak on addition to serum. The slow β_2 peak never appeared on enzyme treatment of partially purified C4. This suggested that this conversion product might be a complex formed by C4 and some other protein in serum.

The presence of EDTA in serum had no influence on the C4 protein patterns seen on addition of $C\bar{1}s$, trypsin or chymotrypsin, whereas the effect of thrombin was inhibited.

EDTA also affected C4 conversion produced by the addition of plasmin to serum. The role played by EDTA in this context is not clear.

As to the effect of $C\bar{1}s$ on preparations of C4, the present findings are in accord with those of Rosenfeld *et al.* (1969) and Budzko & Müller-Eberhard (1970), who found that C4 treated with $C\bar{1}s$ migrated faster than the native protein on crossed immunoelectrophoresis and on immunoelectrophoresis, respectively. The finding that trypsin increased the electrophoretic mobility of C4 does not agree with that of Budzko & Müller-Eberhard (1970), who reported the formation of a slow migrating product on immunoelectrophoresis after treatment of C4 with trypsin. Differences in the electrophoretic media used might explain this discrepancy.

EDTA is known to inhibit the activation of C1s (Lepow, Ratnoff & Levy, 1958) but not the activated enzyme (Ratnoff & Lepow, 1957). The ability of MgEDTA together with the inability of CaEDTA to block C4 conversion are properties common to C1 and the agent responsible for C4 conversion during electrophoresis, the activation of C1 being known to be calcium dependent (Levine *et al.*, 1953).

C1 is also inhibited by heparin and by Sp 54 (Walb, Loos & Hadding, 1971). Recent evidence suggests that Sp 54 interferes with the function of bound C1q (Loos, Borsos & Rapp, 1972). It was interesting to note that the relationship between the doses per weight unit of heparin and Sp 54 needed for complete inhibition of C4 conversion was in good accord with the findings of Walb *et al.* (1971), who compared the anticomplementary effects of these substances in a haemolytic system.

Liquoid has been claimed to inhibit C1r (Naff & Ratnoff, 1968). Carrageenin adsorbs C1q and thereby prevents the binding of C1 to antigen–antibody complexes (Borsos *et al.*, 1965). In the present investigation Carrageenin removed C1q but not C1s from EDTA plasma (unpublished observation).

It therefore seems reasonable that EDTA, heparin, Sp 54, Liquoid and Carrageenin inhibit the conversion of C4 on agarose electrophoresis of serum by their action on C1.

Taylor & Fudenberg (1964) showed that EACA can inhibit the activation of C1s. In the present investigation EACA in concentrations similar to those used by these authors partly inhibited the conversion of C4.

The inhibition of C4 conversion by moderate amounts of $C\bar{1}$ inactivator lends support to the assumption that the conversion is mediated by $C\bar{1}$. Although $C\bar{1}$ inactivator has been found to affect several enzymes, it does not inhibit thrombin (Ratnoff *et al.* 1969; Forbes, Pensky & Ratnoff, 1970).

Soybean trypsin inhibitor inhibits a group of enzymes including plasmin (Breckenridge & Ratnoff, 1965; Back & Steger, 1968) but does not affect $C\bar{1}s$ (Ratnoff & Lepow, 1957). The failure of soybean trypsin inhibitor in blocking C4 conversion in serum on agarose electrophoresis provides further evidence that plasmin is not involved in this reaction. Soybean trypsin inhibitor as well as Trasylol in high concentrations has been shown to influence the activation of $C\bar{1}s$ by activated Hageman factor in hereditary angioneurotic oedema plasma (Donaldson, 1968). Judging from the present findings Hageman factor is not necessary for the conversion of C4 in serum during electrophoresis since serum deficient in this factor gave a normal conversion pattern. Trasylol was found to diminish the slow β_2 peak of the C4 conversion pattern of normal serum, possibly indicating an inhibitory effect on C4 conversion. Clarification of this effect of Trasylol must await further research.

A possible role of C1 in the conversion of C4 on agarose electrophoresis of normal serum is suggested by the absence of C4 conversion on agarose electrophoresis of R1 preparations. The present study shows that enzymes other than $C\bar{1}s$ are able to produce a C4 conversion pattern similar to that seen on analysis of normal serum. Data obtained on the inhibition of C4 conversion support the contention that conversion of C4 on agarose electrophoresis is caused by activation of C1, possibly involving sequential activation of its subcomponents.

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