SUMMARY

Ultracentrifugal experiments indicate that the \( \alpha_2 \)-macroglobulin binding of cationic aspartate aminotransferase, trypsin, and chymotrypsin occurs in conditions approaching physiological.

The order of affinity for \( \alpha_2 \)-macroglobulin is trypsin > chymotrypsin > cationic aspartate aminotransferase.

The binding of cationic aspartate aminotransferase is sensitive to changes in ionic concentration. In general, binding is diminished as the salt concentration rises, but borate in dilute solution enhances the binding effect, and so does a serum factor which is dialysable, ultrafiltrable, and extractable by ether from acidified serum ultrafiltrate.

INTRODUCTION

Boyde and Latner\(^1\) reported that up to 3 zones of aspartate aminotransferase activity could be demonstrated after starch gel electrophoresis of serum. One of these was coincident with the "slow \( \alpha_2 \)" band and is now known to be due to binding of the cationic (mitochondrial) isoenzyme by \( \alpha_2 \)-macroglobulin. Jacobsson\(^2\) showed that part of the normal serum anti-trypsin activity was contained in the \( \alpha_2 \)-globulins. Haverback et al.\(^4\) found that, in admixture with serum, the normal cationic mobility of trypsin and chymotrypsin was reversed and the enzyme migrated with the \( \alpha_2 \)-globulins.

Several groups\(^5\)–\(^8\) have given evidence that the \( \alpha_2 \)-globulin responsible for this binding effect is \( \alpha_2 \)-macroglobulin and there is suggestive evidence that the same serum protein may bind several other enzymes\(^9\)–\(^12\). However, none of the published work contains clear evidence of the binding of any enzyme under physiological conditions.

MATERIALS AND METHODS

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pared as described earlier\textsuperscript{13}. This material was undoubtedly very impure, although free of anionic isoenzyme (specific activity 420 U/g protein). Trypsin and $\alpha$-chymotrypsin were purchased from C. F. Boehringer and Sons, papain (twice recrystallized) from Sigma Chemical Co.

Two preparations of $\gamma$-macroglobulin (19 S $\gamma$-globulin) were used. One was from the serum of patients suffering from myelomatosis (courtesy of Dr. T. J. Muckle), the other was made by dialysing normal serum against water and redissolving the precipitate in 0.9\% sodium chloride. Two preparations were also used of $\alpha_2$-macroglobulin. One was a gift of Professor J. W. Mehl of the University of Southern California to Professor A. L. Latner; the other was prepared by excising the "slow $\alpha_2$" zone after polyacrylamide gel electrophoresis of serum and eluting the protein by macerating the gel in 115 mM sodium chloride containing 2 mM phosphate buffer, pH 7.0, and then mixing the suspension on a turntable for 48 h at 4\°.

**Enzyme assays**

Aspartate aminotransferase was assayed by the method of Boyde\textsuperscript{13}. For the purposes of this paper one unit (U) is taken to be the quantity of enzyme which is capable of converting one \(\mu\)mole of aspartate to oxaloacetate per min under the specified conditions. Trypsin and chymotrypsin were assayed as described by Hummel\textsuperscript{14}.

**Ultracentrifugation**

Four-ml samples of serum, or mixtures containing serum, were subjected to centrifugation for 17 h at 45700 g (Spinco Model L ultracentrifuge, SW39 L head, 24000 rev./min) calculated to give complete sedimentation of a 20 S protein. The rotor chamber thermostat was set at 18\°. This gave an equilibrium rotor temperature of 37\°, attained after 11 h running (36\° after 5 h), or maintained indefinitely if the rotor was pre-warmed in an incubator as in most of the experiments reported here. (The rotor temperature was measured in the sample tubes after interrupting the run and braking as quickly as possible.)

After centrifugation, each specimen was fractionated by aspirating successive 1-ml samples through a needle with the point kept just under the surface of the liquid. Before taking up the 4th fraction, the sediment was resuspended by stirring with a glass rod.

**Electrophoresis**

Polyacrylamide gel electrophoresis, and the method of detecting zones of aspartate aminotransferase activity after electrophoresis, were as described previously\textsuperscript{2}.

**Preparations derived from serum ultrafiltrate**

Twelve-ml lots of normal serum were subjected to ultrafiltration through "Visking" tubing; 10 ml of ultrafiltrate was collected in each case. 10 ml of ultrafiltrate was acidified to pH 1.0 (pH meter) by dropwise addition of concentrated hydrochloric acid, and then shaken with 50 ml diethyl ether. The ether layer was evaporated to dryness (rotary evaporator, 40\°), the residue redissolved in 10 ml distilled water by mechanically rotating the flask for 12 h, and adjusted to pH 7.0 by the dropwise addition of 0.1 M NaOH (pH meter). The aqueous layer (after separa-
TABLE I
DISTRIBUTION OF ENZYME ACTIVITIES AFTER ULTRACENTRIFUGATION

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Composition of mixture</th>
<th>Activity found, % of recovered activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum (ml)</td>
<td>CAA† (mU)</td>
</tr>
<tr>
<td>a</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>b</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>c</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>d*</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>e*</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percentages given are: above—cationic aspartate aminotransferase. below—trypsin or chymotrypsin.
† CAA: cationic aspartate aminotransferase.

RESULTS

When cationic aspartate aminotransferase, 100 mU, was added to serum and subjected to ultracentrifugation, almost all the activity appeared in fraction 4 (sediment). This did not occur in the absence of α2-macroglobulin19. There was barely detectable sedimentation after ultracentrifugation of the isoenzyme in solution in the following: water, 0.9% sodium chloride, 10% bovine albumin, 0.2% γ-macroglobulin, and serum preparations lacking α2-macroglobulin. Addition of α2-macroglobulin to these preparations restored the capacity to sediment cationic aspartate aminotransferase. Anionic isoenzyme was not sedimented under any of the above conditions19.

The sedimentation of trypsin and chymotrypsin was studied by the same ultracentrifugation technique, but in the cold, since at 37° there was excessive digestion of serum proteins. Recoveries were low because of the effects of the antitrypsin activity of serum. Table I shows the distribution of recovered activity after ultracentrifugation. Displayed also in Table I are the results of similar experiments in which both cationic aspartate aminotransferase and trypsin (or chymotrypsin) were mixed with serum. Apparently, the two proteases were able to displace cationic aspartate aminotransferase from combination with α2-macroglobulin. The same effect was demonstrated also by polyacrylamide gel electrophoresis. It made no difference whether the protease or the aminotransferase was added first to the serum. The apparent order of affinity of enzymes for α2-macroglobulin was trypsin > chymotrypsin > cationic aspartate aminotransferase. Papain was also shown to displace the aminotransferase, but its affinity relative to trypsin is not known.

To determine how far the binding phenomenon was dependent on the ionic composition of the medium, serum was exhaustively dialysed against distilled water and then mixed with various salt solutions, before centrifuging in admixture with cationic aspartate aminotransferase. The results are shown in Fig. 1. Borate exhibits a distinct optimum concentration for binding of about 30 mM, whereas in sodium chloride solutions there is inhibition of binding which rises sharply to a limit. For sodium phosphate buffers the rise is more gradual, but the limit is higher.

Fig. 1. Binding of cationic aspartate aminotransferase to α₂-macroglobulin during ultracentrifugation. Variation with ionic concentration. The procedure is described in the text. Results are expressed as the percentage of recovered activity found in the supernatant (fractions 1-3). If no sedimentation occurred the result would be 75%. There was substantial loss of total activity in the presence of the stronger phosphate solutions but this also occurred without ultracentrifugation and the activity could be restored by preincubation with pyridoxal phosphate. Symbols: ■, sodium phosphate buffer, pH 6.5; ●, sodium chloride; ▲, sodium borate buffer, pH 8.4.

Fig. 2. Binding of cationic aspartate aminotransferase to α₂-macroglobulin. Effect of serum ultrafiltrate preparations. The procedures for ultracentrifugation, ultrafiltration, etc., are given in the text. Mixtures were made up containing in 6 ml, 4 ml dialysed serum, 100 mU cationic aspartate aminotransferase, various amounts of sodium chloride, distilled water to volume, and the following: ■, 1.0 serum ultrafiltrate; ○, 1.0 ml ether extract of serum ultrafiltrate; ▲, 1.0 ml “neutralized aqueous layer” (see text); ●, no additional reagent (cf. Fig. 1). Results are expressed as the percentage of recovered activity in the supernatant (fractions 1-3).

Some early difficulties were resolved by the experiments illustrated in Fig. 2. Non-dialysed serum gives more binding than does dialysed serum at the same chloride concentration. The factor responsible for this effect is found in serum ultrafiltrate and in an acid ethereal extract of serum ultrafiltrate.

The binding of trypsin and chymotrypsin was much less sensitive to increasing salt concentration than that of cationic aspartate aminotransferase.

Experiments with purified α₂-macroglobulin gave results similar to those reported here.

DISCUSSION

Previous electrophoretic and chromatographic experiments demonstrated α₂-macroglobulin binding of cationic aspartate aminotransferase only in dilute borate buffers. This led to the suspicion that the phenomenon was not physiological, but due to borate ions complexing with the sugar residues of α₂-macroglobulin (which is a glycoprotein). It is shown above that although borate clearly enhances binding, it is not necessary for the occurrence of a very decided binding effect. According to expectation there is a general effect of increasing ionic concentration in diminishing binding. Further work is in progress on the physico-chemical aspects of the interaction. It seems quite likely that the “serum binding potentiator” will prove to be the anion of an organic acid, perhaps a hydroxy-acid, complexing with...
the sugar residues of \( \alpha_2 \)-macroglobulin in much the same way as is postulated for borate.

Though the presence of a high gravitational field may be expected to alter the equilibrium constant of any chemical reaction, such effects are not usually very large. It is thus reasonable to conclude that \( \alpha_2 \)-macroglobulin binding of trypsin, chymotrypsin, and cationic aspartate aminotransferase can occur in the plasma in life, but we do not know whether this is physiologically important or merely a physico-chemical coincidence. Trypsin bound to \( \alpha_2 \)-macroglobulin retains esterolytic activity and part\(^4,7,8\) or possibly the whole\(^20\) of its proteolytic activity, even in the presence of \( \alpha_2 \)-antitrypsin. It has been proposed that the physiological role of \( \alpha_2 \)-macroglobulin is connected with these facts. Similarly, it may have a role connected with blood coagulation since there is some evidence for \( \alpha_2 \)-macroglobulin binding or inhibition of certain of the enzymes involved\(^9,10,20,21\).

However, quite different proposals can be made, based upon the observed binding of cationic aspartate aminotransferase, and upon the observation\(^23,24\), that the cationic isoenzyme is cleared extraordinarily rapidly from the plasma of dogs after an intravenous injection. It may be that the role of \( \alpha_2 \)-macroglobulin is to facilitate the plasma transport and clearance of cationic aspartate aminotransferase and other cationic proteins. By virtue of their charge alone these proteins might be expected to interact with endothelial membranes so that they would be deposited throughout the blood vessels like scale in water-pipes. Presumably, this would be inimical to health. One would then expect that the raised plasma levels of \( \alpha_2 \)-macroglobulin found in many diseases would reflect an enhanced rate of release of cationic proteins from tissues. Against this hypothesis, there is the recent suggestion that the raised plasma level of \( \alpha_2 \)-macroglobulin in nephrosis is not due to increased synthesis at all, but to the reduced circulating fluid volume\(^24\).

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REFERENCES

