The binding of lithium carmine to a2-macroglobulin

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The colloidal dye lithium carmine was added in vitro to normal human serum. Electrophoretic experiments showed that the dye was associated mainly with a2-globulins, small amounts with the albumin and only traces with the γ-globulins. The main complex was eluted with the macroglobulin peak obtained by gel filtration on Sephadex G-200 and sedimented in the heavy fraction on density gradient ultracentrifugation. The dye-protein complex could be precipitated with an antisemum specific for a2-macroglobulin. Gel filtration of a solution of pure a2-macroglobulin, to which lithium carmine was added, demonstrated that the dye was bound to this protein.

Carmine, a dye prepared from alum-treated cochineal extracts, has widely been used as an ingredient of many histological stains. The dye is not a single definite compound but a mixture of molecules varying in chemical composition and physical state. The active principle, to which the pink color of the dye is ascribed, is carminic acid, a dibasic acid belonging to the anthraquinone group. The staining properties of carmine are to some extent due to the mordant action of aluminium, whereas some of its physical properties may depend on the presence of a small protein moiety derived from the insect.

Orth’s lithium carmine is a dye obtained by treating the dye with lithium carbonate and boiling in a saturated solution of lithium carbonate.

The exact chemical composition of this compound has not been reported. It has been used in histology as a nuclear stain. When injected intravenously, it is in part taken up by the reticuloendothelial system, and in part excreted in the urine.

If the lipophilic dyes are excepted, lithium carmine is the only dye so far reported to associate preferentially with the a2-globulin fraction of human serum.

Considering the recently discovered capacity of a2-macroglobulin (a2M) to bind various macromolecules such as trypsin, chymotrypsin, trypsinogen, plasmin, elastase, growth hormone, possibly insulin, it seemed warranted to investigate a2M with regard to its affinity for the colloidal dye lithium carmine.

Materials and Methods

Human serum from normal donors was stored at 4°C with a trace of sodium azide as an antiseptic. To avoid interference of haemoglobin with the absorbance of lithium carmine, only serum samples free of visible interference of haemoglobin with the absorbance of lithium carmine, only serum samples free of visible...
haemolysis were used. Experiments were performed within three days after sampling.

Crystalline human serum albumin was purchased from Poviet, Amsterdam.

Human IgG was obtained by DEAE-cellulose fractionation using 0.005 M phosphate buffer as an eluant.

Human apM was isolated from a serum belonging to the haptoglobin group HpI-I, following a method described elsewhere.21

A specific antiserum was obtained from a rabbit which had received three intramuscular injections of 4 mg human apM at weekly intervals. This antiserum reacted with pure apM and produced only one precipitation line when tested against normal human serum in immunoelectrophoresis and Ouchterlony plates. For the specific precipitation experiments of the lithium carmine-apM complex, the absence of haemoglobin in the precipitating solution was necessary to obtain reliable absorbancies at 520 m\mu. This was achieved by purifying the immunoglobulins of the antiserum by chromatography on DEAE-cellulose.

Specific anti-ß-lipoprotein serum was a generous gift from Behringwerke, Marburg/Lahn (Germany).

Lithium carmine was purchased from Dr. Karl Hollborn, Leipzig. A solution, containing about 4 mg/ml, was prepared in distilled water. After 2 hours’ mixing, the solution was centrifuged at 20,000 r.p.m. and undissolved material was removed. Of this solution, 2 ml was evaporated in vacuo in the presence of sodium hydroxide pellets, for four days, and the dry lithium carmine was weighed. The solution, which was found to contain 3.3 mg/ml, was used for all binding experiments described in this study. Its absorption spectrum had a maximum at 520 m\mu, with a smaller peak at 560 m\mu.

Extinctions were measured in a Hilger-Watts spectrophotometer.

Quantitative determination of apM was performed according to the single radial diffusion method in antibody-containing plates described by Mancini et al.22

Semi-quantitative estimation of the low-density lipoproteins were carried out in Ouchterlony plates.

Electrophoreses were performed in 50 x 30 x 1 cm Pevikon (PVK) blocks designed to accommodate two samples of 4 ml each. Sodium barbiturate-HCl buffer of pH 8.6 was used at a molarity of 0.03 M in the PVK block and of 0.1 M in the electrode vessels. The samples were applied over a width of 8 cm, by means of a Pasteur pipette. After electrophoresis, at 2 V/cm for 18 hours, the block was cut into 1 cm strips, which were eluted with 10 ml of 0.15 M NaCl. The protein content of each fraction was measured by the micromethod of Lowry et al.23, and the relative concentration of lithium carmine was determined from the extinction at 520 m\mu.

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Gel filtration on Sephadex G-200 was performed in 69 cm columns, having an internal diameter of 2.1 cm. The solvent was 2% (w/v) NaCl in 0.01 M NaHPO₄, and contained 0.01 g/100 ml sodium azide as an antiseptic. The eluate was collected in 2-ml fractions. Extinctions were measured at 280 m\mu and 520 m\mu and used as estimates of the protein and lithium carmine concentrations, respectively. In some experiments a 95 cm column was used, having an internal diameter of 3.6 cm. The fractions had a volume of 5 ml.

Density gradient ultracentrifugations were carried out in the preparative Spinco ultracentrifuge, Model L, using the swinging bucket rotor SW 39. The linear sucrose gradient ranged from 40% to 10% (w/v) in 0.15 M phosphate buffer and had a total volume of 4.2 ml. To avoid sedimentation in droplets, the sample was applied in three superimposed 200-μl-layers. The bottom layer was a mixture of 1 vol of sample and 2 vol of 10% (w/v) sucrose; the second layer was a mixture of 2 vol of sample and 1 vol of the sucrose solution, and the top layer was the sample undiluted. After centrifugation at 40,000 r.p.m., at 4°C, for 16 hours, the bottom of the tubes was pierced with a needle and fractions of about 150 μl were collected dropwise. To 100 μl of each fraction was added 500 μl of 0.15 M NaCl and extinctions were measured at 280 m\mu and 520 m\mu.

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### Results

#### 1. Electrophoresis on PVK-blocks

One sample of lithium carmine (0.2 mg/ml) and 3 samples of normal human serum containing 0.1, 0.5 and 1 mg lithium carmine per ml, respectively, were submitted to electrophoresis (Fig. 1). Free lithium carmine migrated well ahead of the albumin fraction. In the serum containing 0.1 mg lithium carmine/ml, the dye was completely bound to serum proteins, most of it to the apM-globulin fraction, less to albumin, and a trace to the γ-globulins. When the concentration of lithium carmine was raised to 0.5 and 1 mg/ml, the share of the apM-globulin fraction increased markedly, whereas only little additional dye was detected on the γ-globulins, and free lithium carmine appeared in front of the albumin fraction. It was clear that the apM-globulin fraction bound proportionately much more lithium carmine than did albumin.

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2. Gel filtration on Sephadex G-200

One sample of lithium carmine (0.5 mg/ml in 2% NaCl containing 0.01 M Na₂HPO₄), and 3 samples of normal human serum containing 0.1, 0.7 and 1 mg lithium carmine per ml, respectively, were applied successively in 2 ml amounts on the same column of Sephadex G-200 (Fig. 2). Lithium carmine was found to be eluted much after the serum proteins, and its peak was slightly heterogeneous. When 0.1 mg lithium carmine was added per ml serum, the dye was completely bound to the protein, most of the dye being associated with the first peak (macroglobulins), lesser amounts with the third peak (albumin), and traces only with the second peak (mainly IgG). When the dye: protein ratio was raised to 0.7 and 1 mg lithium carmine per ml of serum, an increasing proportion of the dye remained unassociated. Under such conditions most of the protein-bound lithium carmine associated with the peak of the macroglobulins, much less with the third peak and only trace amounts with the second peak.

These experiments, together with the electrophoretic results, led to the conclusion that the main protein accounting for the binding of lithium carmine was an \( \alpha_2 \)-globulin of high molecular weight. Only two proteins are known to possess such properties: the low-density lipoproteins and the \( \alpha_2 \)-macroglobulin.

3. Density gradient ultracentrifugations

One sample of lithium carmine (0.5 mg/ml in 0.15 M NaCl) and 3 samples of normal human serum, containing 0.1, 0.5 and 1 mg lithium carmine per ml, respectively, were applied in 0.4 ml amounts on sucrose density gradients as described under “Methods”. Free lithium carmine sedimented very slowly. The macroglobulin peak proved capable of binding increasing amounts of lithium carmine with increasing concentrations of the dye in the serum. The other fractions, viz. \( \gamma \)-globulins, albumin and free lithium carmine were not completely resolved by this method.

In another experiment, 4 ml normal human serum containing 1 mg lithium carmine/ml, was filtrated through a Sephadex G-200 column. The first peak, containing mainly IgM, \( \alpha_2 \)M and low-density lipoproteins, had a pink colour. It was concentrated to a final volume of 3 ml. A 200 \( \mu \)l sample of this fraction was applied on a density gradient and ultracentrifuged at 40,000 r.p.m. during 16 hrs. A single sharp pink zone was observed in the lower part of the tube. Fractions were collected dropwise and, after appropriate dilutions, the protein and lithium carmine contents were estimated by the extinctions at 280 m\( \mu \) and 510 m\( \mu \), respectively. The \( \alpha_2 \)M was immunologically determined by single radial diffusion in agar plates containing specific anti-\( \alpha_2 \)M serum. The low density lipoproteins were detected
semi-quantitatively by the Ouchterlony method. The results are shown in Fig. 3. All the lithium carmine was found to be associated with the macroglobulin peak containing \(\alpha_2\)M, and virtually none with the lipoproteins. The small peak observed at 510 m\(\mu\) in the lipoprotein region was caused by the normal absorbancy of the lipoproteins, as verified on controls consisting of dye-free macroglobulin samples processed similarly.

Fig. 3. Ultracentrifugation in sucrose density gradient of the first fraction obtained by gel filtration on Sephadex G-200 of normal human serum containing 1 mg lithium carmine per ml. (— • —) : Protein, (— ○ —) : Dye, (— □ —) : quantitative determination of \(\alpha_2\)-macroglobulin (ref. 22), (||| |||) : semi-quantitative estimation of light-density lipoproteins.

4. Specific precipitations of \(\alpha_2\)-macroglobulin

A specific antiserum was used to precipitate the \(\alpha_2\)M from the first Sephadex fraction containing lithium carmine bound to macroglobulins. This fraction was the same as the one submitted to the preceding density gradient experiment.

A specific anti-humin-\(\alpha_2\)M rabbit serum was diluted serially. To 400 \(\mu\)l of each dilution was added 50 \(\mu\)l of the pink Sephadex fraction. After centrifugation, the supernatants were read at 510 m\(\mu\) against the corresponding blanks, which consisted of appropriate dilutions of the antiserum in buffer. The precipitates were washed with 0.9% NaCl, absolute ethanol and ether. The dried precipitates were redissolved in 50 \(\mu\)l 0.1 N NaOH and the protein content was estimated by the Folin-Ciocalteu method. The results (Fig. 4) indicated that about 90% of the dye coprecipitated with the \(\alpha_2\)M.

Fig. 4. Precipitation by a specific antiserum of the \(\alpha_2\)M contained in the same Sephadex fraction as used in Fig. 3. (— • —) : Protein in precipitate (Antigen-antibody complex, Lowry method). (— ○ —) : Dye in supernatant.

filtrated through a Sephadex G-200 column. Only a small amount of the pink colour was associated with the albumin peak, the bulk of the dye being eluted as free lithium carmine. A similar experiment with purified human IgG demonstrated that still less lithium carmine was bound to the protein.

Two ml of an 0.8% (w/v) \(\alpha_2\)M solution containing 1 mg lithium carmine per ml was filtrated through a Sephadex G-200 column (Fig. 5). Lithium carmine was eluted in two fractions, the first one as a very sharp band associated with \(\alpha_2\)M and the second one as a much broader zone corresponding to free dye. Free lithium carmine was found to possess a small but definite absorbancy at 280 m\(\mu\).

Fig. 5. Gel-filtration on Sephadex G-200 of two ml of an 0.8% (w/v) \(\alpha_2\)-macroglobulin containing 1 mg lithium carmine per ml. (— • —) : Protein. (— ○ —) : Dye.
6. Absorption spectra

Owing to the heterogeneity of lithium carmine, it was of interest to determine whether there was any selective binding of different molecular species of the dye to albumin or \( \alpha_2 \)-M. Absorption spectra were measured on a solution of free lithium carmine and on lithium carmine associated with the macroglobulin and albumin fractions obtained by filtration on Sephadex G-200. Although small spectral differences were observed between the three solutions, this experiment provided no evidence for the existence of molecular species of lithium carmine with preferential affinity for either \( \alpha_2 \)-M or albumin.

7. Influence of the binding of lithium carmine to \( \alpha_2 \)-macroglobulin on the trypsin-binding activity of this protein

A 2\% (w/v) agar plate, containing 0.2\% (w/v) human IgG, in 0.03 M barbiturate—HCl buffer of pH 8.6 was prepared. The plate was first dipped for 24 hours in a 60\% ethanol solution containing 5\% acetic acid, in order to precipitate and denature the IgG, following which it was washed for 4 hours with 0.15 M NaCl and then for one night with 0.03 M barbiturate-HCl buffer of pH 8.6. Lithium carmine was added at increasing concentrations, in 50 \( \mu \)l amounts, to six 100-\( \mu \)l-samples of an 0.7\% (w/v) \( \alpha_2 \)-M solution. Pure \( \alpha_2 \)-M and lithium carmine solutions served as controls. These solutions were introduced into the agar gel in 8 \( \mu \)l amounts and were allowed to diffuse for 12 hours. Thereafter, the central reservoir was filled with a 0.05\% (w/v) solution of trypsin (Fig. 6). After 6 hours proteolytic digestion at room temperature, the plate was washed successively with 0.15 M NaCl and distilled water, dried, and stained with Amido-black 10 B. It may be concluded that the binding of lithium carmine to \( \alpha_2 \)-M did not inhibit the anti-trypsin activity of the macromolecule.

Discussion

The chemical composition of carmine has been reported to vary according to the species of insect used for its production and according to the method of manufacture of the pigment\(^2\). In some of our electrophoretic experiments, lithium carmine separated into two partially resolved fractions. Slight heterogeneity of the dye was also apparent on Sephadex G-200. Attempts to improve separation by means of Sephadex G-50, G-25 and G-15 were unsuccessful, due to strong adsorption of the dye to the dextran. Adsorption on Sephadex G-200 seemed much less pronounced.

The molecular weight of lithium carmine has not been reported. The fact that the dye did not filtrate through Visking\(^2\) dialysis membranes and its behaviour in the density gradient and on Sephadex G-200, seem to be compatible with a molecular weight of the order of 10,000.

Bennhold and Ott\(^9\),\(^10\), using free-boundary electrophoresis, reported that lithium carmine added to human serum was bound mainly to \( \alpha \)-globulins and to a lesser extent to albumin and \( \gamma \)-globulins. The present results fully agree with their findings. In addition, our data specify that the dye-binding capacity of the electrophoretic \( \alpha_2 \)-region must be attributed to a single molecular species of protein, viz. \( \alpha_2 \)-M.

Kiyono\(^6\) reported in an extensive study that in vivo injection of lithium carmine in rabbits resulted in the appearance of pink granules in the reticuloendothelial cells of the liver, spleen, bone-marrow, lymph nodes and connective tissues. Seybold\(^7\) demonstrated that lithium carmine, injected in vivo, was recovered in the "mitochondrial" fraction of the liver, together with an increased amount of serum proteins. According to present-day knowledge\(^2\) it may be assumed that lysosomes may have been the cellular organelle involved in this distribution. It would be of interest to determine whether \( \alpha_2 \)-M.
functioning as a carrier protein, is required for the uptake of lithium carmine by the reticuloendothelial system, whether it enters the cells together with the dye, and whether the protein is released or degraded during this process.

Most dyes introduced into the bloodstream become bound to albumin, after which they are either excreted through the kidney, or in the bile, or taken up by the reticuloendothelial system, depending upon the nature of the material. Suzuki studied the renal excretion of lithium carmine, following in vivo injection, and found that it quickly appeared in the urine. It is difficult to estimate the proportions of bound and unbound dye that were achieved during his experiments. Owing to the molecular weight of $a_2M$, significant filtration of the bound lithium carmine through the glomeruli can be excluded. Whether the renal excretion of lithium carmine observed by Suzuki reflected the glomerular passage of excess free dye, or active excretion of free or bound dye by the renal tubular cells, are questions which will require further experimental work.

It has been reported that one mole of $a_2M$ is able to bind two moles of trypsin. No precise estimation of the amount of lithium carmine that is bound per mole of $a_2M$ can be deduced from the results presented in this paper. However, the experiment with trypsin suggests that the binding of lithium carmine to $a_2M$ occurs in a different region of the molecule, although an equilibrium shift cannot be excluded.

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Die Ausschüttung und Funktion von Häutungshormon während eines Zwischenhäutungs-Intervalls bei der Strandkrabbe Carcinus maenas L.

D. Adelung

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Der Häutungszyklus der Strandkrabbe wird mit Hilfe neuer Kriterien in 21 verschiedene Stadien eingeteilt. Als Charakteristika für die einzelnen Stadien dient neben den bisher angewendeten morphologischen Merkmalen der Entwicklungszustand der Regenerate und die Geschwindigkeit, mit der die Krebse den Häutungszyklus durchschreiten.

Für jedes Stadium wird der Häutungshormongehalt mehrerer Tiere bestimmt und eine Hormontiterkurve für einen Häutungszyklus aufgestellt. Aus den Änderungen des Hormongehaltes und den gleichzeitigen morphologischen und physiologischen Veränderungen wird auf die Funktionen des Häutungshormones geschlossen. Vor der Häutung erfolgen zwei Hormonausschüttungen, die eine ca. 6—8 Tage, die andere 1—2 Tage vor der Häutung. Die erste Hormonausschüttung löst wahrscheinlich die Apolyse aus, die zweite bewirkt eine Erhöhung des osmotischen Wertes der Hämolymphe und führt damit zur Auslösung der Häutung im engeren Sinne. Unmittelbar nach der Häutung wird erneut Hormon ausgeschüttet, das vermutlich die Häutungsprozesse in der Cuticula steuert. Nachdem sämtliche mit der vorangegangenen Häutung zusammenhängenden Prozesse abgeschlossen sind, löst eine weitere kurzfristige Hormonausschüttung die Regeneratentwicklung und vielleicht den nächsten Häutungszyklus aus.

Ein Vergleich des Häutungshormongehaltes der Strandkrabbe während eines Häutungszyklus mit demjenigen von Insekten während der Puppenhäutung ergibt einige Übereinstimmungen, z. B. in der Höhe des Hormongehaltes und einige Unterschiede, die sich aus der unterschiedlichen Lebensweise dieser Tiere erklären.

Das Größenwachstum der dekapoden Krebse ist an Häutungen gebunden. Der Zeitpunkt der Häutungen hängt von zahlreichen äußeren und inneren Faktoren ab. So verzögern bei einigen Krebsarten Temperatur, Dauerlicht, die Anwesenheit von Artgenossen oder Nahrungsmangel eine an sich fällige Häutung. Unter konstanten Bedingungen häuten sich die Tiere aber in bestimmten vorhersagbaren

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