The Isolation from Human Blood Plasma of a Substance having Positive Inotropic Action on the Isolated Toad Heart

By C. C. CURTAIN AND WINIFRED G. NAYLER

Baker Medical Research Institute, Commercial Road, Prahran S. 1, Melbourne, Victoria, Australia

(Received 17 December 1962)

The positive inotropic action of blood plasma on isolated hearts has been known for a long time (Bowditch, 1871; Ringer, 1885; Clark, 1913; Hajdu, 1957; Hajdu, Leonard & Akers, 1957*a*; Hajdu, Weiss & Titus, 1957*b*), and the observations recorded by various authors suggest that a number of different plasma components are responsible for positive inotropic activity. Hajdu *et al.* (1957*b*) isolated a substance with digitalis-like activity from mammalian blood and identified it as α -palmitoyl-lysolecithin. Hajdu *et al.* (1957*a*) found that certain plasma protein fractions isolated from hypertensive patients showed a greater inotropic activity on the isolated amphibian heart than those isolated from normotensives. Tanz (1960*a, b*), Tanz & Kerby (1961) and Sayers & Solomon (1960) have found that aldosterone was responsible in part for the positive inotropic activity of human plasma. Nayler & McCulloch (1960*b*) found a heat-stable diffusible factor present in human and animal plasma which possessed positive inotropic activity.

In preliminary investigations of the pharmacological properties of this heat-stable diffusible plasma factor we have shown that it improves the efficiency of the isolated toad heart and that it exerts a marked effect on the rate at which tension is produced by isolated toad ventricular muscle. Seasonal variation in the activity of toad plasma was observed, suggesting a hormonal control over the activity of this plasma factor in the toad.

The present paper describes the isolation of a substance with inotropic activity from heparin-treated human plasma.

EXPERIMENTAL

Human plasma

Blood was obtained by venesection from donors who were bled directly through a 24-gauge stainless-steel needle into silicone-treated glass vessels containing 10000 units of heparin (Boots Pure Drug Co.) for each 100 ml. of blood to be taken. The first 5 ml. of blood passing through the needle was not collected. The cells were separated from the plasma by centrifuging at 4° within 30 min. of collection. The influence of the method of taking blood on the inotropic activity of whole plasma has been investigated by W. G.

Nayler & A. Baumgarten (unpublished work). They found that the inotropic activity remained constant regardless of whether the plasma was collected in untreated glass, silicone-treated glass or polypropylene vessels. Nevertheless, it was felt that for fractionation studies it was essential to use silicone-treated glassware to minimize the risk of producing artifacts by surface-activated plasma enzymes.

The silicone-treated glassware used in the experiments was thoroughly coated by dipping in General Electric Co. Drifilm SC87 and allowed to drain for 30 min. The articles were then rinsed in distilled water and dried at 160° for 120 min.

Solutions

Ringer solution. The Ringer solution was of the following composition (final concentrations, mm): NaCl, 115; NaHCO₃, 20.6; NaH₂PO₄, 3.0; MgSO₄, 1.2; glucose, 16.5; KCl, 3.2; CaCl₂, 0.65. The pH of this solution was adjusted to 7.3 by aeration with O₂ + CO₂ (95:5).

Heparin-treated Ringer solution. This was prepared by adding 10000 units of heparin (Boots Pure Drug Co.) to 100 ml. of the above Ringer solution.

Ammonium acetate solutions. These were prepared by neutralizing redistilled A.R. ammonia with redistilled A.R. acetic acid. It had been found that all the available proprietary A.R.-grade ammonium acetate contained either cardio-stimulant or cardio-depressant substances.

Triethylamine-acetate buffers. These were prepared by adjusting a 5 mm-solution of redistilled triethylamine with a 5 mm-solution of redistilled acetic acid to the desired pH.

Enzymes and bradykinin

Nagarse. Nagarse, a purified proteolytic enzyme preparation from *Bacillus subtilis*, was obtained from the Teikoku Chemical Co., Japan.

Pronase. Pronase, a purified proteolytic enzyme preparation from *Streptomyces grisei*, was obtained from the Kaken Chemical Co., Japan.

Bradykinin. A sample of pure synthetic bradykinin was obtained as a gift from Sandoz Ltd., Basle, Switzerland.

Pharmacological assay

The inotropic activity of plasma and isolated fractions was demonstrated by using the isolated isotonic contracting toad (*Bufo marinus*) heart and the photoelectric apparatus described by Nayler & McCulloch (1960*a*). In essence this consists of a thin rigid wire introduced into a thin-walled Perspex cannula and placed in the aorta so that the beaded end of the wire lies against the endocardium.

At the fine end of the wire a very light shutter lies directly in the path between a constant light-source and a selenium-barrier photoelectric cell. Displacement of this shutter during contraction and relaxation varies the light falling on the photoelectric cell, the output from which is amplified by a linear d.c. amplifier (gain 0-300) and recorded on a direct-linking Evershed and Vignoles recorder. Over the frequency range used in this study the response of this recorder was linear.

After dissection the atrial pace-maker was destroyed by cauterization and the isolated ventricle stimulated with supra-threshold rectangular pulses of 10 msec. duration from a Tektronix square-wave generator. All preparations were equilibrated with Ringer solution for 30 min. before use. During assay the test substance was added directly to the 3 ml. of perfusion fluid in the cannula and the response was standardized against both adrenaline (as adrenaline tartrate, Hermette) and noradrenaline (as Levophed bitartrate monohydrate, Levophed) in the presence and absence of the specific adrenaline antagonist, dichloroisoproterenol.

Chromatography

Gel-filtration chromatography. This was carried out at 4°, in the first instance on columns of Sephadex dextran gel (Pharmacia, Uppsala) (Porath & Flodin, 1959) and subsequently on columns of dispersed cross-linked polyacrylamide gel. This latter differed from the acrylamide gel used by Hjerten & Mosbach (1962) for protein fractionation in that the cross-linking was 5%. At this degree of cross-linking haemoglobin (mol.wt. 68000) was completely excluded from the gel phase, and human serum albumin (mol.wt. 65000) had R_F 0.97 on gel-filtration. To prepare the gel an aqueous solution containing 19% (w/v) of acrylamide, 1% (w/v) of NN' -methylenebisacrylamide and 0.1% of riboflavin was exposed in a depth of not more than 0.75 cm. in a flat tray at a distance of 20 cm. from a pair of Osram 40 w 'white' fluorescent lamps. Polymerization was complete within 20 min., and the gel was broken into small pieces (approx. 0.25 cm. diam.) and washed in running tap water for 24 hr. The gel particles were then dried, spread in a thin layer in a current of air at a distance of 40 cm. from a Mazda 500 w infrared lamp. The dried gel was dispersed in a blender (Semak Vitamiser) and sieved. The water regain of the gel was 5.4 g./g. Except where otherwise stated, the gel used in the filtration experiments was of 40-140 B.S. mesh (dry). All columns were washed for 48 hr. with distilled water before equilibration with the solution to be used in the experiment. For preparation of very large columns acrylamide mixed with 5% (w/w) of NN' -methylenebisacrylamide was purchased as 'Chemical Grouit AM9' from Cyanamid (Australia).

Gel-filtration with short separation times at high reproducible flow rates was carried out with the apparatus illustrated in Fig. 1. A silicone-treated glass chromatography column (1 cm. $\times$ 15 cm.) containing dispersed acrylamide gel in heparin-treated Ringer solution covered by a silicone-treated 0 sintered-glass disk is fitted at the upper end with a securely fixed stopper pierced by four tubes. The first tube is connected to a compressed air cylinder through a reducing valve enabling pressures from 0 to 40 lb./in.² to be applied. The second tube is connected to a solenoid-operated dump valve which opens to the atmosphere; the third tube is connected to a 1 ml. hypodermic syringe with a solenoid-operated plunger. The

fourth tube is connected to a solenoid-operated 5 ml. syringe. The column is also fitted with a pair of platinum electrodes, one just below and the other just above the top of the sinter-protected dispersed gel. If there is no liquid between these electrodes the dump valve is open and there is no pressure in the space at the head of the column. To carry out a rapid separation the 1 ml. syringe is loaded with heparin-treated plasma and the 5 ml. syringe with heparin-treated Ringer solution. A 0.1 sec. pulse of current is applied to the solenoid of the plasma syringe. This delivers the plasma on to the sintered-glass disk, thus covering the top electrode and closing the dump valve; the pressure on the column rises to the pre-set value and the plasma is rapidly pushed into the column. When this happens contact between the electrodes is broken and the dump valve opens, releasing the pressure on the column. The relay controlling the dump valve then activates a timing circuit which operates the solenoid of the 5 ml. syringe after a pre-set time-interval. When the eluting charge is delivered to the top of the column the dump valve again closes and separation of the excluded high-molecular-weight fraction from the retained low-molecular-weight fraction occurs rapidly. With the timer set at its shortest limit it is possible to achieve this separation in 0.60 sec. from the instant of activating the plasma-syringe solenoid. At this speed resolution, even on a column made from 140-210-mesh gel, is poor, but is sufficient to separate insulin and human serum albumin as shown in Fig. 3.

High-voltage paper electrophoresis. This was carried out for 30 min. at a potential gradient of 100 v/cm. in 50 mm-triethylamine-acetate buffer, pH 5.8, on strips (2 cm. $\times$ 30 cm.) of Whatman no. 1 filter paper immersed in refined kerosene maintained at 5°. After electrophoresis the strips

Fig. 1. Diagram of rapid-gel-filtration apparatus. *A*, Starter button; *B*, solenoid-operated 1 ml. syringe; *C*, solenoid-operated 5 ml. syringe; *D*, compressed-air inlet (40 lb./in.²); *E*, solenoid-operated dump valve; *F*, air outlet; *G*, adjustable timer; *H*, vacuum-tube relay; *I*, electrodes; *J*, protective sinter; *K*, acrylamide gel in column. All electronic circuits were conventional. The solenoid-operated equipment was supplied by Goyen Controls, Melbourne.

were washed in light petroleum to remove the kerosene, dried in a current of air at 37° and sprayed with 0.1% (w/v) ninhydrin in butanol.

Preparative high-voltage paper electrophoresis. A portion (0.2 ml.) of the sample was applied in a straight line (3 mm. wide) across a strip (10 cm. wide) of Whatman 3MM filter paper. After electrophoresis at 5° for 30 min. at 100 v/cm. in 5 mM-triethylamine-acetate buffer, pH 5.8, 7 mm. pilot strips were cut from both edges of the sheet, dried in a current of air and sprayed with ninhydrin to locate the bands and estimate their straightness. The bands were cut from the main strip and eluted from the paper with the triethylamine-acetate buffer. The buffer was removed from each fraction by freeze-drying. The residues were dissolved in Ringer solution and tested for inotropic activity.

Ion-exchange chromatography. Unfortunately, all the commercially available ion-exchangers that seemed suitable for the separation of substances with inotropic activity from plasma [Dowex 50, diethylaminoethylcellulose (both from Brown Co. and from Eastman Organic Chemicals) and Sephadex A-50] released substances that depressed the isolated toad heart and which could not be removed even after 128 hr. of washing of the material in columns. We therefore resorted to the synthesis of our own ion-exchange materials from monomers of known purity. The most suitable of these was found to be a dispersed gel prepared from a co-polymer of acrylamide, diethylaminoethyl methacrylate and *NN'*-methylenebisacrylamide.

Diethylaminoethyl methacrylate was prepared by ester-interchange between methyl methacrylate and diethylaminoethanol: 125 g. of methyl methacrylate, 45 g. of diethylaminoethanol, 10 g. of toluene-*p*-sulphonic acid and 10 g. of 3-*tert*-butylcatechol were refluxed for 24 hr. in an atmosphere of nitrogen under a Vigreux column, which was heated at 70° to remove the azeotrope of methanol and methyl methacrylate. The diethylaminoethyl methacrylate was recovered by fractional distillation under vacuum, the fraction coming over at 80° and 10 mm. Hg being kept. Then 46.2 g. of diethylaminoethyl methacrylate, 153.8 g. of acrylamide, 12 g. of *NN'*-methylenebisacrylamide and 10 g. of ammonium persulphate were dissolved in 1 l. of distilled water. Polymerization was initiated by adding, with rapid stirring, 10 ml. of β -dimethylaminopropionitrile, and gelling occurred within 10 min. The gels were broken into small fragments (0.1–0.5 cm.) and washed in running de-ionized water for 36 hr. The gel was then freeze-dried. The fluffy granules were dispersed to a fine powder in a blender (Semak Vitamiser).

The dispersed ion-exchange gel (140–210 mesh) was allowed to swell in distilled water, packed into a column (2 cm. $\times$ 60 cm.) and washed for 24 hr. at a flow rate of 15 ml./hr. with 5 mM-triethylamine-acetate buffer, pH 7.0. The sample for chromatographic separation was dissolved in 5 mM-triethylamine-acetate buffer, pH 7.5, and applied in a volume of 1 ml. to the column. A linear gradient from 5 mM-triethylamine-acetate buffer, pH 7.5, to 0.5 M-triethylamine-acetate buffer, pH 5.2, was fed into the column by a photoelectrically-programmed electrolytic-gradient generator (Curtain, 1962). Fractions (5 ml.) were collected and the buffer was removed from each by freeze-drying. The fractions were tested for inotropic activity and by the ninhydrin reaction of Spackman, Stein & Moore (1958).

RESULTS

Preliminary studies of the separation of the diffusible inotropic factor by gel-filtration

Because of its ability to separate the proteins very rapidly from the lower-molecular-weight substances present in plasma we decided to investigate gel-filtration as a means of isolating the diffusible inotropic factor. Rapid separation was considered an advantage as it would minimize the possibility of forming active material by degradation of the plasma proteins, a possibility that must always exist during the much longer process of dialysis.

Gel-filtration on Sephadex. Samples (1 ml.) of plasma were chromatographed on columns of coarse Sephadex G-25, Sephadex G-50 and Sephadex G-75, which had been washed with heparin-treated Ringer solution. Heparin-treated Ringer solution was used as the eluting fluid. The inotropic activity split into two parts on Sephadex G-75 and Sephadex G-50. One part, comprising 65% of the activity, was displaced through the column with the protein front; the other was slowly eluted from the gel phase. On the Sephadex G-25 all the activity was displaced with the substances excluded from the gel phase. According to the manufacturer's data on the exclusion limits of the gels, the results of these experiments suggest that fresh plasma contains two fractions possessing positive inotropic activity, one with a molecular weight in the 3500–10000 range and the other with a molecular weight exceeding 50000. It is possible that the activity in the high-molecular-weight fraction is the cardio-active globulin system of Hajdu & Leonard (1958). The activity in the low-molecular-weight fraction is presumably identical with the diffusible heat-stable inotropic factor of Nayler & McCulloch (1960*b*). The two active fractions separated on Sephadex were identical with each other and with the diffusible heat-stable inotropic factor of Nayler & McCulloch (1960*b*) in the isolated heart assay. In Ringer solution they immediately increased the amplitude of contractions recorded from stimulated (6 beats/min.) isotonic beating hypodynamic isolated toad hearts. This response was not blocked by the presence (5 μ g./ml.) of the specific adrenaline antagonist, dichloroisoproterenol, and was slowly reversed after further perfusion in pure Ringer solution.

Gel-filtration on columns of dispersed cross-linked polyacrylamide. Washings from Sephadex columns equilibrated with Ringer solution, 0.9% sodium chloride, ammonium acetate solution or phosphate buffer for more than 38 hr. depressed the contractility of the isolated toad hearts used in the biological assay. It was considered that this effect could have resulted from microbial degradation of

the Sephadex, which is a dextran product. Since penicillin and streptomycin, the only antiseptics that did not interfere with the biological assay, were ineffective in preventing the appearance of the depressant action, and since sterilization was impracticable in larger columns, it was decided to investigate filtration through gels less liable to microbial action. Dialyl-cross-linked polyvinylpyrrolidone and poly(vinyl alcohol) cross-linked by reaction with ethylene di-isothiocyanate were tried, but we found that polyacrylamide 5% cross-linked with *NN'*-methylenebisacrylamide was particularly suitable for the fractionation of the two activities. Under none of the experimental conditions employed did the acrylamide gels alone produce cardio-stimulant or cardio-depressant substances.

In the initial experiments with acrylamide gel we used a column (2 cm. $\times$ 40 cm.) of 140–210 mesh gel equilibrated with 80 mM-ammonium acetate solution, pH 7.0. This column had been previously calibrated with a mixture of insulin, lysozyme, trypsin and human serum albumin. Heparin-treated plasma (1 ml.) was allowed to drain into the column under gravity and the chromatogram was then developed with the ammonium acetate solution at the rate of 20 ml./hr. Fractions (5 ml.) were collected, and the residues, after freeze-drying, were each dissolved in 1 ml. of Ringer solution and tested for inotropic activity. The results of this experiment are illustrated in Fig. 2. The inotropic activity is again found in the high-molecular-weight and low-molecular-weight zones of the column effluent. The activity in the high-molecular-weight zone had an elution volume corresponding to that of the plasma proteins; the activity of the low-molecular-weight zone had an elution volume close to that of insulin. As would be expected the amino acids and other low-molecular-weight constituents of plasma were eluted after insulin. About 35% of the activity was present in the low-molecular-weight fraction and 65% in the high-molecular-weight fraction.

Effect of repeated gel-filtration on the isolated inotropically active fractions

The active high-molecular-weight and low-molecular-weight fractions were separately passed through columns identical with that used in the initial separation. No further fractionation was observed and each peak of activity appeared in the same elution volume as in the original separation.

Effect of rate of gel-filtration on the separation of the two inotropically active fractions

A series of separations was carried out at different rates to investigate the possibility that the activity in the low-molecular-weight fraction might arise

from the degradation of plasma proteins. Separations were carried out in (a) 30 sec., (b) 20 sec., (c) 12 sec., (d) 8 sec., (e) 2.5 sec. and (f) 0.6 sec., with the apparatus illustrated in Fig. 1. The experiments illustrated in Fig. 3 show that the activity in the low-molecular-weight fraction is eluted as a

Fig. 2. Chromatography of heparin-treated human plasma on dispersed 5% cross-linked polyacrylamide gel in 80 mM-ammonium acetate solution, pH 7.0. Experimental details are given in the text. The positions of the elution volumes of human serum albumin (A), of trypsin (B), of lysozyme (C) and of insulin (D) are indicated.

Fig. 3. Effect of decreasing the time of separation on the fractionation of the two inotropic activities on 5% cross-linked dispersed acrylamide gel in heparin-treated Ringer solution. Separation times were: (a) 30 sec.; (b) 20 sec.; (c) 12 sec.; (d) 8 sec.; (e) 2.5 sec.; (f) 0.6 sec. Experimental details are given in the text. The elution volumes of human serum albumin (A) and of insulin (B) run on the same column under the same conditions are indicated.

single symmetrical peak in all cases in a progressively smaller elution volume as the rapidity of the separation increases.

Effect of incubation for different times on the separation of the high-molecular-weight and low-molecular-weight fractions of inotropic activity

Plasma from blood collected by using both untreated and silicone-treated glassware was incubated at 37° under sterile conditions for periods of 1, 2, 4, 8, 16 and 24 hr., and the two active fractions were separated on an acrylamide column equilibrated with heparin-treated Ringer solution. The proportion of the fraction with low molecular weight remained at 30 % of the total plasma activity regardless of the time of incubation, or whether the plasma was collected and incubated in untreated or silicone-treated glass tubes.

Large-scale preparation of the fraction with inotropic activity

Direct weighing of the pooled dried active fractions from ten of the separations of 5 ml. lots of plasma on columns (4 cm. $\times$ 60 cm.) of polyacrylamide gel enabled us to estimate that approx. 200 μ g. of active material could be recovered from 1 l. of human plasma. Of this material 1 μ g. possessed the same inotropic activity as 0.09 μ g. of adrenaline. As it was evident that many litres of plasma would have to be processed to provide material for further purification and pharmacological and chemical studies, a method of large-scale preparation was devised. This involved the rapid separation of the proteins from the low-molecular-weight substances in 1 l. of fresh heparin-treated human plasma on a column (15 cm. $\times$ 70 cm.) of 40–110-mesh polyacrylamide gel in 80 mM-ammonium acetate, pH 7.0. The fraction

corresponding to the 2000–10000 molecular-weight range (as determined by previous calibration of the column with oxytocin, insulin and lysozyme) was freeze-dried and the residue was dissolved in 1 ml. of 5 mM-triethylamine-acetate buffer, pH 7.0. This was applied to a column (2.0 $\times$ 100 cm.) of 140–210-mesh polyacrylamide gel equilibrated with 5 mM-triethylamine-acetate, buffer pH 7.0. The column was eluted with 5 mM-triethylamine-acetate buffer, pH 7.0, and 5 ml. fractions were collected. These were freeze-dried and the residues were redissolved in Ringer solution and tested for biological activity. The activity is eluted from the column in a highly reproducible volume, and in routine separation only the fractions in which activity can be predicted need be dried.

Examination by high-voltage electrophoresis of the inotropically active fraction of low molecular weight

The active fraction of low molecular weight separated on the acrylamide gel was submitted to high-voltage paper electrophoresis for 30 min. in 5 mM-triethylamine-acetate buffer, pH 5.8. Three ninhydrin-positive bands were observed: one that barely moved at this pH, a second that was positively charged and a third that was negatively charged. It was established by biological assay of the fractions obtained by preparative high-voltage electrophoresis that all the inotropic activity was associated with the positively charged band.

Ion-exchange chromatography

As preparative high-voltage electrophoresis would not yield sufficient material for our projected biological and clinical studies, ion-exchange chromatography of 200 μ g. samples of the low-molecular-weight active fraction was carried out under the conditions described above.

Three ninhydrin-positive peaks occurred in the column effluent (Fig. 4). The first of these contained the inotropic activity. This peak gave a single ninhydrin-positive band with the same mobilities as the electrophoretically separated fraction on high-voltage paper electrophoresis in 5 mM-triethylamine-acetate buffer at pH 5.0, 6.2 and 7.0. Of the purified substance 1 μ g. was as active as 0.3 μ g. of adrenaline in our biological assay. By direct weighing of the product of five ion-exchange separations we found that 65 μ g. of active material was obtained from approx. 200 μ g. of starting material, representing approximately a threefold purification.

Biological properties of the isolated active substance

Biological activity. The biological activity of the isolated substance appeared to be identical with

Fig. 4. Chromatography of the low-molecular-weight fraction with inotropic activity on diethylaminoethyl acrylate gel. The gradient was from 5 mM-triethylamine-acetate buffer, pH 7.5, to 0.5 M-triethylamine-acetate buffer, pH 5.2, and was linear from 0 to 200 ml. Experimental details are given in the text. □, Inotropic activity; ○, ninhydrin colour.

that of fresh heparin-treated plasma. On addition to our isolated heart preparation in Ringer solution the substance immediately increased the amplitude of the contractions recorded from the heart. The response was not blocked by the addition ($5\text{ }\mu\text{g./ml.}$) of the specific adrenaline antagonist, dichloroisoprotorenol, and was slowly reversed after further perfusion in pure Ringer solution.

Effect of proteolytic degradation in biological activity. 1% (w/v) Pronase and 1% (w/v) Nagarse, each in Ringer solution, were tested for activity in the biological assay. Neither enzyme preparation depressed or stimulated the heart. A portion ($20\text{ }\mu\text{g.}$) of the active substance was incubated with 1 ml. of each enzyme solution and in 1 ml. of Ringer solution at 37° for 1 hr. The substance incubated in the Ringer solution still had full inotropic activity, but the substance incubated in the enzyme solutions was inactive.

Comparison with bradykinin. Pure synthetic bradykinin in concentrations of $0.001\text{--}1\text{ }\mu\text{g./ml.}$ of Ringer perfusate had no effect on the isolated toad heart. In concentrations of $1\text{--}10\text{ }\mu\text{g./ml.}$ a slight depressant effect was noticed.

DISCUSSION

Any claim that the inotropically active principle that we have isolated is a physiological substance existing in the circulation must be critically examined in the light of our current knowledge of the production of pharmacologically active compounds that are released from the blood plasma by various agents. A group of peptides called kinins is known to be released by a surface-activated enzyme system from a protein precursor in blood. The basic structure of this group is a nonapeptide called bradykinin, which was first discovered by Rocha e Silva, Beraldo & Rosenfeld (1949), isolated by Elliot, Lewis & Horton (1960) and synthesized by Boissonnas, Guttman, Jacquenoud, Konzett & Sturmer (1960). Bradykinin itself had no effect on, or slightly depressed, the isolated toad heart in our assay, and this suggests that our substance is not related to the kinins.

Whole plasma itself has an inotropic activity and the isolated substance appears to be responsible for about one-third of the positive inotropic activity of human plasma, regardless of whether the plasma is obtained from blood collected in silicone-treated or untreated glass vessels, and regardless of whether the plasma is fractionated on a gel column and tested immediately or incubated under sterile conditions for 24 hr. It seems unlikely therefore that the active principle is formed during purification as an artifact. The proportion of activity present in the isolated fraction is the same whether Sephadex or acrylamide gel is used for the separa-

tion. These two gels differ markedly in their chemical structure and surface properties. Acrylamide gel is a linear polymer of acrylamide, cross-linked at intervals by methylene bridges. The gel is made hydrophilic by the amide groups, no other polar groups being present. Sephadex appears to consist of dextran chains cross-linked with ether linkages. It derives its hydrophilic nature from the hydroxyl groups of the glucose units of the dextran. It also appears to contain a small number of carboxyl groups (Gelotte, 1960). If the inotropic activity associated with the low-molecular-weight fraction were produced by surface-activation of an enzyme system then we might expect it to be produced in different yields on the different gels.

The experiments carried out at various speeds of separation provide further evidence that the activity in the low-molecular-weight fraction is not formed by contact of the plasma with the gel. The elution volume of a substance on gel-filtration is dependent on the distance that that substance has penetrated into the gel phase in the starting zone, and the shape of the eluted peak will be gaussian only if the initial diffusion process is normal. If the substance responsible for the activity were produced by a reaction with a time comparable with or slower than the time allotted for diffusion in the starting zone, then it would not diffuse into the gel as far as a substance of the same molecular weight pre-existing free in the plasma. Hence we would expect the elution volume of the activity in the original separation to be lower than that observed on repeated chromatography on the same gel. Further, since the reaction would be replenishing the activity lost from the aqueous phase or void volume by diffusion, we would expect the zone of activity obtained in the initial separation to be very broad: in the extreme case where the reaction is much slower than the time taken for the protein zone to leave the column, the activity would extend throughout the column. As none of these effects was observed we conclude that any activity-producing reaction must be complete within considerably less than the shortest separation time used, 0.6 sec.

The results of the proteolysis experiments indicate that intact peptide bonds are necessary for the biological activity of the substance; however, a more definite knowledge of its nature must await structural analysis.

SUMMARY

1. The substances responsible for the positive inotropic activity of normal human blood plasma on the isolated toad heart were separated into a high-molecular-weight (greater than 60000) and low-molecular-weight (4000–10000) fractions by gel-filtration on Sephadex or 5% cross-linked poly-

acrylamide gel. A highly active substance was isolated by ion-exchange chromatography on a 5 % cross-linked polymer of acrylamide, *NN'*-methylene-bisacrylamide and diethylaminoethyl methacrylate.

2. The initial gel-filtration experiments were carried out under a wide variety of conditions and resulted in the same yield of high-molecular-weight and low-molecular-weight fractions with inotropic activity.

3. It was concluded that it was unlikely that the low-molecular-weight fraction with inotropic activity was an artifact produced from the plasma proteins by the method of isolation.

4. The inotropic activity of the isolated substance was destroyed by incubation with protease.

The authors acknowledge the technical assistance of Miss S. Freame and Miss J. Harris. They are also indebted to Mr S. Hart and Mr J. Bremner for advice on and help with the construction of chromatographic apparatus.

REFERENCES

- Boissonnas, R. A., Guttman, S. T., Jacquenoud, P. A., Konzett, H. & Sturmer, B. (1960). *Experientia*, **16**, 326.
- Bowditch, H. P. (1871). *Anstalt. zu Leipzig*, **6**, 139.
- Clark, A. J. (1913). *J. Physiol.* **47**, 66.
- Curtain, C. C. (1962). *J. Chromat.* **7**, 24.
- Elliot, D. F., Lewis, G. P. & Horton, E. W. (1960). *Biochem. J.* **74**, 15P.
- Gelotte, B. (1960). *J. Chromat.* **3**, 330.
- Hajdu, S. (1957). *J. Pharmacol.* **120**, 90.
- Hajdu, S. & Leonard, E. (1958). *Circulation Res.*, **6**, 740.
- Hajdu, S., Leonard, E. & Akers, R. P. (1957a). *Circulation Res.* **5**, 319.
- Hajdu, S., Weiss, H. & Titus, E. (1957b). *J. Pharmacol.* **120**, 99.
- Hjerten, S. & Mosbach, R. (1962). *Analyt. Biochem.* **3**, 109.
- Nayler, W. G. & McCulloch, M. (1960a). *Aust. J. exp. Biol. med. Sci.* **38**, 117.
- Nayler, W. G. & McCulloch, M. (1960b). *Aust. J. exp. Biol. med. Sci.* **38**, 127.
- Porath, J. & Flodin, P. (1959). *Nature, Lond.*, **183**, 1657.
- Ringer, S. (1885). *J. Physiol.* **6**, 361.
- Rocha e Silva, M., Beraldo, T. & Rosenfeld, G. (1949). *Amer. J. Physiol.* **156**, 261.
- Sayers, G. & Solomon, N. (1960). *Endocrinology*, **66**, 719.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1150.
- Tanz, R. D. (1960a). *J. Pharmacol.* **128**, 168.
- Tanz, R. D. (1960b). *Abstr. Proc. Endocrine Meetings, Miami*.
- Tanz, R. D. & Kerby, C. F. (1961). *J. Pharmacol.* **131**, 56.

Biochem. J. (1963) **89**, 75

Formation and Breakdown of Collagen and Elastin in the Human Uterus during Pregnancy and Post-Partum Involution

BY J. F. WOESSNER, JUN. AND T. H. BREWER*

Laboratories for Cardiovascular Research, Howard Hughes Medical Institute, Miami, Fla. and Departments of Biochemistry and Obstetrics and Gynecology, University of Miami School of Medicine, Coral Gables, Fla., U.S.A.

(Received 11 February 1963)

The post-partum involuting uterus presents one of the most favourable systems for an investigation of the mechanisms of protein catabolism in the mammalian organism. Involution involves an extremely rapid breakdown of muscle protein and of the connective-tissue framework of the uterus. Harkness & Moralee (1956) found that the half-life of uterine collagen was only 24 hr. during involution in the rat. The possible mechanism of collagen breakdown in the rat uterus was studied by Woessner (1962a), and it was postulated that the breakdown, which apparently proceeded to the free amino acid level, was due to the action of pro-

teolytic enzymes. Difficulties in obtaining sufficient tissue for enzyme studies led us to investigate the human uterus.

The rate and extent of collagen changes in the human uterus during pregnancy and post-partum involution have been studied by Montfort & Pérez-Tamayo (1961) and Morrión & Seifter (1962). We have extended these studies to show that elastin, as well as collagen, undergoes a marked increase in pregnancy and a rapid decrease during involution. Moreover, there is a striking effect of successive pregnancies on the uterus and its connective tissue. The proteolytic enzyme activity of the uterus and the nature of uterine collagen in both normal and involutionary states have been examined in a search for clues to the mechanism of collagen breakdown.

* Present Address: Department of Obstetrics and Gynecology, University of California Medical Center, San Francisco 22, Calif., U.S.A.