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Separation of Pancreatic Cardiodepressant Activity into Peptide and Salt Activity[1] (39910)

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Introduction. One of several mechanisms proposed to explain irreversibility in shock is the elaboration of circulating cardiodepressant substances in shock states. Recently, Lefer (8) proposed one such factor which originates in the ischemic pancreas and may be crucial to the development of irreversibility in shock states. The chemical composition of this factor has been reported to be organic in nature (1, 8). Lefer (8) has reported the molecular weight to be in the range of 500–1000, and its plasma concentration during shock in the picomolar range. It is inactivated by trypsin and contains free amino groups. We have previously reported a similar compound in preincubated pancreatic homogenates that appears to be peptidic in nature, due to its uv absorbance at 280 nm, reaction with *o*-phthalaldehyde as well as ninhydrin, and its susceptibility to destruction by ashing. However, Wangensteen *et al.* (2), repeating the methods of Lefer reported that the biological activity of the samples tested by Lefer were due to a contaminant, excess Na^+, rather than a specific cardiodepressant substance. In this study, we have attempted to separate biological activities of salt and peptidic fractions of the initially active cardiodepressant source. As a source, we used incubated pancreatic homogenate, which contains high amounts of such activity (1, 9).

Methods. The methods for obtaining a biologically active preparation are described in detail in a previous report (1). In brief, pancreata were excised from nonshocked anesthetized dogs, washed in cold Krebs–Henseleit (KH) solution, minced, homogenized, and incubated (37°) for a period of 3 hr. The homogenate was then heated (80° for 10 min) to denature the larger proteins, centrifuged, and a clear supernatant was recovered. The supernatant was ultrafiltered through a UM-2 filter (1000 MW, Amicon Co.), and the filtrate was recovered. This filtrate contained high amounts of cardiodepressant activity (CDA).

Gel filtrations. One gram of lyophilized sample [either bovine serum albumin (BSA) or UM-2 ultrafiltrate] was redissolved in 20 ml of deionized water and applied to a Sephadex G-10 column (2.5 × 150-cm bed; Pharmacia Fine Chemicals). The column was then eluted at 25° with 0.5% acetic acid at a rate of 68 ml/hr. Fractions (6.8 ml) were collected and monitored for their absorbance at 280 nm. Aliquots (0.5 ml) of each fraction were used for Na^+, K^+, Cl^-, and Ca^{2+} analysis (see below). The remainder of each fraction was lyophilized to dryness and reconstituted to 15 ml with KH solution for biological testing. A sample (0.5 ml) of KH-reconstituted gel fractions was removed and analyzed for Na^+, K^+, Cl^-, and Ca^{2+} content (see below). The elution pattern of the column was calibrated using glycine and hexaglycine, the latter eluting in the void volume and the former being retarded.

CDA analysis. The assay for cardiodepressant activity is described fully in a previous publication (1). In brief, a right ventricular papillary muscle was removed from the heart of an anesthetized (pentobarbital, 20 mg/kg) cat and suspended in an oxygenated (95% O_2, 5% CO_2) KH bath (37 ± 0.01°). The muscle was stimulated at twice-threshold voltage, 1/sec for 16 msec, and developed tension was recorded. The assay

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involves replacing the control bathing medium with tested material and recording the increase or decrease in developed tension. One CDA unit is defined as a 1% decrease in developed steady-state tension in the assayed medium compared to the previous steady-state control reading. When a tested solution produced a stimulation of the muscle, the results are reported as negative CDA units. The pH of each reconstituted sample was tested and adjusted to 7.40 ± 0.05. Previous experience indicated that developed tension was depressed by a solution pH outside the range of 7.25–7.60.

Salt measurements. Na^+ and K^+ concentrations of the column eluate fractions and KH-reconstituted fractions were analyzed via flame photometry using an Instrumentation Lab flame photometer, Model 343. CL^- concentration was assayed via colorimetric analysis using a Technicon autoanalyzer. Standard and sample Cl^- concentrations were checked by titration. Ca^{2+} concentrations were measured with a Perkin Elmer atomic adsorption spectrophotometer, Model 460. With each assay, four standard concentrations of each species were used to calibrate the machines.

Results. In order to test the separating ability of the gel filtration procedure, 1 g of BSA dissolved in 20 ml of 0.9% NaCl solution was applied to the Sephadex G-10 column and eluted with 0.5% acetic acid. The BSA appeared, as indicated by the large uv-absorbing peak in fractions 18–23 (122–156 ml), followed by the Na^+ in fractions 23–29 (156–197 ml). There was no detectable Na^+ in fractions 18–23. Thus, use of the Sephadex G-10 column allowed for a clear separation of salt from albumin.

One gram of lyophilized IPH ultrafiltrate was then applied to this column and eluted with 0.5% acetic acid. The A_{280} profile obtained is illustrated in Fig. 1. Salt concentrations and CDA activity of each of the KH-reconstituted fractions are listed in Table I. The first ultraviolet (uv)-absorbing peak appeared in fractions 16–23 (109–156 ml), followed by several retarded uv-absorbing peaks. Each fraction was lyophilized, reconstituted with KH, tested for CDA, and assayed for various ionic concentrations. Table I presents these data. Two groups of fractions had measurable CDA activity. The first group was the void volume fractions (18–22; 122–150 ml), and contained high amounts of peptidic material. These fractions after KH reconstitution also contained constituent salts in concentrations similar to those of control KH, indicating the absence of salts coeluting with the peptidic material. The second CDA group (fractions 24–28) contained some uv-ab-

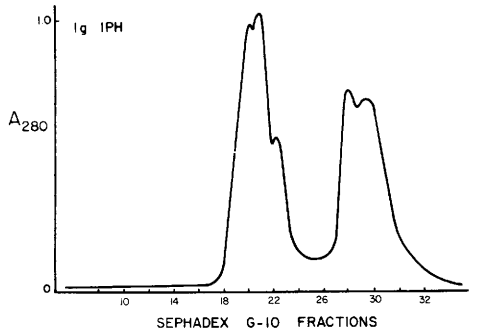

FIG. 1. Gel filtration of 1 g of lyophilized UM-2 ultrafiltrate of IPH on a Sephadex G-10 column (2.5 × 150 cm) with 0.5% acetic acid (6 × 8 ml/hr). Ultraviolet absorbance was monitored by a LUV Model 280 (Laboratory Data Control). Fractions (6.8 ml) were automatically collected.

TABLE I. SALT COMPOSITION AND CDA ACTIVITY OF KH-RECONSTITUTED SEPHADEX G-10 FRACTIONS OF 1-g IPH ULTRAFILTRATE.

Fraction No.	CDA[a] (U)	Na^+ (mequiv/liter)	K^+ (mequiv/liter)	Cl^- (mequiv/liter)	Ca^{2+} (mg/dl)
17	0	143	6.4	130	8.1
18	16	141	6.2	129	8.2
19	41	139	6.3	131	7.6
20	48	142	6.1	129	8.4
21	80	148	6.2	130	8.6
22	70	143	6.1	127	11.2
23	−26	148	6.1	126	11.6
24	15	201	7.2	124	10.8
25	40	251	9.7	204	10.8
26	12	172	19.8	191	13.0
27	59	297	37.7	393	14.1
28	60	290	22.4	345	11.2
29	−50	143	6.7	131	8.4
30	−77	152	6.9	137	8.1
31	−13	140	6.3	128	8.0
KH	0	144	6.4	131	8.2

[a] Cardiodepressant activity (CDA) reported as percentage decrease in developed tension of the isolated cat papillary muscle after the replacement of the bathing medium with the tested solution. Negative activity indicates stimulation of the muscle.

sorbing material as well as high amounts of Na^+, K^+, Cl^-, and Ca^{2+}.

In order to test the ability of high Na^+ concentrations to inhibit the isolated papillary muscle, we added increasing amounts of NaCl to the bathing medium (Fig. 2). The CDA activity was determined, the bathing solution was saved, and Na^+ concentrations were measured. Raising the Na^+ concentrations depressed the papillary muscle. The range of Na^+ concentrations was 140 to 220 mequiv/liter, producing CDA levels from 0 to 40 U.

Discussion. The concept of circulating cardiodepressant substances produced during shock states has been proposed for some time. In 1943, Blalock (3) reported the presence of toxic substances in lymph produced by gross trauma; Katzenstein *et al.* (4) reported similar substances in the lymph subsequent to hind-limb tourniquet shock. The splanchnic organs have long been suspected as the site of production for these toxic compounds (5, 6). A circulating cardiotoxic factor was reported by Gomez and Hamilton (7) to be present in the blood of dogs subjected to hemorrhagic shock. Recently, Lefer (8) reported the appearance of a circulating cardiodepressant factor in dogs, cats, and humans in a variety of shock states. He reported the major source of this factor to be the ischemic, hypoxic pancreas (8). The lines of evidence supporting this concept were reviewed by Lefer (8); the data suggested that the pancreas is the major, if not only, source of this factor in shock states, i.e., Glenn and Lefer (9) reported that pancreatic homogenates produced this biological activity and homogenates of liver, spleen, and intestine did not. We have confirmed that incubated pancreatic homogenates can produce this activity and further demonstrated that nonincubated pancreatic homogenates did not contain such activity (1).

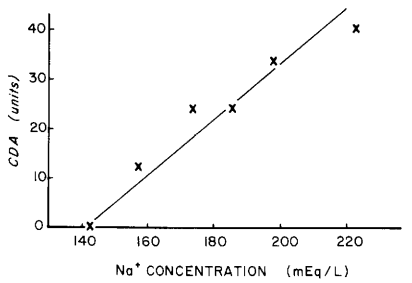

FIG. 2. Effect of raising NaCl concentration upon PM developed tension. The Na^+ concentrations of each solution were measured after the recording of the CDA activity of each sample.

The preliminary isolation technique for this factor (8) included deproteinization, ultrafiltration, and gel filtration on Biogel P-2 (Biorad Laboratories). Using these procedures, Lefer reported the active substance to be a low-molecular-weight (500–1000) peptide. Wangensteen *et al.* (2), using the same techniques, were unable to confirm Lefer's results, and reported that the biological activity observed by Lefer was solely due to the presence of excess salt (NaCl) in the assay system. These authors could not obtain depressant activity in plasma isolated from normal or shocked animals (2). Additionally, Wangensteen *et al.* (2) observed that, when depression in the papillary muscle occurred, the Na^+ and Cl^- concentrations were elevated. Their conclusion was that cardiodepressant activity of shock plasma had no pathophysiological significance in circulatory shock.

We used Sephadex G-10 gel filtration to separate peptidic material from salt material in the incubated pancreatic homogenates that we used as a source of the cardiodepressant activity. To test the ability of Sephadex G-10 to separate protein from salt, we applied 1 g of BSA in 0.9% NaCl. Sephadex G-10 was able to clearly separate salt from BSA, BSA eluting in the void volume and salts being retarded. Eluting the IPH, which contained large amounts of CDA, a similar separation occurred (Fig. 1). CDA was associated with the first peptidic peak and with the retarded salt peak. The application of NaCl-augmented KH to the papillary muscle depressed the tension development of the muscle, indicating that the CDA activity in fractions 24–28 (Table I) can be explained entirely by the presence of excess NaCl.

It is of interest to note that Wangensteen *et al.* (2) presented similar data on the effect of raised Na^+ concentrations upon the developed tension of the papillary muscle. They reported approximately the same

magnitude of effect as we have seen.

Thus, from an incubated pancreatic homogenate which contains large amounts of CDA activity, two separable types of CDA activity were obtained. One type of CDA appeared to be due to the concentration of salts, especially NaCl, in the incubation medium. The second type appeared to be due to the peptidic material present in the void volume of the Sephadex G-10 elution.

Summary. The cardiodepressant activity of shock plasma has been ascribed to the presence of a circulating cardiodepressant factor of pancreatic origin. It has been suggested that the activity observed was due to the presence of excess salt in the assay system, rather than a specific pancreatic cardiodepressant peptide (PCP). We wish to report that we obtained cardiodepressant activity (CDA) from an incubated pancreatic homogenate (IPH) (1:6, w/v, in Krebs–Henseleit solution). Heat-precipitable proteins of the IPH were removed by heating (80°) and centrifugation, the clear supernatant was subjected to ultrafiltrations, and the fraction below a molecular weight of 1000 was applied to a Sephadex G-10 column. This material was separated into 40 fractions, and each fraction was lyophilized and reconstituted with 15 ml of Krebs–Henseleit buffer. The fractions were then assayed for Na^+, K^+, Cl^-, and Ca^{2+} concentrations as well as CDA activity in the isolated cat papillary muscle (PM) assay system. The IPH was resolved into several uv-absorbing peaks (A_{280}). The CDA was associated with the nonretarded peptide peak and the salt peak. No salts, as detected by flame photometries, were present in the nonretarded peptide peak. Raising the Na^+ concentration in the KH buffer and testing it in the PM system revealed that CDA increased proportionally to NaCl content. Thus, it appears that CDA of IPH can be separated into a peptidic depressant fraction which is distinct from the salt depressant fraction.

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