THE ISOLATION AND CHARACTERIZATION OF SALMON ULTIMOBRANCHIAL CALCITONIN

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ABSTRACT

ALTHOUGH THE FIRST PREPARATIONS OF THE HYPOCALCEMIC HORMONE, CALCITONIN (CT), WERE EXTRACTED FROM RAT THYROID GLANDS, HISTOLOGICAL EVIDENCE SHOWED THAT THE "C" CELLS WHICH PRODUCE THE HORMONE ARE NOT RESTRICTED TO THIS GLAND, BUT ALSO OCCUR IN THE PARATHYROID AND THYMUS TISSUES OF MAMMALS. THESE CELLS ARISE EMBRYOLOGICALLY FROM THE LAST BRANCHIAL POUCH AND IN NON-MAMMALS THEY FORM A SEPARATE ULTIMOBRANCHIAL GLAND WHICH ALSO CONTAINS HYPOCALCEMIC ACTIVITY. THE WORK DESCRIBED IN THIS THESIS PROVIDES EVIDENCE THAT THIS ACTIVITY RESULTS FROM POLYPEPTIDES STRUCTURALLY SIMILAR TO THOSE ISOLATED FROM MAMMALIAN THYROID TISSUES AND EXPLORES THE RELATIONSHIP BETWEEN THE STRUCTURAL AND FUNCTIONAL DIFFERENCES OF THE TWO TYPES OF CT.

A SURVEY OF FOUR MAMMALIAN THYROID TISSUES (HUMAN, BOVINE, PORCINE AND MURINE) AND FOUR NON-MAMMALIAN ULTIMOBRANCHIAL TISSUES (TURKEY, CHICKEN, SALMON AND DOGFISH) DEMONSTRATED THAT THESE TISSUES CONTAINED HYPOCALCEMIC POLYPEPTIDES WITH MOLECULAR WEIGHTS OF ABOUT 4000 AS DETERMINED BY GEL FILTRATION. WHEN EXTRACTS WERE PREPARED USING AN ORGANIC SOLVENT MIXTURE DEVELOPED FOR THE THYROID CT'S THE ULTIMOBRANCHIAL TISSUES YIELDED MORE HYPOCALCEMIC ACTIVITY ON A FRESH WEIGHT BASIS AND THE FINAL PRODUCT HAD A HIGHER SPECIFIC ACTIVITY. SALMON ULTIMOBRANCHIAL TISSUE WAS COLLECTED ON A LARGE SCALE AND EXTRACTED TO PROVIDE

MATERIAL FOR CHEMICAL CHARACTERIZATION. A SERIES OF THREE GEL FILTRATION STAGES ON SEPHADEX G-50 ALTERNATING WITH TWO ION-EXCHANGE CHROMATOGRAPHY STAGES ON SE-SEPHADEX C-25 AT TWO PH'S PROVIDED A 300,000 FOLD PURIFICATION AND YIELDED 15 MG OF PURE SALMON CT. AMINO ACID ANALYSIS AND PARTIAL CHARACTERIZATION OF TRYPTIC PEPTIDES INDICATED THAT THE ULTIMOBRANCHIAL HORMONE WAS A 32 AMINO ACID POLYPEPTIDE WITH A DISULFIDE BRIDGE AT THE C-TERMINUS. ALTHOUGH THESE FEATURES ARE ALSO COMMON TO ALL THE MAMMALIAN CT'S, THERE ARE A NUMBER OF UNIQUE FEATURES IN THE SALMON CT STRUCTURE.

THESE STRUCTURAL DIFFERENCES WERE ALSO REFLECTED IN THE BIOLOGICAL ACTIVITY OF THE HORMONE. SALMON CT WAS NEARLY 50 TIMES MORE ACTIVE THAN HUMAN CT IN THE STANDARD BIOASSAY AND THE RESPONSE TO THE SALMON HORMONE WAS PROLONGED. TESTS IN PLASMA BOTH IN VIVO AND IN VITRO INDICATED THAT SALMON CT WAS MUCH MORE STABLE THAN THE THYROID CT'S SUGGESTING A POSSIBLE REASON FOR ITS GREATER POTENCY. A SURVEY OF COHN FRACTIONS FROM HUMAN PLASMA SHOWED THAT FRACTIONS III-O, IV-1 IV-4 CONTAINED ENZYMES CAPABLE OF RAPIDLY DEGRADING PORCINE CT. IN FURTHER STUDIES ON FRACTION IV-1 A SELECTIVE "CALCITONINASE" WAS PURIFIED BY CHROMATOGRAPHY ON DEAE-SEPHADEX, SEPHADEX G-200 AND CM-SEPHADEX. THIS ENZYME RAPIDLY INACTIVATED PORCINE CT, BUT HAD NO SIGNIFICANT EFFECT ON SALMON OR HUMAN CT. SYNTHETIC PORCINE CT WAS DIGESTED WITH THE ENZYME AND THE RESULTANT PEPTIDES WERE ISOLATED AND IDENTIFIED. THE NATURE OF THESE PEPTIDES INDICATED THAT THE

ENZYME WAS A PEPTIDASE WITH A SPECIFICITY FOR THE CARBOXYL SIDE OF ARGININE RESIDUES. SIMILAR DIGESTIONS OF PURE NATIVE SALMON CT PRODUCED NO PEPTIDES PROVIDING AT LEAST A PARTIAL EXPLANATION FOR THE GREATER STABILITY OF THIS HORMONE. THESE EXPERIMENTS ALSO SHOWED THAT THE ENZYME WOULD NOT SPLIT AT ALL ARGININE RESIDUES AND WOULD NOT CLEAVE BONDS ASSOCIATED WITH OTHER BASIC RESIDUES. THE DATA INDICATED THAT THE ENZYME HAD A MOLECULAR WEIGHT OF ABOUT 30,000 AND PROBABLY WAS DERIVED FROM A PRECURSOR WITH A MOLECULAR WEIGHT OF ABOUT 100,000. TESTS OF THE ACTION OF THE ENZYME ON FIBRINOGEN SHOWED THAT IT WAS NOT THROMBIN. COMPARISON OF AVAILABLE DATA WITH THAT FOR OTHER PLASMA ENZYMES INDICATED SIMILARITIES TO THE KALLIKREIN FAMILY OF ENZYMES, BUT THE "CALCITONINASE" DOES NOT APPEAR TO BE IDENTICAL WITH ANY OF THE WELL STUDIES MEMBERS OF THIS GROUP.

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I. INTRODUCTION

THE FIRST CLEAR EVIDENCE FOR ENDOCRINE CONTROL OF THE CALCIUM CONCENTRATION IN PLASMA CAME FROM THE WORK OF MACCALLUM AND VOEGTLIN IN 1909 (71) WHICH LINKED THE TETANY FOLLOWING PARATHYROIDECTOMY TO A FALL IN BLOOD CALCIUM. IT WAS 16 YEARS BEFORE THE ENDOCRINE FUNCTION OF THE PARATHYROIDS WAS CONFIRMED BY EXTRACTION OF AN ACTIVE PARATHYROID HORMONE (PTH) BY COLLIP IN 1925 (23) AND A FURTHER 34 YEARS UNTIL THE HORMONE WAS ISOLATED BY AURBACH (6) AND RASMUSSEN AND CRAIG (101) IN 1959. ALTHOUGH A FAIRLY CLEAR PICTURE OF THE FUNCTION OF THE HORMONE HAS EMERGED (42, 93, 5, 120) THE CHEMISTRY OF THE HORMONE HAS PROVED EXTREMELY DIFFICULT, AND ONLY THE AMINO ACID COMPOSITION OF PORCINE PTH (70) AND A PARTIAL SEQUENCE OF BOVINE PTH (95) ARE AVAILABLE AT PRESENT.

THE RATE AT WHICH KNOWLEDGE OF THE SECOND CALCIUM REGULATING HORMONE, CALCITONIN (CT), IS ACCUMULATING IS VERY MUCH GREATER. ALTHOUGH IT WAS ONLY RECOGNIZED BY COPP ET AL (30, 27) IN 1961, SYNTHETIC HORMONE WAS PREPARED IN 1968. TWO YEARS AFTER THE RECOGNITION OF CT, THE PREPARATION OF ACTIVE EXTRACTS FROM RAT THYROID BY HIRSCH ET AL (56) LED A NUMBER OF LABORATORIES TO BEGIN WORK ON ISOLATION OF THE HORMONE FROM MAMMALIAN THYROID TISSUE (127, 99, 98, 62, 41, 9). THIS WORK RESULTED IN THE SEQUENCING (8, 96, 82) AND SYNTHESIS (50, 107) OF THE HORMONE FROM PORCINE THYROID

TISSUE AND EVENTUALLY TO SEQUENCES FOR HUMAN CT (81) AND BOVINE CT (17).

WHILE THE WORK ON THE CHEMISTRY OF THE MAMMALIAN HORMONE WAS IN PROGRESS, STUDIES ON ORIGIN, CONTROL AND FUNCTION PROCEDED APACE AND CONSIDERABLE INFORMATION ON THESE PROPERTIES OF THE HORMONE ARE NOW AVAILABLE (57, 102, 126, 125). STUDIES ON THE CELL OF ORIGIN FOR CT LED TO EVIDENCE THAT CT WAS PRESENT IN NON-MAMMALS LACKING PTH, AND IT IS NOW APPARENT THAT THIS "SECOND" HORMONE IS PHYLOGENETICALLY MORE PRIMATIVE THAN PTH. CT MAY WELL HAVE DEVELOPED IN EARLY MARINE VERTEBRATES AS A MEANS OF MAINTAINING CALCIUM CONCENTRATIONS IN THE BODY FLUIDS AT LEVELS LOWER THAN THE SURROUNDING SEAWATER (25), WHILE PTH ACTS PRIMARILY TO MAINTAIN HIGH LEVELS OF CIRCULATING CALCIUM IN LAND VERTEBRATES FACED WITH AN ENVIRONMENT HIGH IN PHOSPHATES AND RELATIVELY LOW IN CALCIUM (24).

THE INTERACTION OF THESE TWO HORMONES IS PROBABLY RESPONSIBLE FOR THE CLOSE REGULATION OF PLASMA CALCIUM LEVELS IN MAN WHERE THE DIURNAL FLUCTUATIONS ARE GENERALLY LESS THAN ± 5% (21). INCREASES IN PLASMA CALCIUM LEVELS OF THIS MAGNITUDE (5%) HAVE BEEN SHOWN TO RESULT IN SIGNIFICANT RISES IN THE SECRETION RATE OF PTH (94) AND IN ITS CIRCULATING LEVEL (4). SIMILARLY, DECREASES IN PLASMA CALCIUM LEVELS RESULT IN AN INCREASE IN BOTH THE SECRETION (20) AND THE CIRCULATING LEVEL OF CALCITONIN (4). AN INCREASE IN PTH IN TURN ACTS TO RELEASE CALCIUM STORED IN BONE (100), TO INCREASE CALCIUM ABSORPTION IN THE GUT (33), TUBULAR REABSORPTION OF CALCIUM IN THE KIDNEY (90) AND TO INCREASE URINARY EXCRETION OF

PHOSPHATE (23). ALL OF THESE ACTIONS TEND TO INCREASE PLASMA CALCIUM LEVELS AND LOWER PLASMA PHOSPHATE LEVELS. AN INCREASE IN CALCITONIN ON THE OTHER HAND APPARENTLY ACTS PRIMARILY TO INHIBIT BONE RESORPTION (2, 100) RESULTING IN A DECREASE IN PLASMA CALCIUM. THE TIME COURSE OF THE RESPONSE TO PTH IS RATHER SLOW AND IT IS GENERALLY NEARLY AN HOUR BEFORE THE EFFECTS OF ITS ADMINISTRATION ON PLASMA CALCIUM LEVELS CAN BE OBSERVED. THE MAXIMAL EFFECT MAY NOT OCCUR FOR SEVERAL HOURS AND MAY PERSIST FOR 24 HOURS OR MORE (24). IN CONTRAST CALCITONIN ACTS RAPIDLY AND THE MAXIMAL RESPONSE MAY OCCUR IN LESS THAN ONE HOUR WITH A RETURN TO NORMAL IN A MATTER OF A FEW HOURS (31). THE PICTURE OF CALCIUM HOMEOSTASIS THAT THIS SUGGESTS IS ONE OF EXTREMELY FINE CONTROL BASED ON TWO NEGATIVE FEEDBACK SYSTEMS ACTING IN OPPOSITION TO MAINTAIN PLASMA CALCIUM AT A PRECISE SET POINT. ONE COMPONENT ACTS OVER THE LONG TERM TO RAISE PLASMA CALCIUM AND THE SECOND ACTS RAPIDLY TO LOWER PLASMA CALCIUM AGAINST THE BACKGROUND OF THE SLOWER COMPONENT THUS ACHIEVING THE PRECISE CONTROL OBSERVED IN THE INTACT ANIMAL.

AS ELEGANT AS THIS SYSTEM IS THERE IS STILL SOME DOUBT ABOUT ITS VALIDITY AS IT HAS PROVED DIFFICULT TO DEMONSTRATE A PHYSIOLOGICAL NEED FOR CALCITONIN. UNTIL RECENTLY THE PTH SYSTEM HAD PROVED CAPABLE OF MAINTAINING PLASMA CALCIUM LEVELS UNDER ALL STRESSES EXCEPT SUCH UNNATURAL PROCEDURES AS INFUSION OF CALCIUM AND PERITONEAL LAVAGES WITH HIGH CALCIUM SOLUTIONS (114). GRAY AND MUNSON (46) HAVE NOW SHOWN THAT THYROIDECTOMIZED RATS WITH FUNCTIONING PARATHYROIDS MAY SHOW SIGNIFICANT PLASMA

CALCIUM ELEVATION FOLLOWING HIGH CALCIUM MEALS. THUS THERE ARE OBVIOUS PARALLELS WITH THE INSULIN-GLUCAGON SYSTEM. HOWEVER, THIS STILL APPEARS TO BE A RELATIVELY MINOR ROLE, AND THE FACT THAT CT CAN BE DETECTED IN SIGNIFICANT QUANTITIES IN ANIMALS WITH NORMAL PLASMA CALCIUM LEVELS (4) SUGGESTS THAT IT MAY HAVE ADDITIONAL IMPORTANCE. AMONG THE POSSIBILITIES SUGGESTED HAVE BEEN A ROLE IN INCREASING BONE DEPOSITION IN FRACTURE HEALING IN ADULTS (35) AND IN THE FORMATION OF NEW BONE IN UTERO AND IN EARLY LIFE (43). THIS SECOND POSSIBILITY IS SUPPORTED BY THE OBSERVATIONS THAT THE RESPONSE TO CT IS VERY AGE DEPENDENT AND THAT YOUNG ANIMALS HAVE A MUCH GREATER SENSITIVITY TO CT THAN OLD (31). THIS CAN BE READILY EXPLAINED IN TERMS OF CALCITONIN'S INHIBITION OF BONE RESORPTION, BUT THIS DOES NOT DETRACT FROM ITS POSSIBLE ROLE. THERE IS ALSO EVIDENCE THAT CT INFLUENCES THE RATE OF CALCIUM TRANSPORT ACROSS THE MEMBRANES OF VARIOUS CELLS (12, 104) SUGGESTING THAT IT MAY PLAY A ROLE IN THE CONTROL OF INTRACELLULAR AS WELL AS EXTRACELLULAR CALCIUM LEVELS. ELUCIDATION OF THE ROLES OF THESE ENDOCRINE SYSTEMS IN CALCIUM METABOLISM AND HOMEOSTASIS CLEARLY REQUIRES CHARACTERIZATION OF THE IMPORTANCE OF THESE AND OTHER POSSIBLE ACTIONS OF CT.

THE ANALYSIS OF THE PHYLOGENETIC ORIGIN OF VARIOUS HORMONES HAS PROVIDED CONSIDERABLE INSIGHT INTO THEIR FUNCTION (45, 111), BUT LITTLE INFORMATION ALONG THESE LINES WAS AVAILABLE FOR CT. SUCH ANALYSIS SHOULD INDICATE HOW THE HORMONE FUNCTIONS IN OTHER SPECIES AND THE IMPORTANCE OF SUCH FUNCTIONS IN MAMMALS COULD THEN BE ASSESSED. IN 1967 PEARSE AND

CARVALHEIRA (91) SHOWED THAT THE PARAFOLLICULAR OR "C" CELLS OF THE MAMMALIAN THYROID ARISE EMBRYOLOGICALLY FROM THE TERMINAL BRANCHIAL POUCH WHICH IN LOWER VERTEBRATES FORMS A SEPARATE GLAND, THE ULTIMOBRANCHIAL. IN THE SAME YEAR COPP ET AL (28, 29, 32) AND TAUBER (124) EXTRACTED POTENT HYPOCALCEMIC FACTORS FROM THE ULTIMOBRANCHIAL GLANDS OF SEVERAL NON-MAMMALIAN VERTEBRATES, INCLUDING THE CHICKEN AND THE DOGFISH, BUT THE RELATIONSHIP BETWEEN THESE ULTIMOBRANCHIAL FACTORS AND THE MAMMALIAN CALCITONINS WAS ESSENTIALLY UNKNOWN.

THE WORK DESCRIBED IN THE PRESENT STUDY BEGAN AT THIS POINT, AND WAS AIMED AT A CHEMICAL CHARACTERIZATION OF THE ULTIMOBRANCHIAL FACTORS TO ESTABLISH THEIR RELATIONSHIP TO THE MAMMALIAN CALCITONINS. THE BEST APPROACH APPEARED TO BE A SURVEY OF CERTAIN BASIC FEATURES OF THE HYPOCALCEMIC FACTORS FROM A NUMBER OF SPECIES, FOLLOWED BY A DETAILED STUDY OF ONE OF THE ULTIMOBRANCHIAL FACTORS TO ALLOW COMPARISON OF ITS STRUCTURE TO THAT OF PORCINE CT. AT THE TIME THIS WORK WAS BEGUN THE AMINO ACID SEQUENCE OF THE PORCINE MOLECULE WAS STILL UNKNOWN, BUT INTENSIVE STUDIES WERE UNDERWAY IN A NUMBER OF LABORATORIES SUGGESTING THAT THIS INFORMATION WOULD SOON BECOME AVAILABLE. THE DIRECTION OF ANY FURTHER STUDIES DEPENDED ON THE OUTCOME OF THIS COMPARATIVE SEQUENCE WORK, AND WHETHER THERE WERE SIGNIFICANT DIFFERENCES OR SIMILARITIES BETWEEN THE ULTIMO-BRANCHIAL AND THYROID CALCITONINS. THE FINAL CHAPTER DESCRIBES A SERIES OF EXPERIMENTS DESIGNED TO CLARIFY THE RELATIONSHIP OF THE STRUCTURAL DIFFERENCES FOUND TO THE DIFFERENCES IN BIOLOGICAL ACTIVITY OBSERVED.

II. MOLECULAR WEIGHTS OF CALCITONINS EXTRACTED FROM ULTIMOBRANCHIAL AND THYROID TISSUES.*

A. Introduction.

The work of Copp (28, 29) and Tauber (124) had indicated that the ultimobranchial bodies of certain lower vertebrates contained hypocalcemic activity similar to that of CT from the mammalian thyroid, but further evidence was necessary to confirm that these factors were chemically similar to the mammalian hormones. There was considerable information suggesting that porcine CT had a molecular weight of about 3000 (49, 99) and it was felt that comparison of the molecular weights of ultimobranchial factors and thyroid calcitonins would provide such evidence. This type of comparison could be made fairly easily by chromatography of extracts from the various glands on a Sephadex column which had been calibrated with markers of known molecular weights (3) using the bioassay for hypocalcemic activity to determine the elution volume of the active molecules.

Preliminary chromatography of extracts of porcine thyroid and dogfish and chicken ultimobranchial glands on Sephadex G-75 (32) had shown that all of the active molecules were eluted very near the total volume (V_T) of the column, and since the relationship between elution volume and the log of the molecular weight becomes non-linear near V_T (3) the less porous Sephadex G-50 was chosen for molecular weight

*Part of the work described in this chapter was previously published (86).

ESTIMATION. UNFORTUNATELY THE ELUTION VOLUME-MOLECULAR WEIGHT RELATIONSHIP FOR THIS SEPHADEX HAD NOT BEEN DESCRIBED ADEQUATELY IN THE LITERATURE AND THE NUMBER OF MARKERS AVAILABLE IN THE PROPER WEIGHT RANGE WAS LIMITED. IT WAS THEREFORE NECESSARY TO CHECK THE SMALL MARKERS WHICH WERE AVAILABLE AGAINST OTHER LARGER MARKERS ON THE BETTER CHARACTERIZED SEPHADEX G-75 TO VERIFY THAT THEY WOULD YIELD THE DESIRED LINEAR RELATIONSHIP.

THE MOLECULAR WEIGHT STUDIES REQUIRED ACTIVE EXTRACTS FROM THE THYROID AND ULTIMOBRANCHIAL GLANDS OF A VARIETY OF SPECIES, AND PREPARATION OF THESE EXTRACTS PROVIDED AN OPPORTUNITY TO EVALUATE EXTRACTION PROCEDURES AND DETERMINE THE POTENTIALS OF VARIOUS ULTIMOBRANCHIAL TISSUES AS POSSIBLE SOURCES OF CT FOR FURTHER DETAILED STUDIES. THE EARLIER WORK ON ULTIMOBRANCHIAL TISSUES HAD BEEN BASED ON ACID EXTRACTIONS, A PROCEDURE WHICH IS USEFUL ON A SMALL SCALE BECAUSE OF ITS SIMPLICITY BUT WHICH PRODUCES A NUMBER OF PROBLEMS WHEN SCALED UP. RASMUSSEN ET AL (103) DISCUSSED SUCH PROBLEMS AS PARTIAL HYDROLYSIS AND INCOMPLETE DISSOCIATION OF PEPTIDES FROM THE TISSUE IN THEIR REPORT ON THE EXTRACTION OF PTH, AND THESE SAME PROBLEMS APPLY TO THE EXTRACTION OF CT. AN EVEN GREATER DRAWBACK TO USE ON A LARGE SCALE IS THAT SUCH EXTRACTIONS YIELD LARGE VOLUMES OF AQUEOUS SOLUTIONS WHICH ARE DIFFICULT TO HANDLE AND SINCE THE METHOD IS RELATIVELY UNSELECTIVE, GENERALLY CONTAIN NUMEROUS PROTEIN COMPONENTS. GUDMUNDSSON ET AL (48) HAD DEVELOPED A SOLVENT EXTRACTION PROCEDURE FOR PORCINE CT WHICH PRODUCED A HIGHLY PURIFIED

PRODUCT IN A CONVENIENT FORM, AND PRELIMINARY WORK (32) INDICATED THAT IT WOULD ALSO BE SUITABLE FOR ULTIMOBRANCHIAL MATERIALS. THIS RELATIVELY SELECTIVE PROCEDURE HAD THE ADDED ADVANTAGE OF PROVIDING FURTHER EVIDENCE OF THE SIMILARITY OF THE CALCITONINS FROM VARIOUS SOURCES. SELECTION OF THE SPECIES TO BE STUDIED WAS BASED PRIMARILY ON THE AVAILABILITY OF ANIMALS, THE EASE OF GLAND REMOVAL AND THE SPECIFIC ACTIVITY OF THE GLANDS AS SEEN IN ACID EXTRACTIONS SINCE ONE OF THE SPECIES WOULD EVENTUALLY BE SELECTED FOR FURTHER STUDY AND LARGE QUANTITIES OF STARTING MATERIAL WOULD BE NEEDED. AN EFFORT WAS ALSO MADE TO SELECT A FAIRLY BROAD SPECTRUM OF SPECIES SO THAT THE INFORMATION OBTAINED WOULD BE GENERALLY APPLICABLE.

B. Extractions.

1. Methods.

a. Isolation and preparation of glands.

THE INITIAL STEP PRIOR TO HORMONE EXTRACTION WAS THE COLLECTION OF SUFFICIENT QUANTITIES OF THYROID AND ULTIMOBRANCHIAL TISSUE FROM THE VARIOUS SPECIES TO YIELD ADEQUATE PRODUCT FOR STUDY. THE TWO AVIAN SPECIES STUDIED, TURKEY (*Meleagra gallapavo*) AND DOMESTIC FOWL (*Gallus domesticus*) ARE BOTH RAISED COMMERCIALLY AND THE GLANDS COULD BE COLLECTED AT THE PROCESSING PLANT AS THEY FORM A PART OF THE VISCERA WHICH ARE NORMALLY DISCARDED. THE ULTIMOBRANCHIAL GLANDS IN BOTH BIRDS ARE BILATERIAL AND LIE ALONG THE COMMON CAROTID ARTERIES NEAR THE ORIGIN OF THE SUBCLAVIAN ARTERY. THEIR RELATIONSHIP TO THE TWO PARATHYROID GLANDS AND THE THYROID IS

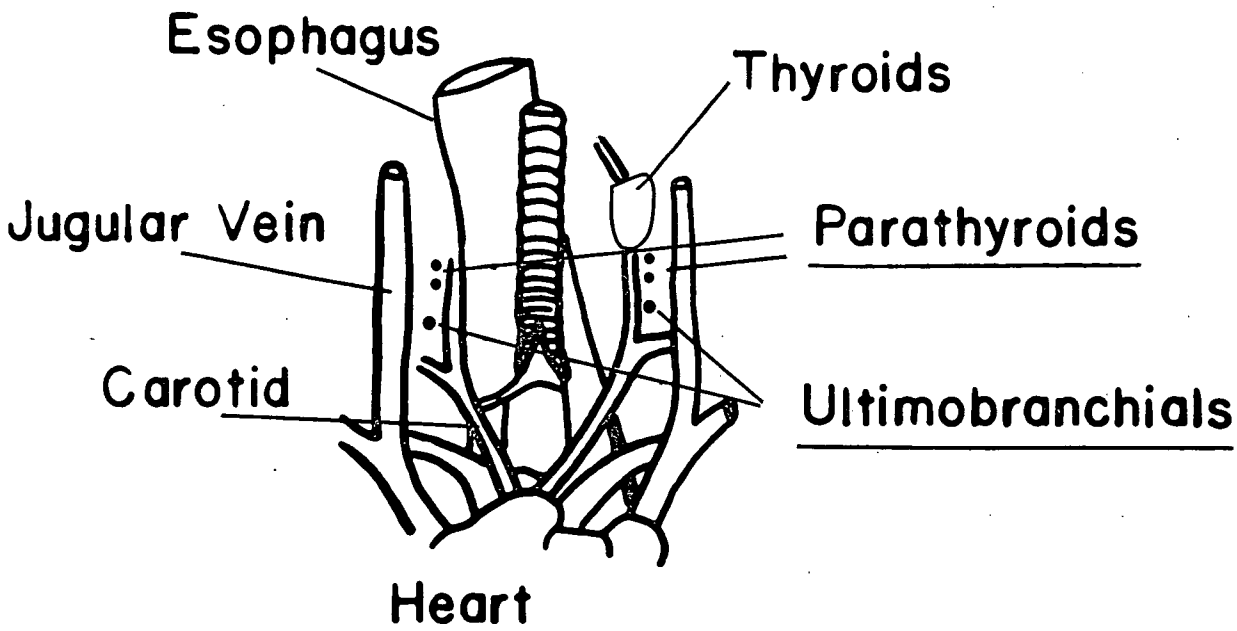


FIGURE 1. LOCATION OF AVIAN ULTIMOBRANCHIAL GLANDS.

INDICATED IN FIGURE 1. THE GLANDS WERE SEPARATED FROM SURROUNDING TISSUE AND FROZEN ON DRY ICE.

THE SALMON GLANDS WERE ALSO COLLECTED AT A COMMERCIAL PROCESSING PLANT. WHEN THE HEADS OF THE FISH ARE REMOVED THE TRANSVERSE SEPTUM IS EXPOSED AS THE POSTERIOR END OF THE HEAD PORTION ALLOWING RELATIVELY EASY ISOLATION OF THE GLANDULAR MATERIAL, WHICH FORMS A DIFFUSE BAND OF TISSUE ON THE ANTERIOR SURFACE OF THE SEPTUM. THE MAJORITY OF THE GLANDULAR TISSUE LIES BETWEEN THE DIAPHRAGM AND THE SINUS VENOSUS SLIGHTLY VENTRAL TO THE ESOPHAGUS. BECAUSE OF THE DIFFUSE NATURE OF THE GLAND THE ENTIRE SEPTUM WAS REMOVED, FROZEN AND EXTRACTED. MOST OF THE SALMON PROCESSED WERE CHUM, SOCKEYE OR COHO (ONCERHYNCHUS KETA, KISUTCH OR NERKA) AND NO EFFORT WAS MADE TO DIFFERENTIATE BETWEEN THESE SPECIES.

DOGFISH (SQUALUS SUCKLEYI) ARE COMMON IN LOCAL WATERS AND WERE CAUGHT AND PROCESSED COMMERCIALLY FOR A TIME. DURING THIS PERIOD GLANDS WERE COLLECTED FROM THE CARCASSES OF PROCESSED FISH, BUT WHEN THE FEDERAL SUBSIDY FOR THIS TYPE OF FISHING WAS TERMINATED IT BECAME NECESSARY TO COLLECT GLANDS FROM WHOLE FISH PACKED IN ICE AND DELIVERED TO THE LABORATORY. THIS SITUATION WAS LESS THAN SATISFACTORY, BUT SINCE SQUALUS WAS THE MOST PRIMATIVE GENUS SHOWN TO PRODUCE HYPOCALCEMIC FACTORS IT WAS ESSENTIAL THAT IT BE STUDIED. THE ULTIMO-BRANCHIAL GLAND CONSISTS OF NUMEROUS VASCULAR FOLLICLES LOCATED BENEATH THE FLOOR OF THE PHARYNX ON THE LEFT SIDE AT THE JUNCTURE WITH THE ESOPHAGUS. IT IS POSITIONED BETWEEN THE PHARYNX AND THE PERICARDIUM IN THE TRIANGLE FORMED BY THE

BASIBRANCHIAL AND CERATOBRANCHIAL CARTILAGES AND THE CORACO-BRANCHIAL MUSCLE. AS WITH THE SALMON ISOLATION IT IS DIFFICULT AND LARGE AMOUNTS OF CONNECTIVE TISSUE ARE PRESENT IN THE MATERIAL EXTRACTED.

BECAUSE OF THE HIGH PROTEOLYTIC ENZYME ACTIVITY IN THE THYROID IT WAS IMPERATIVE THAT THESE GLANDS BE FROZEN IMMEDIATELY AFTER REMOVAL. FOR THIS REASON THE SAMPLE OF HUMAN THYROID EXTRACT WAS OBTAINED DURING A PARTIAL THYROIDECTOMY ON A PATIENT AT THE VANCOUVER GENERAL HOSPITAL AND FROZEN ON DRY ICE. THE RAT THYROIDS WERE FROZEN ON DRY ICE IMMEDIATELY AFTER REMOVAL FROM THE ANIMAL.

PORCINE AND BOVINE EXTRACTS WERE PREPARED FROM COMMERCIAL ACETONE DRIED, DEFATTED THYROID POWDERS PURCHASED FROM WILSON LABORATORIES, CHICAGO, ILLINOIS.

IN ALL CASES WHERE FRESH OR FROZEN GLANDS WERE USED THE TISSUES WERE HOMOGENIZED, DRIED AND DEFATTED PRIOR TO EXTRACTION. TWO METHODS WERE USED FOR THIS. THE FIRST METHOD WAS A STANDARD ACETONE DRYING PROCEDURE IN WHICH GLANDS WERE HOMOGENIZED IN A WARING BLENDER IN COLD (4^{0} C) ACETONE. THE HOMOGENATE WAS THEN CENTRIFUGED AND THE SUPERNATE DISCARDED. THE PRECIPITATE WAS WASHED WITH FRESH, COLD ACETONE AND RECENTRIFUGED. THIS WASHING PROCEDURE WAS REPEATED A NUMBER OF TIMES UNTIL THE ADDITION OF WATER TO THE SUPERNATE NO LONGER CAUSED CLOUDING, INDICATING THE ABSENCE OF LIPID FROM THE ACETONE. THE REMAINING MATERIAL, WHEN DRIED IN A STREAM OF AIR IN A BUCHNER FUNNEL, PRODUCED A LIGHT, BUFF-COLORED POWDER READY FOR EXTRACTION.

ACETONE DRYING REQUIRED LARGE AMOUNTS OF TIME AND MATERIALS, AND WAS NOT REALLY SUITABLE FOR LARGE SCALE EXTRACTIONS. THIS WAS ESPECIALLY TRUE FOR THE AVIAN GLANDS WHICH WERE EMBEDDED IN ADIPOSE TISSUE THAT COULD ONLY BE REMOVED WITH GREAT DIFFICULTY. OTHER METHODS WERE EXAMINED, AND THE MOST ATTRACTIVE OF THESE APPEARED TO BE THAT OF E. LEVIN (69), WHICH USED 1,2-DICHLOROETHANE TO EXTRACT THE LIPID. AFTER THE GLANDS HAD BEEN HOMOGENIZED IN THE SOLVENT, THE MIXTURE WAS DISTILLED UNDER REDUCED PRESSURE AND A LOW-BOILING, SOLVENT-WATER AZEOTROPE REMOVED. THE REMAINING SOLVENT CONTAINING THE LIPID WAS REMOVED FROM THE PELLET-LIKE GLANDULAR MATERIAL BY FILTRATION AND REDISTILLED ALONG WITH THE IMMISIBLE SOLVENT LAYER FROM THE AZEOTROPIC MIXTURE TO RECOVER THE SOLVENT. THE PELLETS WERE WASHED WITH SOLVENT ON THE FILTER FUNNEL AND AIR DRIED.

B. EXTRACTION PROCEDURES.

THE DRIED, DEFATTED POWDERS WERE EXTRACTED, USING MODIFICATIONS OF THE METHOD DESCRIBED BY GUDMUNDSSON ET AL (48). IN THE INITIAL EXTRACTION AND PRECIPITATION STAGE WHICH WAS USED IN ALL EXPERIMENTS, 100 G OF POWDER IS STIRRED GENTLY FOR 12 HOURS AT ROOM TEMPERATURE IN 1.5 L OF BUTANOL: ACETIC ACID: WATER (75: 7.5: 21) AND THEN FILTERED. THE RESIDUE WAS STIRRED AGAIN IN 750 ML OF SOLVENT FOR 4 HOURS AND FILTERED. THE FILTRATE FROM THE FIRST TWO STEPS WAS POOLED WITH 300 ML OF SOLVENT FROM A THIRD WASH AND COOLED TO -10^{0} C. FIVE VOLUMES

OF ACETONE PREVIOUSLY COOLED TO -10°C (APPROXIMATELY 12.5 L) WAS STIRRED INTO THE POOLED FILTRATE AND THE MIXTURE ALLOWED TO STAND FOR 48 HOURS AT -10° C. THE MAJORITY OF THE LIQUID COULD THEN BE ASPIRATED OFF AND DISCARDED. THE REMAINING SLURRY WAS CENTRIFUGED AT -10° C AND THE SUPERNATE DISCARDED. THE PRECIPITATE WAS WASHED TWICE IN ABOUT 2 L OF -10° C ACETONE AND DRIED IN THE CENTRIFUGE BOTTLES UNDER A STREAM OF NITROGEN.

THE RESULTANT PRECIPITATE COULD THEN BE TREATED IN SEVERAL ALTERNATE WAYS. THE PROCEDURE OF GUDMUNDSSON ET AL (48) WAS FOLLOWED ON THE BOVINE AND PORCINE THYROID POWDERS. THE DRIED PRECIPITATE WAS EXTRACTED WITH 250 ML OF 70% ETHANOL, PH 5.5, FOR 2 HOURS AND CENTRIFUGED. THE PRECIPITATE WAS THEN RESUSPENDED IN 125 ML AND THE PROCESS REPEATED. THE TWO SUPERNATES WERE POOLED AND CONCENTRATED IN VACUO TO A VOLUME OF APPROXIMATELY 150 ML. TRICHLOROACETIC ACID (TCA), 100% W/V, WAS THEN ADDED TO A CONCENTRATION OF 12 G/100 ML, AND THE MIXTURE ALLOWED TO STAND FOR 12 HOURS AT 4°C. THE PRECIPITATE WAS WASHED WITH 10% TCA (W/V) AND RESUSPENDED IN 50 ML OF 0.5 M ACETIC ACID. THE SUSPENSION WAS THEN ADDED TO AN EQUAL VOLUME OF IRA-400 ANION EXCHANGE RESIN, 20-40 MESH, ACETATE FORM*, TO REMOVE THE TCA. WHEN THE SOLUTION HAD CLEARED THE SLURRY WAS FILTERED AND THE RESIN WASHED WITH 10 ML OF 0.5 M ACETIC ACID. THE FILTRATE WAS LYOPHILIZED TO YIELD THE FINAL EXTRACT. GUDMUNDSSON USED ACETONE: ETHER WASHES TO REMOVE TCA.

*THE RESIN WAS REGENERATED USING 3 WASHES IN 5 VOLUMES OF 1 M SODIUM HYDROXIDE, FOLLOWED BY 3 DISTILLED WATER WASHES. IT WAS THEN CONVERTED TO THE ACETATE FORM BY 3 WASHES IN 5 M ACETIC ACID AND PREPARED FOR TCA ABSORPTION BY 3 WATER WASHES.

THIS SAME PROCEDURE WAS USED ON SMALLER QUANTITIES OF DRIED-DEFATTED POWDERS FROM DOGFISH AND CHICKEN ULTIMOBRANCHIAL GLANDS. THE METHOD WAS EFFECTIVE BUT THE LATER STEPS WERE TECHNICALLY DIFFICULT ON SMALLER QUANTITIES AND YIELDS WERE POOR.

THE NECESSITY FOR WORKING ON A SMALLER SCALE LED TO THE FOLLOWING MODIFICATIONS. THE ACETONE PRECIPITATE WAS WASHED TWICE WITH COLD(0°C) ACETONE AND MOST OF THE ACETONE REMOVED IN A STREAM OF AIR. THE MOIST POWDER WAS THEN EXTRACTED WITH TEN VOLUMES OF 0.1 M FORMIC ACID AT ROOM TEMPERATURE FOR 4 HOURS AND THE SUPERNATE COLLECTED. TWO FURTHER EXTRACTIONS USING FIVE VOLUMES AND TWO VOLUMES OF 0.1 M FORMIC ACID FOR 2 HOURS AND 0.5 HOURS RESPECTIVELY WERE PERFORMED AND THE THREE SUPERNATES POOLED. LYOPHILIZATION YIELDED TAN POWDERS WHICH DISSOLVED READILY IN THE BUFFERS USED FOR COLUMN CHROMATOGRAPHY. UNLESS SPECIFIED THIS PROCEDURE WAS USED ON ALL PREPARATIONS DESCRIBED.

c. BIOLOGICAL ASSAY PROCEDURE.

THE BIOLOGICAL ACTIVITY OF THE VARIOUS EXTRACTS WAS DETERMINED BY A MODIFICATION OF THE METHOD OF KUMAR ET AL (66) USING 80-90 G, MALE LONG-EVANS RATS. MATERIALS TO BE ASSAYED WERE SUITABLY DILUTED IN AN INJECTION VEHICLE OF 0.1% BOVINE SERUM ALBUMIN, IN 0.1 M SODIUM ACETATE BUFFER AT pH 4.5, AND INJECTED INTO THE TAIL VEIN. BLOOD SAMPLES WERE COLLECTED FROM THE TAIL VEIN 60 MINUTES AFTER INJECTION AND DUPLICATE PLASMA CALCIUM DETERMINATIONS OBTAINED BY A

METHOD SIMILAR TO THAT OF KEPNER AND HERCULES (64) WHICH HAD BEEN AUTOMATED USING A TECHNICON AUTOANALYSER AND A TURNER FLOUROMETER EQUIPT WITH A FLOW CELL AS DESCRIBED BY NEWSOME (83).

THE MEASURED PLASMA CALCIUM LEVELS WERE THEN COMPARED WITH THOSE OF A SIMILAR GROUP OF CONTROL RATS INJECTED WITH VEHICLE ONLY. THE DIFFERENCE BETWEEN THE MEAN PLASMA CALCIUM LEVEL IN MG% OF THE CONTROL GROUP AND THE PLASMA CALCIUM LEVEL OF EACH EXPERIMENTAL BLOOD SAMPLE WAS CALCULATED AND CALLED THE RESPONSE. USING THIS RESPONSE AS THE DEPENDENT VARIABLE AND THE LOG OF THE DOSE AS THE INDEPENDENT VARIABLE IT WAS POSSIBLE TO CALCULATE A SAMPLE REGRESSION OF Y ON X FOR EACH SERIES OF DILUTION AS DESCRIBED BY SNEDECOR (116). THESE CALCULATIONS PRODUCED THE FAMILY OF LOG DOSE-RESPONSE CURVES SHOWN IN FIGURE 4 WHICH WERE USED IN EVALUATING THE BIOASSAYS.

COMPARISON OF THESE CURVES TO A CURVE ESTABLISHED FOR THE HOUSE STANDARD (PORCINE CT, AL0831, ARMOUR PHARMACEUTICAL CO.) AND CHECKED AGAINST THE MRC RESEARCH STANDARD A, (WHICH DEFINES THE MRC UNIT) YIELDED VALUES FOR THE ACTIVITY OF EACH SAMPLE IN MRC MILLIUNITS/MG (MU/MG). BECAUSE OF VARIATIONS IN THE SLOPES OF THE CURVES AND THE INHERENT INACCURACIES OF THE BIOASSAY SYSTEM, IT IS PREFERABLE TO EXPRESS THE ACTIVITY OF A PREPARATION AS A RANGE OF POSSIBLE VALUES RATHER THAN ATTEMPTING TO ASSIGN A SINGLE ACTIVITY.

THE RANGES USED WERE BASED ON THE 0.95 CONFIDENCE LIMITS OF THE ESTIMATED RESPONSE ($\hat{Y}$) AND WERE CALCULATED AS FOLLOWS.

1. RESPONSES (Y) OF 1.0 AND 2.0 MG% CALCIUM WERE SELECTED

AS REPRESENTATIVE VALUES.

2. THE LOG DOSE (X) WHICH CORRESPONDED TO AN ESTIMATED Y($\hat{Y}$) OF 1.0 WAS NOTED.

3. THE STANDARD ERROR OF THE ESTIMATED Y WAS THEN CALCULATED AS

$$s_{\hat{Y}} = s_{YX}\sqrt{1/N + x^2/\Sigma x^2}$$

4. THE 0.95 CONFIDENCE LIMITS OF THE POPULATION MEAN (μ_Y) FOR THE GIVEN X WAS THEN

$$\hat{Y} - T_{.05}s_{\hat{Y}} \leq \mu \leq \hat{Y} + T_{.05}s_{\hat{Y}}$$

WHERE $T_{.05}$ IS DETERMINED FROM THE DEGREES OF FREEDOM (N-2). (116)

5. THESE TWO VALUES WERE THEN TRANSPOSED TO THE RESPONSE AXIS OF THE STANDARD CURVE AND TWO CORRESPONDING ACTIVITIES IN MU READ.

6. DIVISION OF THESE ACTIVITIES BY THE LOG DOSE OBTAINED IN STEP 2 ABOVE YIELDS A RANGE OF ACTIVITY IN MU/MG REPRESENTING THE 0.95 CONFIDENCE LIMITS AT THIS POINT, IF THE STANDARD CURVE IS ASSUMED TO CONTAIN NO ERROR. THIS IS A REASONABLE ASSUMPTION SINCE THIS CURVE IS THE BASIS FOR ALL ACTIVITY ESTIMATES USED IN THE LABORATORY AND THESE ESTIMATES ARE USED PRIMARILY FOR COMPARISON.

7. A REPETITION OF STEPS 2 THROUGH 6 USING Y = 2.0 GIVES AN ADDITIONAL RANGE OF ACTIVITIES. THE FINAL RANGE EXPRESSED IS THE EXTREME VALUES OBTAINED FROM THE TWO CALCULATIONS.

MOST OF THE STATISTICAL CALCULATIONS ABOVE WERE ROUTINELY PERFORMED BY COMPUTER. THE PROGRAM USED FOR THIS CONTAINED

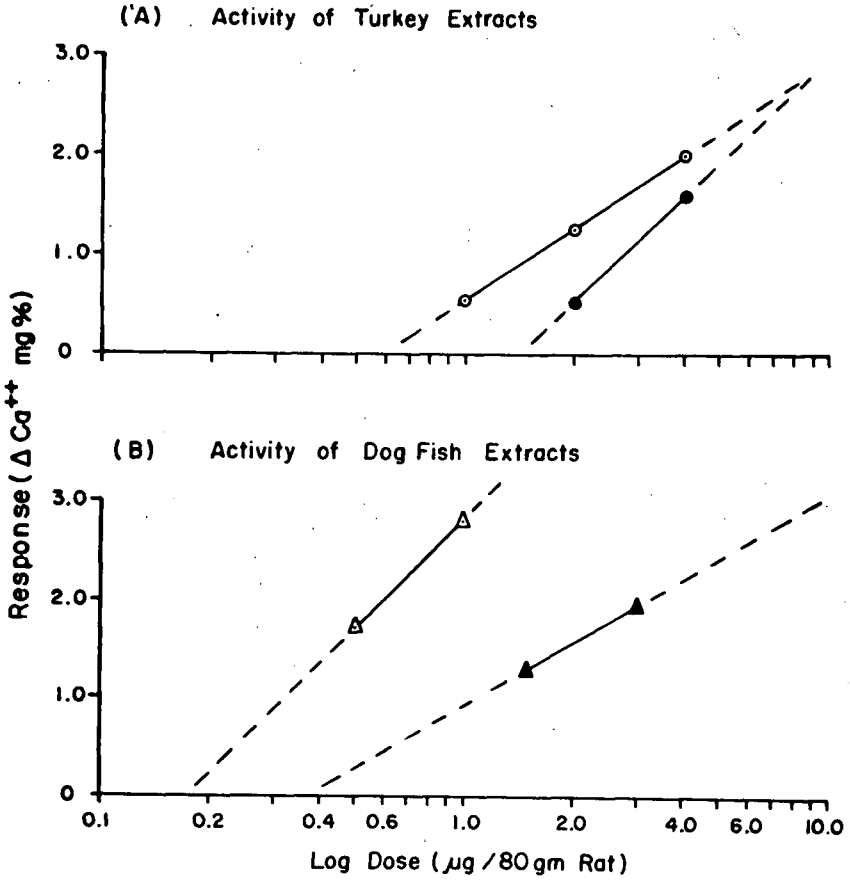


FIGURE 2. (A) COMPARISON OF REGRESSIONS OF LOG DOSE ON RESPONSE FOR TURKEY ULTIMOBRANCHIAL EXTRACTS FROM ACETONE AND DICHLOROETHANE DRIED GLANDS.
●– ACETONE, O– DICHLOROETHANE

(B) COMPARISON OF REGRESSIONS OF LOG DOSE ON RESPONSE FOR DOGFISH ULTIMOBRANCHIAL EXTRACTS PREPARED USING FORMIC ACID OR TCA.
▲– TCA PRECIPITATION, △– FORMIC ACID EXTRACTION

MODIFICATIONS BY THE AUTHOR AND ITS DETAILS ARE INCLUDED IN APPENDIX A.

2. Results and Discussion.

FIGURE 2(A) SHOWS THE LOG DOSE-RESPONSE CURVES FOR TWO SAMPLES OF TURKEY ULTIMOBRANCHIAL EXTRACTS. THE EXTRACTIONS WERE CARRIED OUT IN PARALLEL EXCEPT FOR THE INITIAL DRYING STAGE. THE BIOLOGICAL ACTIVITY OF THE ACETONE DRIED MATERIAL RANGES FROM 540 TO 840 MU/MG WHILE THAT OF THE DICHLOROETHANE DRIED MATERIAL WAS SOMEWHAT LOWER, RANGING FROM 420-530 MU/MG. THE LOWER BIOLOGICAL ACTIVITY OF THE DICHLOROETHANE MATERIAL WAS CONSIDERED A MAJOR DISADVANTAGE AT THIS STAGE AND ALTHOUGH THE PROBLEM DID NOT SEEM INSURMOUNTABLE, A COMPLETE INVESTIGATION WAS NOT CARRIED OUT. ONE ATTEMPT AT REDUCING HEAT DENATURATION BY USING A DICHLOROMETHANE AZEOTROPE WITH A LOWER BOILING POINT PRODUCED INCONCLUSIVE RESULTS. THE MATERIAL OBTAINED FROM THIS PROCEDURE HAD EXCELLENT PHYSICAL PROPERTIES AND APPEARED TO BE FREE OF LIPID AFTER A SINGLE OPERATION. FURTHER WORK MIGHT WELL PROVE IT TO BE A VALUABLE TECHNIQUE PARTICULARLY FOR FATTY STARTING MATERIALS.

FIGURE 2(B) SHOWS THE LOG DOSE-RESPONSE CURVES FOR TWO SAMPLES OF DOGFISH ULTIMOBRANCHIAL EXTRACTS. THE EXTRACTIONS WERE CARRIED OUT IN PARALLEL USING THE ACETONE DRYING TECHNIQUE AND THE SOLVENT EXTRACTION OF GUDMUNDSSON *ET AL* (48) UP TO THE STAGE OF TCA PRECIPITATION. ONE SAMPLE WAS THEN PRECIPITATED WITH TCA WHILE THE SECOND WAS EXTRACTED WITH 0.1 M FORMIC ACID AS DESCRIBED EARLIER. THE BIOLOGICAL ACTIVITY OF THE TCA POWDER (3600-4600 MU/MG) WAS MORE THAN DOUBLE THAT OF THE

TABLE I

SPECIFIC BIOLOGICAL ACTIVITIES OF THYROID AND ULTIMOBRANCIAL EXTRACTS

SOURCE	ACTIVITY RANGE (MU/MG)	CENTER OF RANGE (MU/MG)	RELATIVE POTENCY (PORCINE = 1)
HUMAN THYROID	5	5	0.02
BOVINE THYROID*	20-25	22.5	0.1
MURINE THYROID	100-150	125	0.5
PORCINE THYROID*	210-280	245	1
TURKEY UB	540-840	690	3
DOGFISH UB	800-1300	1050	4
SALMON UB	2500-4300	3400	14
CHICKEN UB*	13,000-17,000	15,000	60

*INCLUDES TCA PRECIPITATION STEP.

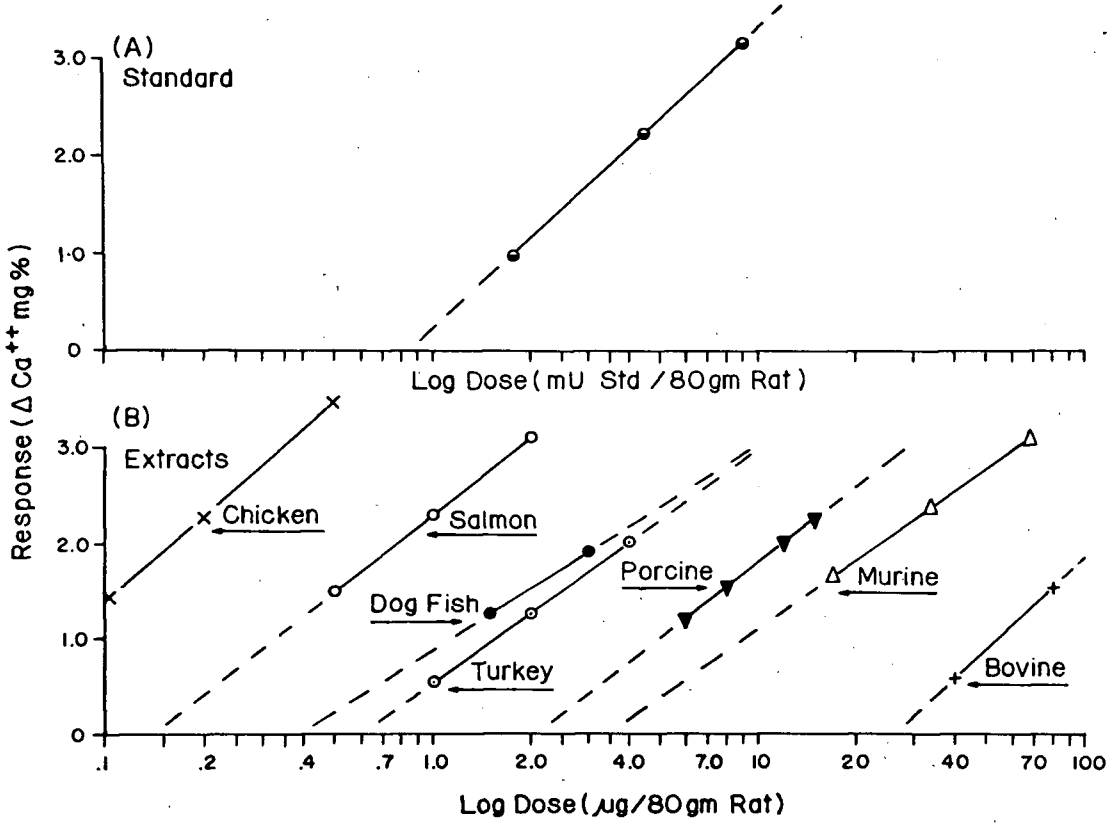


FIGURE 3. (A) REGRESSION OF LOG DOSE ON RESPONSE FOR HOUSE STANDARD. (ARMOUR PORCINE CT, AL0831)

(B) REGRESSIONS OF LOG DOSE ON RESPONSE FOR EIGHT EXTRACTS FROM THYROID AND ULTIMOBRANCHIAL TISSUES. CALCULATED ACTIVITY RANGES ARE SHOWN IN TABLE I.

FORMIC ACID POWDER (800-1300), BUT BECAUSE OF THE SIMPLICITY OF THE PROCEDURE, TOTAL RECOVERY WAS MUCH GREATER WITH THE FORMIC ACID EXTRACTION. EXPRESSED IN TERMS OF MRC MILLIUNITS PER GRAM OF FRESH TISSUE (MU/G TISSUE) THE ACTIVITIES WERE AS FOLLOWS:

TCA POWDER	1000-1300 MU/G TISSUE
FORMIC ACID POWDER	4500-7300 MU/G TISSUE

ON THE BASIS OF THIS LOSS OF ACTIVITY IT WAS DECIDED THAT THE ADDITIONAL COMPLICATIONS OF THE TCA PRECIPITATION COULD NOT BE JUSTIFIED, PARTICULARLY SINCE THE NEXT STAGE OF PURIFICATION WAS TO BE A GEL FILTRATION STEP WHICH WOULD REMOVE THE LOW MOLECULAR WEIGHT CONTAMINANTS EQUALLY AS WELL AS THE TCA STEP.

FIGURE 3(B) SHOWS A SERIES OF LOG DOSE-RESPONSE CURVES FOR BIOASSAYS ON EXTRACTS FROM SEVEN SPECIES PLOTTED ON THE SAME AXIS. THE CURVE IN FIGURE 3(A) IS THE HOUSE STANDARD WHICH IS USED TO CALCULATE THE BIOLOGICAL ACTIVITY OF A PREPARATION IN MU/MG. THE ACTIVITY RANGES FOR THE EXTRACTS AND THEIR RELATIVE POTENCIES ARE SUMMARIZED IN TABLE 1. THE VALUE INCLUDED FOR THE HUMAN MATERIAL IN THE TABLE IS AN EXTREMELY ROUGH ESTIMATE BASED ON A SINGLE POINT. BETTER DATA COULD NOT BE OBTAINED BECAUSE OF THE LOW YIELD AND LIMITED STARTING MATERIAL. IT IS OF SOME INTEREST, HOWEVER, FOR COMPARISON AND IT IS TYPICAL OF THE YIELD FROM HUMAN THYROID. THE CHICKEN, PORCINE AND BOVINE EXTRACTS WERE PREPARED USING THE TCA PRECIPITATION PROCEDURE AND THEREFORE PROBABLY HAVE HIGHER SPECIFIC ACTIVITIES THAN WOULD HAVE BEEN OBTAINED HAD FORMIC ACID EXTRACTION BEEN USED.

TABLE II

BIOLOGICAL ACTIVITY YIELDS FROM THYROID AND ULTIMOBRANCHIAL TISSUES

SOURCE	CENTER OF RANGE (MU/MG)*	MG EXTRACT / G TISSUE	MU / G TISSUE
HUMAN THYROID	5	1.0	5
BOVINE THYROID	22.5	(0.5)**	11
MURINE THYROID	125	5.2	650
PORCINE THYROID	245	(0.7)**	170
TURKEY UB	690	3.3	2300
DOGFISH UB	1050	5.6	5900
SALMON UB	3400	1.3	4400
CHICKEN UB	15,000	0.76	11,500

* SEE TABLE I

** ESTIMATE ASSUMING DRY, DEFATTED WEIGHT EQUALS 8% OF FRESH WEIGHT.

SPECIFIC ACTIVITIES OF CRUDE EXTRACTS SUCH AS THESE ARE GENERALLY USEFUL FOR CALCULATION OF HORMONE CONTENT OF THE FRESH GLANDS OR AS AN INDEX OF THE DEGREE OF PURIFICATION ACHIEVED IN LATER STAGES. THESE CALCULATIONS WILL BE DISCUSSED LATER. THEY CAN ALSO PROVIDE AN ESTIMATE OF THE PURITY OF THE MATERIAL AND OF THE EXPECTED YIELDS OF PURE HORMONE IF THE ABSOLUTE SPECIFIC ACTIVITY OF THE MOLECULES ARE COMPARABLE. ON THIS BASIS, KNOWING THAT PURE PORCINE CT HAD A SPECIFIC ACTIVITY OF 200-250 U/MG, THE SALMON EXTRACT SHOULD CONTAIN 1 TO 2% HORMONE. AS WILL BE SHOWN LATER, THIS IS RATHER AN OVER-ESTIMATE.

TABLE II SHOWS THE CALCULATED ACTIVITIES OF THE FRESH TISSUES USED IN THE EXTRACTS. BOVINE AND PORCINE VALUES ARE ESTIMATES BASED ON THE ASSUMPTION THAT THE DRY, DEFATTED POWDERS REPRESENT APPROXIMATELY 8% OF THE FRESH WEIGHT. THE RELATIVE ACTIVITIES OF THE TISSUES FROM THE EIGHT SPECIES ARE COMPARABLE WITH THOSE OBTAINED IN ACID EXTRACTION (32) THOUGH THE ABSOLUTE VALUES ARE GENERALLY LOWER. THERE ARE TWO POSSIBLE EXPLANATIONS FOR THIS FACT. FIRST THE SOLVENT EXTRACTION METHOD IS MORE COMPLEX AND THERE ARE NUMEROUS OPPORTUNITIES FOR SIGNIFICANT LOSSES TO OCCUR, WHERE AS A SIMPLE ACID EXTRACTION INVOLVES ALMOST NO LOSSES DUE TO HANDLING. SECOND, THE PRIMARY PURPOSE OF THESE EXTRACTIONS WAS TO ESTABLISH THE FEASABILITY OF LARGER SCALE EXTRACTIONS, AND THE EMPHASIS WAS PLACED ON MINIMIZING THE TIME SPENT IN COLLECTING THE GLANDS. THUS NO GREAT EFFORT WAS MADE TO REMOVE ALL NON-GLANDULAR TISSUE AND THE STARTING MATERIALS

WERE GENERALLY MUCH CRUDER THAN THOSE USED TO ESTIMATE ACTUAL ACTIVITY LEVELS IN GLANDS.

THE MAJOR VALUE OF THE DATA, HOWEVER, IS ITS USEFULNESS IN ESTIMATING THE AMOUNT OF STARTING MATERIAL NECESSARY TO PROVIDE SUFFICIENT HORMONE FOR STRUCTUAL WORK. ALL OF THE ULTIMOBRANCHIAL TISSUE APPEARED TO BE EXCELLENT SOURCES OF HORMONE SINCE THEY CONTAINED FROM 10 TO 50 TIMES THE ACTIVITY OF THE PORCINE THYROID. THE CHICKEN ULTIMOBRANCHIALS WERE PARTICULARLY ATTRACTIVE SINCE THEY WERE 2 TO 3 TIMES MORE ACTIVE THAN EITHER THE DOGFISH OR SALMON GLANDS. THIS COULD BE DUE, AT LEAST IN PART, TO THE DIFFUSE NATURE OF THE GLANDULAR TISSUE IN THE TWO FISH AS OPPOSED TO THE DISCRETE GLAND WHICH EXISTS IN THE CHICKEN. THIS EXPLANATION DOES NOT HOLD, HOWEVER, IN THE TURKEY WHICH HAS A DISCRETE GLAND AND A CONSIDERABLY LOWER ACTIVITY PER UNIT WEIGHT.

ON THE BASIS OF THE EXPERIMENTS OUTLINED IN THIS CHAPTER IT WAS DECIDED THAT ALL FOUR OF THE ULTIMOBRANCHIAL SOURCES CONTAINED SUFFICIENT QUANTITIES OF HORMONE TO ALLOW PURIFICATION AND ISOLATION, AND THAT THE SOLVENT EXTRACTION PROCEDURE FOLLOWED BY A FORMIC ACID EXTRACTION WAS A SUITABLE METHOD FOR PREPARING THESE HORMONES. THE FINAL DECISION TO CARRY ON WITH THE SALMON HORMONE WAS THEREFORE BASED ON OTHER FACTORS. THE DOGFISH WAS ELIMINATED BECAUSE OF THE RELATIVE DIFFICULTY IN ACQUIRING STARTING MATERIAL AND THE AVIAN SOURCES WERE PASSED OVER BECAUSE THE LARGE AMOUNTS OF FATTY TISSUE ASSOCIATED WITH THE GLANDS MADE THE ACETONE DRYING PROCEDURE EXPENSIVE AND TIME CONSUMING. THIS LEFT THE SALMON AS THE

SPECIES OF CHOICE.

THE SALMON HAD THE SECOND HIGHEST ACTIVITY PER WEIGHT OF TISSUE AND THE SECOND HIGHEST SPECIFIC ACTIVITY IN THE EXTRACT. (THE HIGHER ACTIVITY OF THE CHICKEN EXTRACT MIGHT BE EXPLAINED BY THE TCA PRECIPITATION STEP WHICH WAS INCLUDED IN THE CHICKEN EXTRACTION.). THE TISSUE WAS EASY TO WORK WITH, AND COULD BE OBTAINED IN LARGE QUANTITIES ON THE PRODUCTION LINE OF A LOCAL CANNERY. THUS IT MET ALL THE REQUIREMENTS FOR A STARTING MATERIAL FOR LARGE SCALE EXTRACTION.

ASSUMING AN ACTIVITY APPROXIMATELY EQUAL TO THAT OF THE PURE PORCINE HORMONE (200-250 U/MG) IT WAS CALCULATED THAT 1 KG OF SALMON ULTIMOBRANCHIAL TISSUE SHOULD CONTAIN ABOUT 20 MG OF HORMONE. ALLOWING FOR A 50% LOSS DURING PURIFICATION THIS MEANT THAT 2 KG OF TISSUE WOULD BE NEEDED TO PROVIDE THE 10 TO 20 MG OF PURE HORMONE NECESSARY FOR STRUCTURAL WORK. ALTHOUGH THIS REPRESENTED ABOUT 10 TONS OF SALMON, IT WAS STILL A GROSS UNDERESTIMATION.

C. Calibration of Sephadex Columns.

1. Methods.

THE PROCEDURE USED IN PREPARING THE CALIBRATED SEPHADEX G-75 COLUMN WAS ESSENTIALLY THAT DESCRIBED BY ANDREWS (3). A 1.2 x 80 CM COLUMN OF SEPHDEX G-75 WAS PREPARED USING 0.1 M FORMIC ACID AS AN ELUANT. APPROXIMATELY 1 MG OF THE MARKER PROTEIN WAS THEN DISSOLVED IN 0.5 ML OF 2 M UREA IN 0.1 M FORMIC ACID. THE UREA INSURED THAT THE PROTEIN WOULD DISSOLVE AND INCREASED THE DENSITY OF THE SOLUTION ALLOWING IT TO BE

LAYERED BENEATH THE ELUANT ON THE TOP OF THE COLUMN. ONCE THE SAMPLE WAS APPLIED ELUANT WAS ALLOWED TO FLOW THROUGH THE COLUMN UNDER GRAVITY, AND 3 ML FRACTIONS OF ELUATE WERE COLLECTED. THE ABSORBANCE OF EACH FRACTION AT 280 NM WAS MEASURED WITH A BAUSCH AND LOMB SPECTRONIC 600 SPECTROPHOTOMETER AND WHEN PLOTTED AGAINST THE TOTAL VOLUME COLLECTED IT PRODUCED A TYPICAL ELUTION PROFILE CONTAINING A SINGLE PEAK. THE CENTER OF THIS PEAK WAS DESIGNATED AS THE ELUTION VOLUME (V_E) FOR THAT PROTEIN AND THIS VALUE WAS PLOTTED AGAINST THE LOG OF THE MOLECULAR WEIGHT OF THE PROTEIN. (SEE FIGURE 4).

THE MARKERS USED WERE GAMMA GLOBULIN (160,000 M.W.), MYOGLOBIN (17,800 M.W.) CYTOCHROME C (12,400 M.W.), GLUCAGON (3485 M.W.), BACITRACIN (1411 M.W.), INSULIN (5722 M.W.) AND ACTH (4542 M.W.). THE FIRST FOUR MARKERS HAD BEEN USED BY ANDREWS AND WERE CONSIDERED A CHECK ON THE OTHER THREE. THE FIRST FIVE MARKERS WERE OBTAINED FROM MANN RESEARCH LABORATORIES, INC. NEW YORK. THE INSULIN WAS RECRYSTALIZED BOVINE INSULIN, GRADE B FROM CALBIOCHEM, LOW ANGELES. THE ACTH WAS CHROMATOGRAPHICALLY PURE PORCINE ACTH FROM SIGMA CHEMICAL CO., ST. LOUIS.

THE CALIBRATION PROCEDURE FOR THE SEPHADEX G-50 COLUMN WAS IDENTICAL TO THAT DESCRIBED FOR THE G-75 COLUMN EXCEPT FOR THE SEPHADEX USED, THE COLUMN SIZE (1.5 x 80 CM) AND THE MARKERS. THE SAME LOW MOLECULAR WEIGHT MARKERS WERE USED (BACITRACIN, GLUCAGON, ACTH AND INSULIN), BUT BOVINE SERUM ALBUMIN (65,000 M.W.) WAS USED TO INDICATE THE VOID VOLUME

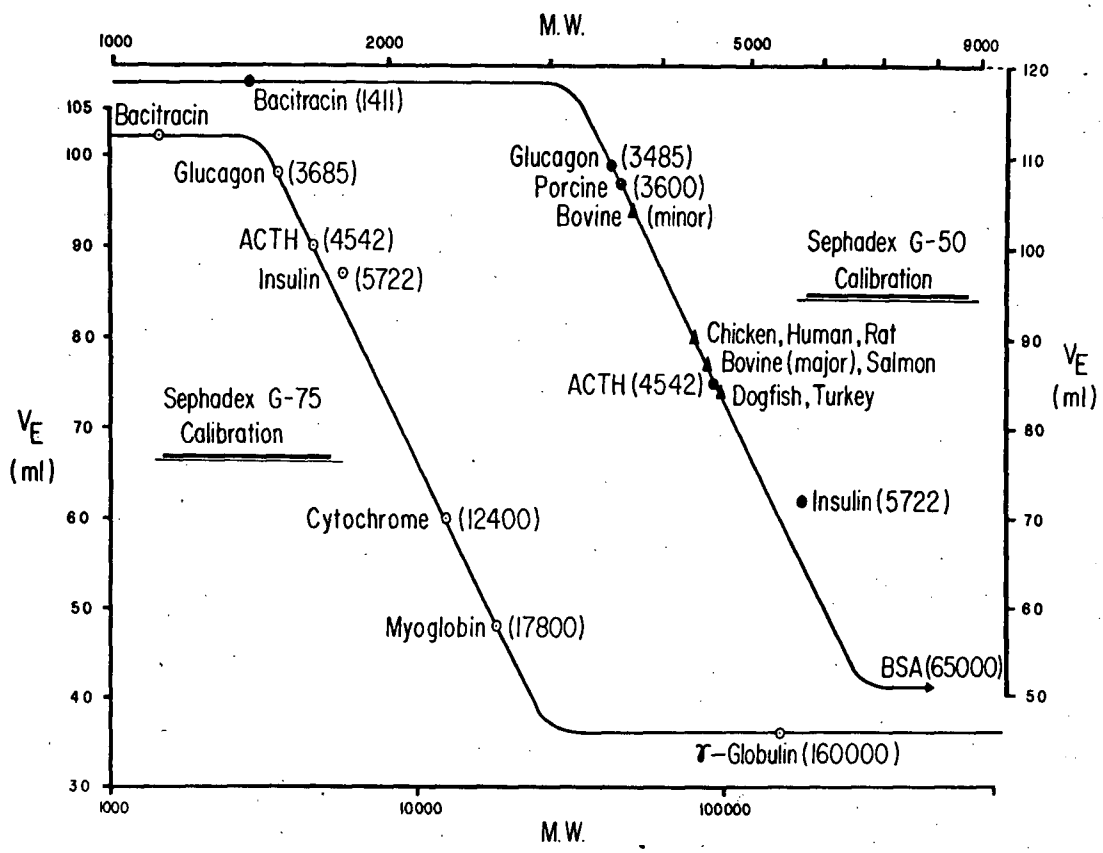


FIGURE 4. CALIBRATION CURVES FOR SEPHADEX G-50 AND G-75, INCLUDING ELUTION VOLUMES OF HYPOCALCEMIC ACTIVITY FROM EXTRACTS. G-75 COLUMN, 1.2 x 80 CM; ELUANT, 0.1 M FORMIC ACID; TEMPERATURE, 4°C; FLOW RATE 5 ML/HR; FRACTION SIZE, 3.0 ML. G-50 COLUMN, 1.5 x 80 CM; OTHER CONDITIONS THE SAME.

○, G-75 MARKERS; ●, G-50 MARKERS; ▲, HYPOCALCEMIC ACTIVITY.

(V_o) OF THIS COLUMN. THE ALBUMIN WAS GRADE A, CRYSTALLINE FROM CAL BIOCHEM, LOS ANGELES.

2. RESULTS AND DISCUSSION.

THE RESULTS OF THIS CALIBRATION ARE SHOWN IN FIGURE 4 WHERE THE ELUTION VOLUMES (V_E) OF EACH MARKER ARE PLOTTED VERSUS THE LOG OF THE MOLECULAR WEIGHT OF THAT MARKER. THE LINEARITY OF THIS RELATIONSHIP BETWEEN M.W. 3000 AND 30,000 HAS BEEN REPORTED PREVIOUSLY AND GLUCAGON, CYTOCHROME C AND MYOGLOBIN HAVE BEEN SHOWN TO FIT THE LINE WELL.(3). IN THIS EXPERIMENT THESE RESULTS WERE REPRODUCED AND ACTH WAS ALSO SHOWN TO LIE ALONG THE LINE. INSULIN, HOWEVER, DID NOT BEHAVE AS PREDICTED AND HAD A V_E SOMEWHAT LARGER THAN EXPECTED. ON THE G-75 COLUMN INSULIN HAD AN APPARENT MOLECULAR WEIGHT OF SLIGHTLY BELOW 5000 A VALUE SOME 10-15% BELOW ITS REAL MOLECULAR WEIGHT OF 5722. THIS ANOMALOUS BEHAVIOR PROBABLY RESULTS FROM THE FACT THAT GEL FILTRATION METHODS ACTUALLY MEASURE MOLECULAR SIZE WHICH IS INFLUENCED BY FACTORS OTHER THAN MOLECULAR WEIGHT AS WILL BE DISCUSSED IN A LATER CHAPTER.

FIGURE 4 ALSO SHOWS THE CALIBRATION OF THE G-50 COLUMN. AGAIN THERE IS A LINEAR RELATIONSHIP BETWEEN THE LOG OF THE MOLECULAR WEIGHT AND THE ELUTION VOLUME, THOUGH WITH G-50 THE RANGE IS MUCH SMALLER EXTENDING ONLY FROM ABOUT 3000 TO 9000 M.W. AS EXPECTED, HOWEVER, THE RESOLUTION IN THIS RANGE WAS GREATER THAN WAS SEEN WITH G-75. INSULIN BEHAVED MUCH AS IT HAD ON G-75. WHEN ITS REAL MOLECULAR WEIGHT WAS PLOTTED VERSUS ITS ELUTION VOLUME THE POINT LAY TO THE RIGHT OF THE LINE ESTABLISHED BY THE OTHER MARKERS. IF ITS APPARENT

MOLECULAR WEIGHT AS SEEN ON G-75 WAS USED INSTEAD, THE RESULTANT POINT LAY VERY NEAR THE LINE. IN THE ABSENCE OF OTHER MARKERS IN THIS WEIGHT RANGE IT SEEMED JUSTIFIABLE TO USE THIS APPARENT MOLECULAR WEIGHT TO PROVIDE A POINT AT THE LOWER END OF THE LINE. THE RELIABILITY OF THE UPPER END OF THE LINE WAS ENHANCED WHEN THE MOLECULAR WEIGHT OF PORCINE CT, WHICH HAD ORIGINALLY BEEN CONSIDERED AN UNKNOWN, WAS CONFIRMED BY THE SEQUENCE WORK OF BELL (9) AND POTTS (96).

D. Estimation of Molecular Weights of Hypocalcemic Factors.

1. Methods.

TO OBTAIN ESTIMATES OF THE MOLECULAR WEIGHTS OF THE ACTIVE FACTORS IN THE EXTRACTS, SAMPLES OF EACH EXTRACT WERE APPLIED TO THE CALIBRATED G-50 COLUMN IN A MANNER SIMILAR TO THAT USED FOR THE MARKERS. THE SAMPLES WERE APPLIED IN 0.1 M FORMIC ACID MADE 8 M IN UREA. THESE SAMPLES WERE ELUTED AND THE ABSORBANCE AT 280 NM OF EACH 3 ML FRACTION DETERMINED. RECORDS OF THE ELUTION PROFILES ARE SHOWN IN FIGURES 5 AND 6.

THE ELUTION VOLUME OF THE ACTIVE MOLECULES WAS DETERMINED BY A BIOASSAY PROCEDURE SIMILAR TO THAT DESCRIBED PREVIOUSLY. THE ASSAY WAS USED ONLY AS A QUALITATIVE METHOD TO INDICATE PRESENCE OR ABSENCE OF HYPOCALCEMIC FACTORS; HOWEVER, AND NO ATTEMPT WAS MADE TO MEASURE THE ACTUAL AMOUNT OF ACTIVITY PRESENT. ROUTINELY THE COLUMN FRACTIONS WERE SURVEYED BY TAKING 0.1 ML ALIQUOTS FROM EVERY SECOND FRACTION BETWEEN V_0 AND V_T (50 TO 150 ML) AND DILUTING THEM WITH 0.2 ML OF SODIUM ACETATE, BSA VEHICLE. EACH 0.3 ML SAMPLE WAS THEN

INJECTED INTO A RAT AND THE RATS PLASMA CALCIUM IN MG% DETERMINED AFTER ONE HOUR. THE DIFFERENCE (Ca, MG%) BETWEEN THE TEST RAT'S PLASMA CALCIUM AND THE MEAN PLASMA CALCIUM OF A GROUP OF CONTROL RATS INJECTED WITH VEHICLE ONLY WAS THEN USED AS AN INDEX OF HYPOCALCEMIC ACTIVITY. ANY DROP IN PLASMA CALCIUM WHICH EXCEEDED 1 MG% WAS CONSIDERED INDICATIVE OF A HYPOCALCEMIC FACTOR SINCE VARIATIONS OF THIS MAGNITUDE ARE ALMOST NEVER SEEN IN VEHICLE INJECTED RATS. IF ANY AMBIGUITIES WERE SEEN THE SURVEY WAS REPEATED SAMPLING FROM EVERY FRACTION. BECAUSE OF THE LOW ACTIVITY IN THE HUMAN THYROID EXTRACT LARGER SAMPLES WERE REQUIRED FOR INJECTION. 0.4 ML ALIQUOTS WERE TAKEN AND 0.1 ML OF A CONCENTRATED VEHICLE ADDED (0.4 M SODIUM ACETATE pH 4.5 CONTAINING 0.4% BOVINE SERUM ALBUMIN.) GENERALLY THE ACTIVE REGION OF ANY COLUMN WAS BETWEEN 3 AND 5 FRACTIONS WIDE, AND SINCE THE ASSAY USED WAS ONLY QUALITATIVE IT WAS IMPOSSIBLE TO KNOW WHICH FRACTION WAS MAXIMALLY ACTIVE. FOR THIS REASON THE ELUTION VOLUME SHOWN FOR EACH HORMONE IS THAT VOLUME WHICH CORRESPONDS TO THE CENTER OF THE ACTIVE REGION.

ONE ADDITIONAL SAMPLE OF BOVINE THYROID EXTRACT WAS CHROMATOGRAPHED ON THE COLUMN. (SEE FIGURE 7). THIS SAMPLE WAS PREPARED USING THE FORMIC ACID EXTRACTION PROCEDURE RATHER THAN THE TCA PRECIPITATION.

2. RESULTS AND DISCUSSION.

ONCE THE G-50 COLUMN HAD BEEN CALIBRATED IT WAS POSSIBLE TO ELUTE SAMPLES OF THE VARIOUS EXTRACTS FROM THE COLUMN AND TO DETERMINE THE ELUTION VOLUME OF THE ACTIVE MOLECULES IN

TABLE III

MOLECULAR WEIGHT ESTIMATES FOR CALCITONINS FROM ELUTION VOLUMES ON A SEPHADEX G-50 COLUMN

SPECIES	ELUTION VOLUME (ML)	ESTIMATED MOLECULAR WEIGHT
PORCINE	108	3600
BOVINE I	105	3700
BOVINE II	87	4500
MURINE	90	4300
HUMAN	90	4300
CHICKEN	90	4300
SALMON	87	4500
TURKEY	84	4600
DOGFISH	84	4600

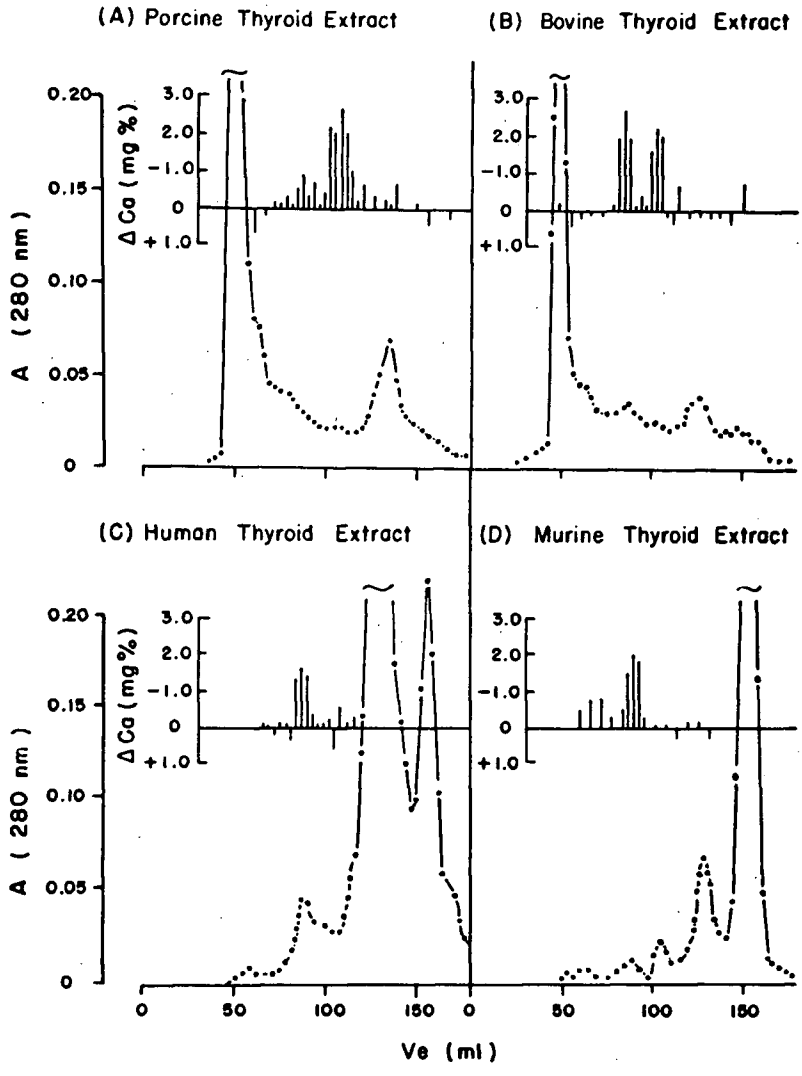


FIGURE 5. ELUTION PROFILES (A280 NM) OF THYROID EXTRACTS ON SEPHDEX G-50. CONDITIONS AS INDICATED IN FIGURE 4. ΔCa IS THE DROP IN PLASMA CALCIUM AS SEEN IN THE BIOASSAY.

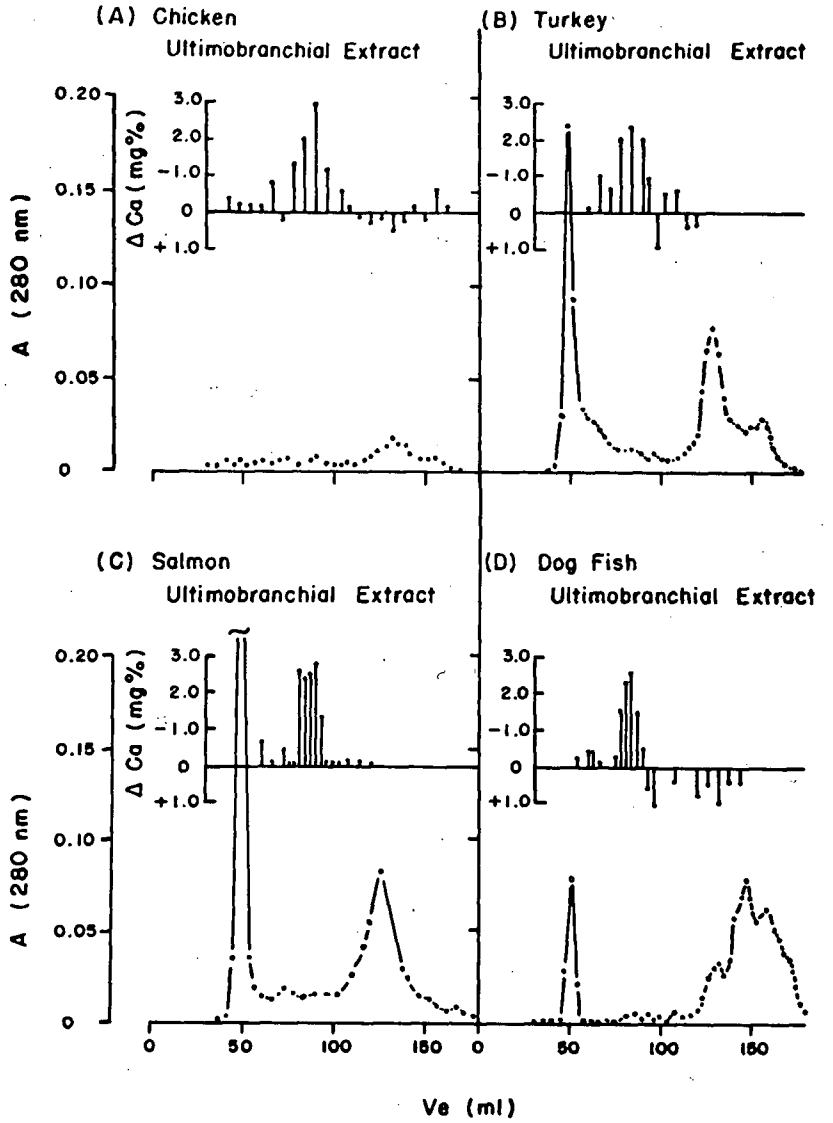


FIGURE 6. ELUTION PROFILES (A280 NM) OF ULTIMOBRANCHIAL EXTRACTS ON SEPHADEX G-50. CONDITIONS AS INDICATED IN FIGURE 5.

EACH OF THE EXTRACTS USING THE BIOASSAY. THE ELUTION PROFILES AND BIOASSAYS OF THE ELUANT FRACTIONS FROM THE THYROID EXTRACTS ARE SHOWN IN FIGURE 5 AND THOSE OF THE ULTIMOBRANCHIAL EXTRACTS IN FIGURE 6. THE ELUTION VOLUMES OF THE HORMONES ARE SUMMARIZED IN TABLE III. THIS TABLE ALSO SHOWS THE MOLECULAR WEIGHT ESTIMATES FOR EACH HORMONE AS CALCULATED FROM THE CALIBRATION CURVE IN FIGURE 4 WHERE THE POSITIONS OF THE HORMONES ARE INDICATED ON THE G-50 CALIBRATION CURVE. THE ESTIMATED MOLECULAR WEIGHTS OF THE MOLECULES VARIED FROM ABOUT 3600 TO 4600 WITH THE MAJORITY NEARER THE HIGH FIGURE. THE BOVINE EXTRACT, HOWEVER, WAS UNUSUAL IN THAT IT YIELDED TWO ACTIVE REGIONS, ONE WITH A MOLECULAR WEIGHT OF 3700 AND A SECOND WITH A WEIGHT OF 4500.

THE REASON FOR THE PRESENCE OF TWO ACTIVE REGIONS IN THE BOVINE ELUATE IS NOT CLEAR. SINCE THIS WORK WAS COMPLETED BREWER ET AL (17) HAS ISOLATED AND CHARACTERIZED BOVINE THYROID CT AND HIS PUBLISHED WORK TO DATE MAKES NO MENTION OF ANY SIMILAR OBSERVATION. HE DID FIND TWO ACTIVE MOLECULES DURING ION-EXCHANGE CHROMATOGRAPHY, BUT THESE DIFFERED ONLY IN CHARGE DUE TO THE OXIDATION OF METHIONINE, A DIFFERENCE WHICH WOULD PROBABLY BE UNDETECTABLE BY GEL FILTRATION. THIS STILL DOES NOT RULE OUT THE POSSIBILITY THAT ANOTHER BOVINE CT OF SLIGHTLY DIFFERENT STRUCTURE MIGHT EXIST, AND POSSIBLY HAS BEEN OVERLOOKED OR UNREPORTED BY BREWER, JUST AS KEUTMAN ET AL (65) HAVE SHOWN THE PRESENCE OF A MINOR COMPONENT IN SALMON ULTIMOBRANCHIAL EXTRACT WHICH WAS OVERLOOKED IN THE PRESENT STUDY. A SECOND EXPLANATION COULD BE THE FORMATION

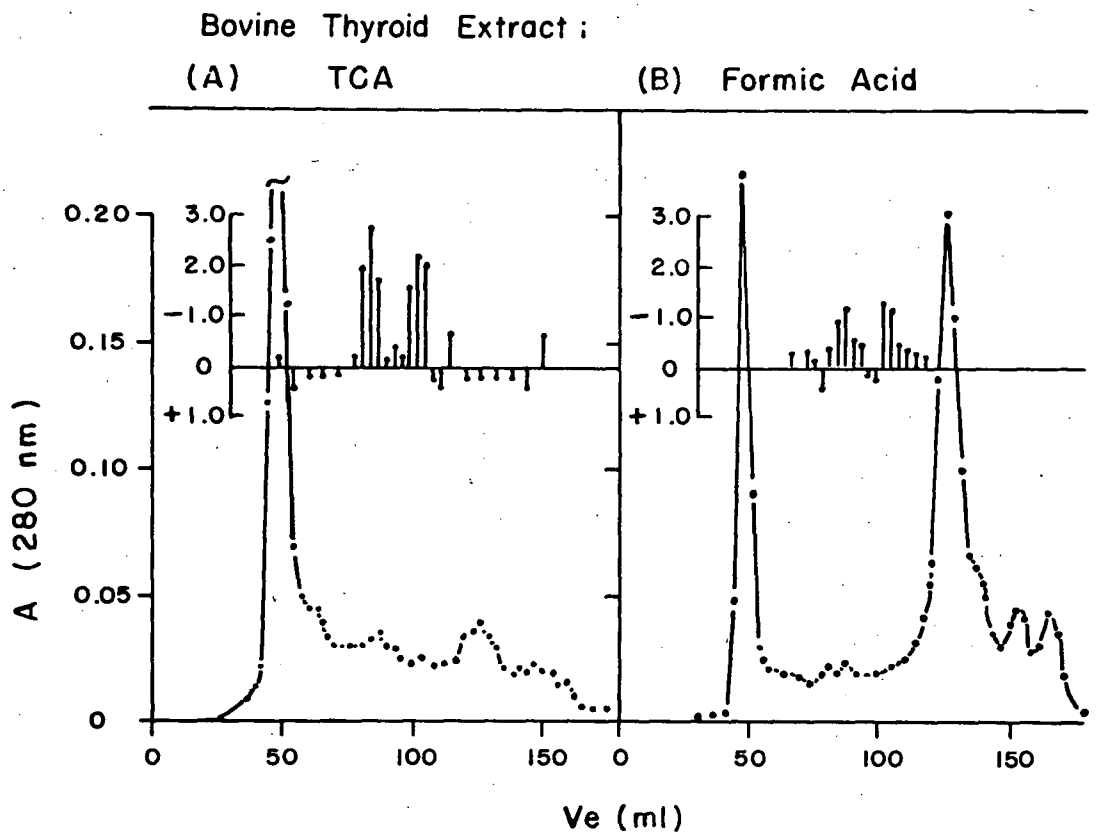


FIGURE 7. COMPARISON OF ELUTION PROFILES ON G-50 OF TWO BOVINE THYROID EXTRACTS PREPARED BY DIFFERENT METHODS. CONDITIONS AS INDICATED IN FIGURE 5. TCA PRECIPITATION AND FORMIC ACID EXTRACTION PROCEDURES ARE DESCRIBED IN THE TEXT.

OF A DIMER SUCH AS THAT REPORTED BY NEHER ET AL (80) IN EXTRACTS OF HUMAN THYROID MEDULLARY CARCINOMA TISSUE. THIS DOES NOT SEEM TOO LIKELY, HOWEVER, SINCE THE ACID CONDITIONS MAINTAINED THROUGHOUT THIS EXTRACTION AND CHROMATOGRAPHY SHOULD HAVE PREVENTED THE DISULFIDE INTERCHANGE NECESSARY FOR THE FORMATION OF SUCH A DIMER. ALSO THE APPARENT MOLECULAR WEIGHT OF THE LARGER ACTIVE SPECIES IN THE BOVINE EXTRACT WAS MUCH SMALLER THAN WOULD HAVE BEEN EXPECTED FOR SUCH A DIMER. THE ONLY OTHER EXPLANATION WHICH SEEMS REASONABLE IS THE EXISTENCE OF AN INTERACTION BETWEEN PART OF THE BOVINE HORMONE AND SOME OTHER SMALL MOLECULE CAUSING THE HORMONE TO BEHAVE AS THOUGH IT WERE LARGER. BECAUSE OF THIS DEVIATION FROM THE PATTERN OF THE OTHER EXTRACTS, IT WAS DECIDED THAT FURTHER EXPERIMENTS SHOULD BE CARRIED OUT TO VERIFY THE PRESENCE OF THE TWO ACTIVE COMPONENTS. A NEW EXTRACT WAS PREPARED FROM BOVINE THYROID POWDER USING THE FORMIC ACID EXTRACTION STEP RATHER THAN THE TCA PRECIPITATION, AND THE EXTRACT WAS CHROMATOGRAPHED ON THE CALIBRATED G-50 COLUMN. THE RESULTANT ELUTION PROFILE AND BIOASSAY DATA AS SHOWN IN FIGURE 7(B) CONFIRMED THE RESULTS SEEN PREVIOUSLY. THIS EXPERIMENT ALSO SERVED AS A CHECK ON THE REPRODUCIBILITY OF THE DATA OBTAINED FROM THE G-50 COLUMN DURING THE PERIOD OF ITS USE, SINCE THE BOVINE TCA EXTRACT WAS THE SECOND UNKNOWN ELUTED AND THE BOVINE FORMIC ACID EXTRACT WAS THE LAST. BY COMPARING FIGURE 7(A) TO 7(B) IT CAN BE SEEN THAT THE ELUTION VOLUMES OF THE ACTIVE COMPONENTS VARY BY ABOUT ± 3 ML (THE VOLUME OF THE FRACTIONS COLLECTED) AFTER ABOUT

6 MONTHS OF COLUMN USAGE. IN THE REGION OF THE UNKNOWNS THIS REPRESENTS A VARIATION IN MOLECULAR WEIGHT OF ABOUT ±150. IT SHOULD BE NOTED THAT THIS VARIATION APPLIES ONLY TO REPRODUCIBILITY AND NOT TO THE ACCURACY OF THE ESTIMATES SINCE MANY OTHER FACTORS INFLUENCE ACCURACY (EG. DIFFERENCES IN MOLECULAR SHAPE AS DISCUSSED FOR INSULIN.)

ONE ADDITIONAL FACT WHICH IS ILLUSTRATED BY THE ELUTION PROFILES OF FIGURES 7(A) AND 7(B) IS THE DIFFERENCE BETWEEN THE TCA PRECIPITATED EXTRACTS AND THOSE PREPARED WITH FORMIC ACID. WITH THE TCA EXTRACT THE PEAK AT THE V_0 IS RELATIVELY LARGE AND THAT AT V_T IS RELATIVELY SMALL. THIS IS TO BE EXPECTED SINCE TCA IS GENERALLY USED TO PRECIPITATE LARGE PROTEINS, AND THE SAME PATTERN CAN BE SEEN IN THE OTHER TCA EXTRACTS. THE REVERSE IS TRUE OF THE FORMIC ACID EXTRACTS, BUT IN BOTH CASES THE ABSORBANCE AT 280 NM IN THE REGION OF THE ACTIVE MOLECULES IS QUITE LOW INDICATING THAT SEPHADEX G-50 CHROMATOGRAPHY SHOULD PROVIDE AN EXCELLENT PURIFICATION OF EITHER TYPE OF EXTRACT. THUS IF SEPHADEX CHROMATOGRAPHY WERE TO BE THE NEXT STAGE OF PURIFICATION THERE WAS LITTLE OR NO ADVANTAGE IN USING THE MORE COMPLEX TCA PRECIPITATION PROCEDURE. THIS WAS PARTICULARLY TRUE OF THE ULTIMOBRANCHIAL MATERIALS WHICH GENERALLY HAD RELATIVELY SMALL PEAKS AT V_T IN ANY CASE.

THE RESULTS OF THIS SERIES OF EXPERIMENTS INDICATED THAT THE HYPOCALCEMIC FACTORS EXTRACTED FROM THE ULTIMOBRANCHIAL GLANDS OF THE FOUR LOWER VERTEBRATES STUDIED WERE IN FACT POLYPEPTIDES SIMILAR IN SIZE TO THE CALCITONINS ISOLATED FROM

MAMMALIAN THYROID, AND THAT CT WAS A WELL EXTABLISHED HORMONE ARISING AT A VERY EARLY STAGE OF VERTEBRATE EVOLUTION. THERE WAS AN APPARENT VARIATION IN MOLECULAR WEIGHTS FROM ABOUT 3600 TO 4600, THOUGH IT BECAME CLEAR WITH FURTHER WORK THAT THE ACTUAL VARIATION WAS PROBABLY MUCH LESS THAN THIS. THE REASONS FOR THIS APPARENT VARIATION WILL BE EXPLORED IN CHAPTER III.

III. ISOLATION AND CHARACTERIZATION OF SALMON CALCITONIN.*

A. Introduction.

The studies described in the preceeding chapter showed the feasibility of large scale purifications of salmon ultimobranchial CT, and work was begun to allow determination of its primary structure. The earlier work had shown the value of Sephadex G-50 as a purification step and further purifications based on the charge characteristics of the molecule were anticipated. Once pure hormone was obtained and its amino acid composition determined, well established procedures for the production and sequencing of overlapping peptide fragments such as those used earlier in sequence studies on porcine CT (96, 10, 83) and human CT (82) could be used to elucidate the primary structure of salmon CT. Knowing this structure and the biological activities of the hormone it was reasonable to assume that certain characteristics and properties of the calcitonins and of the species producing them would be clarified.

B. Preliminary Experiments.

1. Extractions.

The extract for the pilot purification was prepared by the solvent extraction procedure described in Chapter II. The starting material was 590 g of septal tissue from chum, sockeye and coho salmon which had been frozen immediately

*Part of the work described in this chapter was previously published (87).

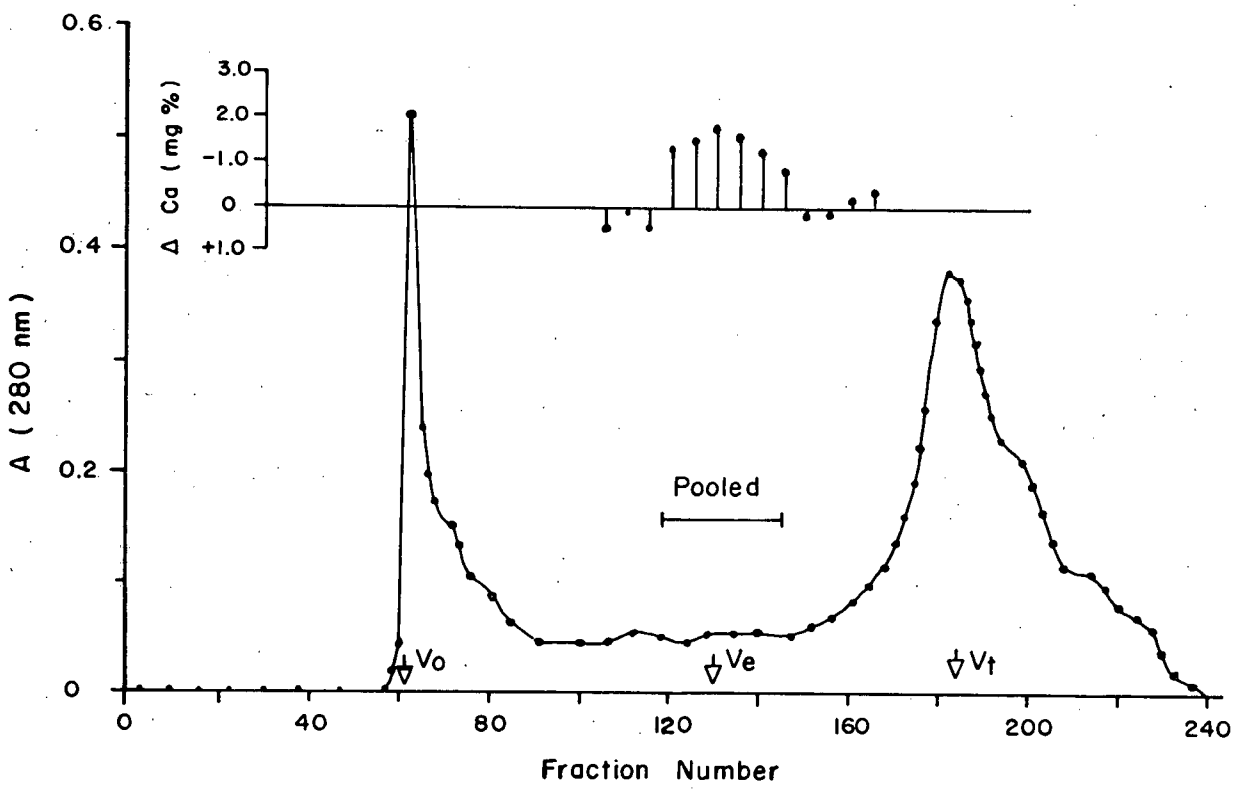


FIGURE 8. ELUTION PROFILE OF SALMON ULTIMOBRANCHIAL SOLVENT EXTRACT ON SEPHADEX G-50: PRELIMINARY STAGE 1. COLUMN, 5 x 150 CM; ELUANT, 0.1 M FORMIC ACID; TEMPERATURE, 4° C; FLOW RATE, 12 ML/HR; FRACTION SIZE, 15.0 ML.

AFTER COLLECTION. ACETONE DRYING REDUCED THE WEIGHT OF THE TISSUE TO 85 G. SOLVENT EXTRACTION OF THE DRY POWDER YIELDED 2.4 G OF EXTRACT WITH A SPECIFIC ACTIVITY OF 3 MRC U/MG.

2. STAGE 1 CHROMATOGRAPHY.

THE FIRST GEL FILTRATION STAGE WAS CARRIED OUT ON A 5 X 130 CM COLUMN OF SEPHADEX G-50 AT 4^0 C USING A 0.1 M FORMIC ACID ELUANT, AND COLLECTING 15 ML FRACTIONS AT A RATE OF 12 ML/HR. THE ABSORBANCE AT 280 NM WAS DETERMINED FOR THE ELUATE FRACTIONS AS AN INDEX OF PROTEIN CONCENTRATION AND THEY WERE SURVEYED FOR BIOLOGICAL ACTIVITY AS DESCRIBED IN CHAPTER II, USING 0.3 ML INJECTIONS OF SUITABLY DILUTED ELUATE. THE 2.4 G OF EXTRACT, WAS DISSOLVED IN 20 ML OF 0.1 M FORMIC ACID AND APPLIED TO THE COLUMN IN TWO 10 ML VOLUMES. THE V_E OF THE CT WAS TAKEN AS THE CENTER OF THE BIOLOGICALLY ACTIVE REGION AND USED TO CALCULATE A VALUE FOR K_D. AN INDEX OF MOLECULAR WEIGHT WHICH IS INDEPENDENT OF COLUMN SIZE AND IS EQUAL TO $(V_E-V_0)/(V_T-V_0)$ WHERE THE VOLUME TERMS HAVE THE DEFINITIONS USED IN CHAPTER II.

THE ELUTION PROFILE AND ACTIVITY SURVEY FOR THE SAMPLES WERE SIMILAR TO THOSE SEEN IN CHAPTER II AS SHOWN IN FIGURE 8. THE ACTIVE REGIONS OF THE ELUATES, FRACTIONS 118 TO 145, WERE POOLED AND LYOPHILIZED TO YIELD 130 MG OF SLIGHTLY OFF-WHITE POWDER WITH A SPECIFIC ACTIVITY OF 40 MRC U/MG. DETAILS OF RECOVERY AND DEGREE OF PURIFICATION ARE INCLUDED IN THE SUMMARY. THE K_D OF THE ACTIVE REGION WAS APPROXIMATELY 0.6.

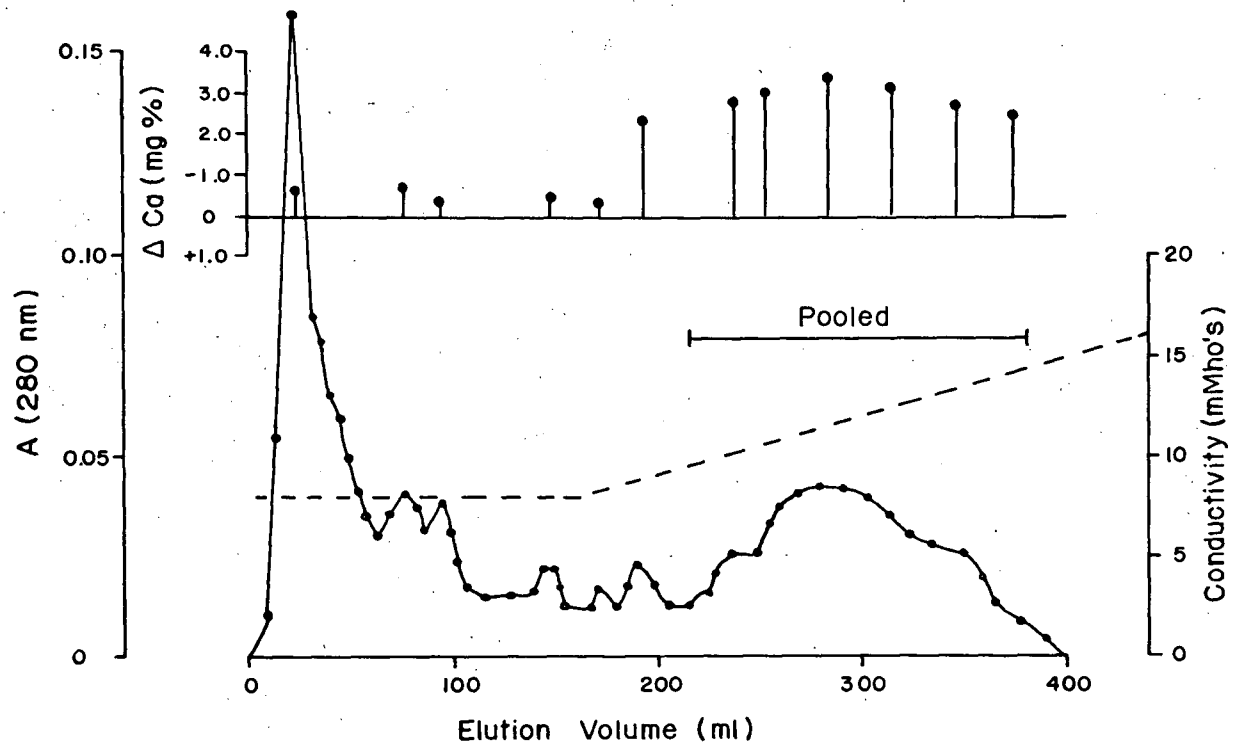


FIGURE 9. ELUTION PROFILE OF STAGE 1 PRODUCT ON SE-SEPHADEX C-25: PRELIMINARY STAGE 2. COLUMN, 1.2 x 20 CM; ELUANT, LINEAR GRADIENT OF pH 3.4 AMMONIUM FORMATE FROM 0.2 M TO 0.5 M; TEMPERATURE, 4^{0} C; FLOW RATE, 10 ML/HR.

3. Stage 2 Chromatography.

A 1.2 x 20 cm column of SE-Sephadex C-25 was prepared and equilibrated at 4°C with 0.2 M ammonium formate buffer at pH 3.4. 95 mg of stage 1 powder was dissolved in the starting buffer and applied to the column. After washing with 50 ml of starting buffer, the material remaining on the column was eluted with a linear gradient of ammonium formate buffer from 0.2 M (pH 3.4; conductivity, 8.0 mMhos; volume, 150 ml) to 0.5 M (pH 3.4; conductivity, 18.0 mMhos; volume, 150 ml). The eluate was collected at a rate of 10 ml/hr and absorbance at 280 nm and biological activity followed as before. The conductivity of each fraction was also determined using a Radiometer model CDM 2d conductivity meter.

The elution profile, conductivity and biological activity of the eluate fractions are shown in Figure 9. The biological activity came off the column in a broad peak centered on a conductivity of about 12 mMhos. The entire active region from fraction 15 to 28 was pooled and lyophilized. As the ammonium formate buffer was not sufficiently volatile to allow complete desalting by lyophilization, specific activity was not determined at this stage.

4. Stage 3 chromatography.

Bio-Gel P-10, a polyacrilamide gel with properties similar to Sephadex G-50, was used to desalt and further purify the stage 2 product. A 1.2 x 200 cm column was

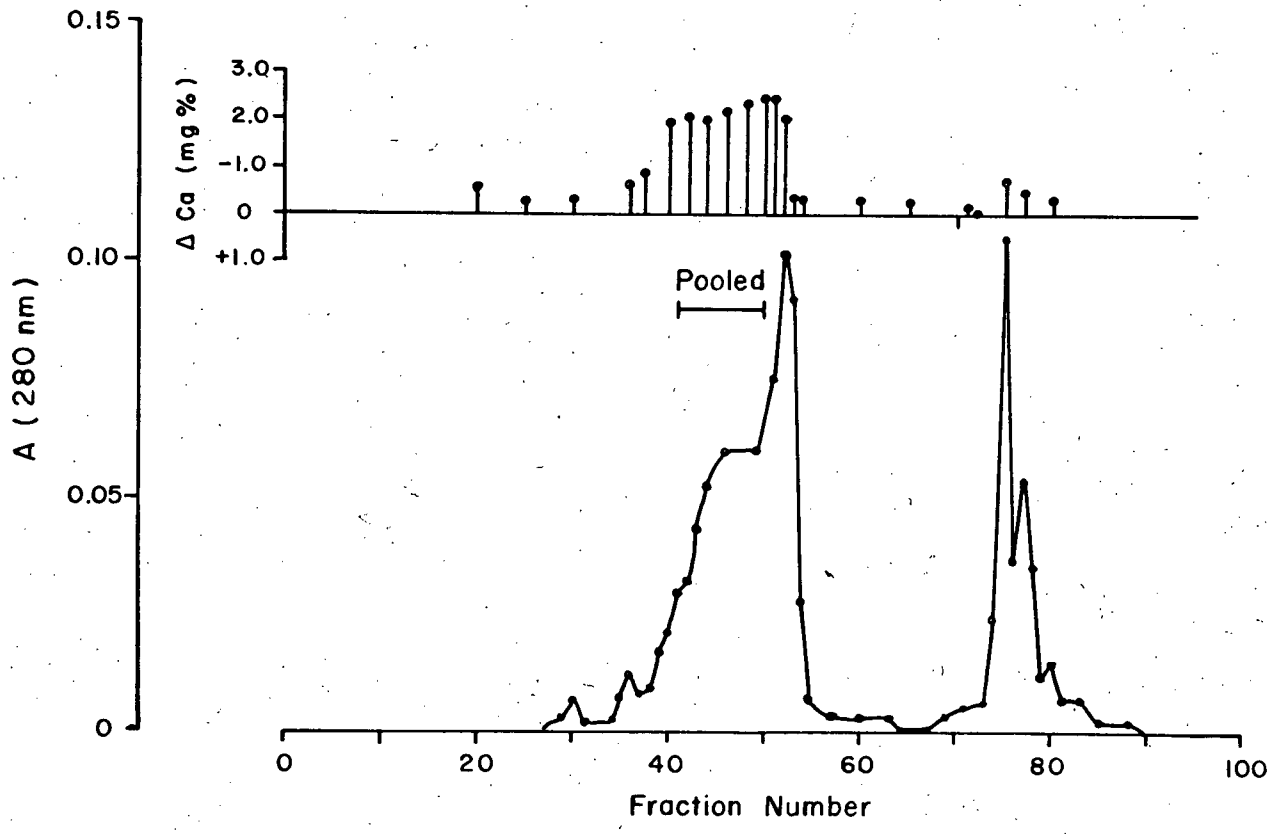


FIGURE 10. ELUTION PROFILE OF STAGE 2 PRODUCT ON BIO-GEL P-10: PRELIMINARY STAGE 3. COLUMN, 1.2 x 200 CM; ELUANT, 0.4 M ACETIC ACID; TEMPERATURE, 4° C; FLOW RATE, 6 ML/MR; FRACTION SIZE, 3.4 ML.

PREPARED IN 0.4 M ACETIC ACID TO PROVIDE MAXIMUM RESOLUTION AND THE POOLED ACTIVITY FROM STAGE 2 WAS APPLIED TO THE COLUMN IN 1 ML OF ELUANT MADE 4 M IN UREA. THE ELUATE WAS COLLECTED IN 3.4 ML FRACTIONS AT A RATE OF 6 ML/HR.

AS SEEN IN FIGURE 10 THE BIOLOGICAL ACTIVITY WAS ASSOCIATED WITH THE TWO LARGE, UNRESOLVED PEAKS BUT A DETAILED BIOASSAY OF THE REGION SHOWED THAT ONLY THE BROAD SHOULDER ON THE LEADING SIDE WAS ACTIVE. FRACTION NUMBER 46 AT THE CENTER OF THE SHOULDER WAS LYOPHILIZED AND AN ALIQUOT OXIDIZED WITH PERFORMIC ACID AS DESCRIBED IN SECTION E. SAMPLES OF BOTH OXIDIZED AND UNOXIDIZED MATERIAL WERE HYDROLYSED AND AMINO ACID ANALYSES PERFORMED. THE MOLE RATIOS OF SEVERAL AMINO ACIDS WERE FAR BELOW UNITY INDICATING THAT THE PEAK COULD NOT CONTAIN A SINGLE MOLECULE OF THE EXPECTED MOLECULAR WEIGHT. THE QUANTITIES OF CYSTINE AND CYSTEIC ACID WERE QUITE LOW AND SINCE TWO RESIDUES OF $\frac{1}{2}$-CYSTINE WERE EXPECTED IN THE NATIVE MOLECULE IT WAS CLEAR THAT THE HORMONE WAS ONLY A MINOR COMPONENT OF THE PEAK. FRACTION 46 CONTAINED APPROXIMATELY 1.5 MG OF LYOPHILIZED MATERIAL (0.45 MG/ML) REPRESENTING 0.33 µMOLES OF PURE MATERIAL WITH A MOLECULAR WEIGHT OF 4500. AS THE FRACTION CONTAINED ONLY 0.1 µMOLES OF $\frac{1}{2}$-CYSTINE, EQUIVALENT TO 0.05 µMOLES OF HORMONE, IT WAS ESTIMATED THAT THE PEAK CONTAINED ABOUT 15% HORMONE. THE SPECIFIC ACTIVITY OF THE MATERIAL WAS ASSAYED AS ABOUT 700 MRC U/MG SUGGESTING THAT THE PURE HORMONE MIGHT HAVE A SPECIFIC ACTIVITY OF NEARLY 5000 MRC U/MG, AND INDICATING THAT MUCH LARGER QUANTITIES OF TISSUE WOULD BE REQUIRED TO YIELD

FIGURE 11

SUMMARY OF PRELIMINARY PURIFICATION OF SALMON CT

PROCEDURE	WEIGHT	SPECIFIC ACTIVITY (MRC U/MG)	APPROX. ACTIVITY (MRC U)
SEPTAL TISSUE	590 G		
↓ DRY AND DEFAT WITH ACETONE			
ACETONE POWDER	85 G		
↓ EXTRACT WITH BuOH:HAc:H_2O			
FILTRATE / RESIDUE			
↓ PRECIPITATE WITH 5 VOL. OF ACETONE AT -20^0C			
RESIDUE (DISCARDED) / PRECIPITATE			
↓ EXTRACT WITH 0.1M FORMIC ACID			
SUPERNATE / RESIDUE (DISCARDED)			
↓ LYOPHILIZED			
FINAL EXTRACT	2400 MG	3	7000
↓ MOLECULAR SIEVING ON SEPHADEX G-50			
STAGE 1 ACTIVITY	130 MG	40	5200
↓ 95 MG, ION EXCHANGE ON SE-SEPHADEX, PH 3.4			
STAGE 2 ACTIVITY			
↓ MOLECULAR SIEVING ON BIO-GEL P-10			
STAGE 3 ACTIVITY	8.3 MG	400	3700

SUFFICIENT HORMONE FOR ANALYSIS.

THE REMAINDER OF THE ACTIVE REGION, FRACTIONS 41-50, WAS POOLED AND LYOPHILIZED YIELDING 3.8 MG OF POWDER. THE TOTAL YIELD OF ACTIVITY AT THIS STAGE WAS ABOUT 3700 MRC U FROM AN INITIAL SAMPLE CONTAINING 3800 MRC U. THIS MATERIAL WAS USED IN A SERIES OF TEST COLUMNS TO DETERMINE THE BEST CONDITIONS FOR FURTHER PURIFICATION. EVENTUALLY CHROMATOGRAPHY ON AN SE-SEPHADEX COLUMN EQUILIBRATED AT A HIGHER pH PROVED MOST EFFECTIVE.

5. SUMMARY OF PURIFICATION.

FIGURE 11 SUMMARIZES THE MAJOR STAGES USED IN THE PRELIMINARY EXPERIMENTS. BIOLOGICAL ACTIVITY WAS NOT ASSAYED DURING THE INITIAL EXTRACTION, BUT AT THE FINAL STAGE THE EXTRACT HAD AN ACTIVITY OF ABOUT 3 U/MG FOR A TOTAL OF ABOUT 7000 U. AFTER SEPHADEX CHROMATOGRAPHY ABOUT 75% OF THE ACTIVITY WAS RECOVERED. 3800 U WERE APPLIED TO THE ION EXCHANGE COLUMN AND 3700 U RECOVERED AFTER THE FINAL GEL FILTRATION STAGE FOR A RECOVERY OF 97%. THE OVERALL RECOVERY FROM THE EXTRACT TO THE 15% PURE PRODUCT OF STAGE 3 WAS THEREFORE ABOUT 73%. THE MATERIAL AT THIS STAGE REPRESENTED A 50,000 FOLD PURIFICATION OVER THE FRESH GLANDS AND REQUIRED A FURTHER SEVEN-FOLD PURIFICATION.

C. <u>PREPARATION OF PURE SALMON CALCITONIN</u>.

1. EXTRACTION.

THE PRELIMINARY EXPERIMENTS MADE IT CLEAR THAT MUCH LARGER QUANTITIES OF GLANDS WOULD HAVE TO BE PROCESSED AND

AS COLLECTION TECHNIQUES IMPROVED TO THE POINT WHERE OVER 200 POUNDS OF SEPTAL TISSUE WERE COLLECTED FROM 500 TONS OF SALMON IN A SEASON, LABORATORY SCALE DRYING AND EXTRACTION WERE NO LONGER FEASIBLE. QUANTITIES OF FROZEN SEPTAL TISSUE WERE SHIPPED TO CANADA PACKERS LTD., TORONTO FOR SOLVENT EXTRACTION AND TO ARMOUR PHARMACEUTICAL CO., KANKAKEE, ILL. FOR EXTRACTION BY A METHOD THEY HAD DEVELOPED FOR PORCINE CT (15).

THE STARTING MATERIAL WHICH EVENTUALLY YIELDED PURE SALMON CALCITONIN IN SIGNIFICANT QUANTITY WAS AN EXTRACT PREPARED BY ARMOUR. THIS MATERIAL HAS BEEN REPORTED TO HAVE A SPECIFIC ACTIVITY OF 230 MRC U/MG (65); HOWEVER, WHEN ASSAYED AS DESCRIBED IN CHAPTER II A SOMEWHAT LOWER SPECIFIC ACTIVITY (150-200 MRC U/MG) WAS OBTAINED. 50 KG OF SEPTAL TISSUE YIELDED 4 G OF THIS MATERIAL--A TOTAL OF BETWEEN 600,000 AND 900,000 MRC UNITS.

2. STAGE 1 CHROMATOGRAPHY.

THE PROCEDURE DESCRIBED IN THE PRELIMINARY EXPERIMENT WAS SCALED UP TO HANDLE THE ENTIRE 4 G SAMPLE ON A SINGLE COLUMN. THE STARTING MATERIAL WAS DISSOLVED IN 40 ML OF 2 M UREA IN 0.1 M FORMIC ACID AND APPLIED TO A 10 X 150 CM COLUMN OF SEPHADEX G-50. THE CONSTRUCTION OF THIS COLUMN IS DESCRIBED IN APPENDIX B. THE SAMPLE WAS ELUTED WITH 0.1 M FORMIC ACID AT $4^{\circ}C$ AND COLLECTED AT A RATE OF 250 ML/HR. ABSORBANCE AT 280 NM AND BIOLOGICAL ACTIVITY OF THE SAMPLES WERE FOLLOWED AS DESCRIBED PREVIOUSLY WITH SAMPLES SUITABLY DILUTED PRIOR TO INJECTION. SPECIFIC

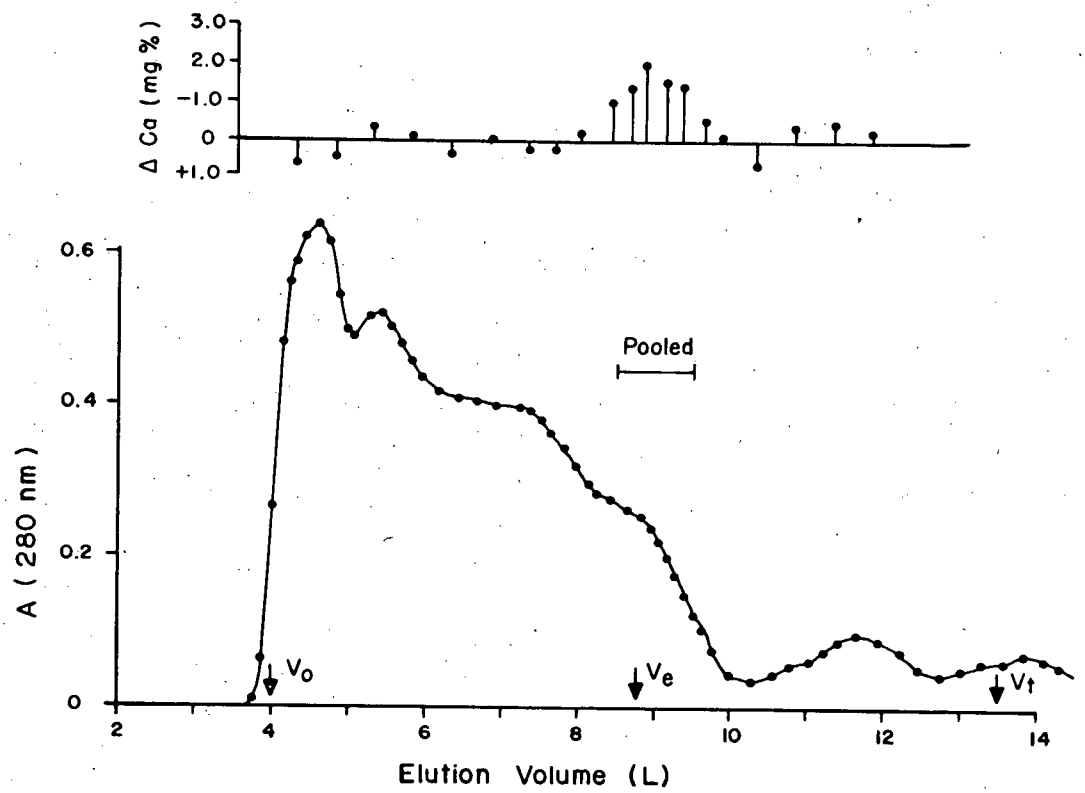


FIGURE 12. ELUTION PROFILE OF SALMON ULTIMOBRANCHIAL ACID EXTRACT ON SEPHADEX G-50: STAGE 1. COLUMN, 10 x 150 CM; ELUANT, 0.1 M FORMIC ACID; TEMPERATURE, 4^{0} C; FLOW RATE, 250 ML/HR.

ACTIVITY WAS DETERMINED IN THE BIOASSAY DESCRIBED IN CHAPTER II ON AN ALIQUOT OF THE POOLED SAMPLE WHICH WAS LATER LYOPHILIZED AND WEIGHED.

THE ELUTION PROFILE AND ACTIVITY SURVEY OF THE COLUMN IS SHOWN IN FIGURE 12. THE PROFILE DIFFERED MARKEDLY FROM THAT SEEN FOR THE SOLVENT EXTRACTS USED PREVIOUSLY, BUT VARIED ONLY SLIGHTLY FROM THAT SEEN BY KEUTMANN ET AL (65) IN A SIMILAR EXPERIMENT. THE K_D VALUE OF THE BIOLOGICAL ACTIVITY (0.6), HOWEVER, WAS NEARLY IDENTICAL TO THAT SEEN IN OTHER EXPERIMENTS ON THE SALMON HORMONE.

THE ACTIVE REGION EXTENDED FROM AN ELUTION VOLUME OF 8.3 L TO 9.6 L, BUT TO AVOID CONTAMINATION FROM THE LARGE LEADING PEAK ONLY THE REGION FROM 8.5 TO 9.5 L WAS POOLED. AFTER LYOPHILIZATION THIS POOL YIELDED 350 MG OF MATERIAL WITH A SPECIFIC ACTIVITY OF ABOUT 650 MRC U/MG (SEE FIGURE 18). THE LOW RECOVERY ON THIS COLUMN (30% TO 40%) WAS DUE PRIMARILY TO THE NARROW REGION POOLED. DETAILS ON RECOVERY AND DEGREE OF PURIFICATION ARE GIVEN IN THE SUMMARY.

3. STAGE 2 CHROMATOGRAPHY.

THE COLUMN USED IN THIS STAGE WAS SIMILAR TO THAT DESCRIBED IN THE PRELIMINARY EXPERIMENT THOUGH SLIGHTLY LARGER TO HANDLE THE GREATER QUANTITY OF MATERIAL. A 2.0 x 20 CM COLUMN OF SE-SEPHADEX C-25 WAS EQUILIBRATED AT 4° C WITH 0.2 M AMMONIUM FORMATE AT PH 3.4. THE 350 MG OF MATERIAL FROM STAGE 1 WAS APPLIED TO THE COLUMN IN 10 ML OF STARTING BUFFER AND ELUTED WITH 400 ML OF STARTING BUFFER

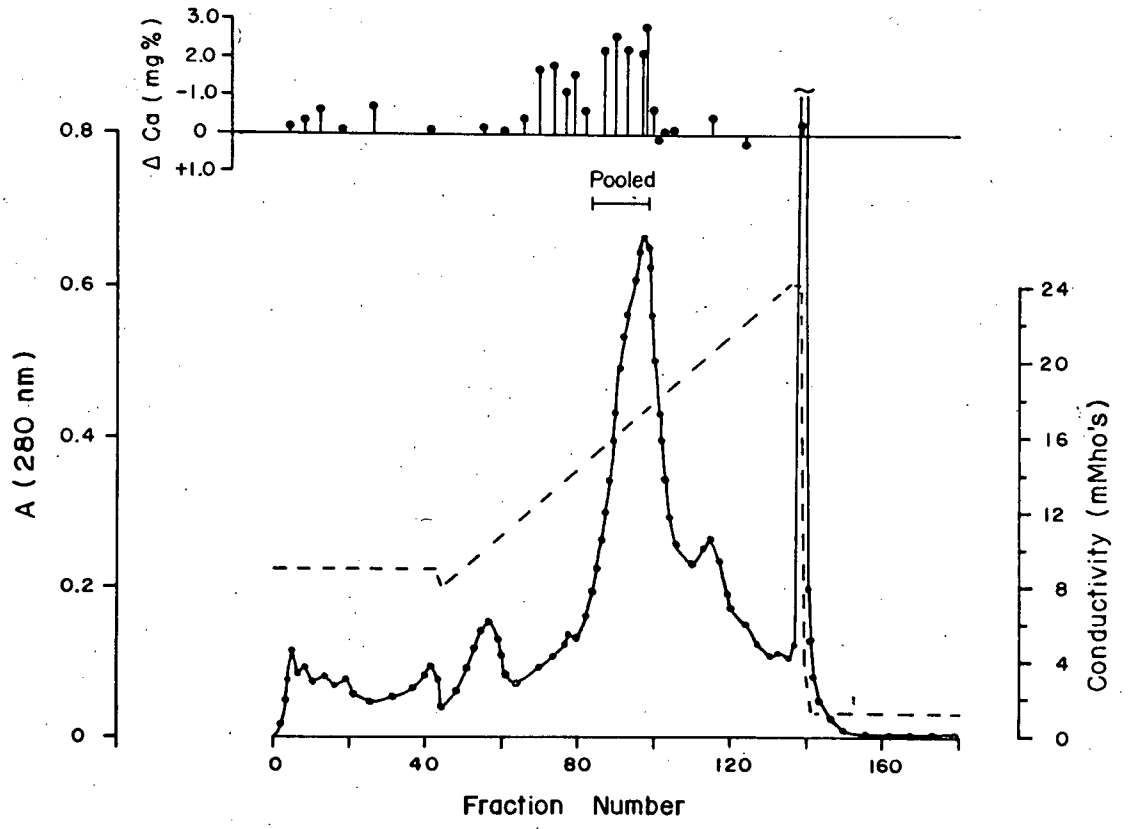


FIGURE 13. ELUTION PROFILE OF STAGE 1 PRODUCT ON SE-SEPHADEX C-25: STAGE 2. COLUMN, 2 X 20 CM; ELUANT, LINEAR GRADIENT OF pH 3.4 AMMONIUM FORMATE FROM 0.2 M TO 0.6 M, FOLLOWED BY 1 M AMMONIUM HYDROXIDE; TEMPERATURE 4° C; FLOW RATE, 20 ML/HR; FRACTION SIZE, 12 ML.

FOLLOWED BY A LINEAR GRADIENT OF pH 3.4 AMMONIUM FORMATE BUFFER FROM 0.2 M (CONDUCTIVITY, 8.0 mMHOS; VOLUME, 500 ML) TO 0.6 M (CONDUCTIVITY, 24.0 mMHOS; VOLUME, 500 ML). AFTER THE GRADIENT WAS COMPLETED THE COLUMN WAS WASHED WITH 500 ML OF 1 M AMMONIUM HYDROXIDE TO ENSURE RECOVERY OF ALL APPLIED MATERIALS. THE ELUATE WAS COLLECTED IN 12 ML FRACTIONS AT A RATE OF 20 ML/HR. ABSORBANCE AT 280 NM, CONDUCTIVITY AND BIOLOGICAL ACTIVITY WERE FOLLOWED AS PREVIOUSLY DESCRIBED.

FIGURE 13 SHOWS THE RESULTS OF THE STAGE 2 ELUTION. ONLY ONE MAJOR PEAK WAS ELUTED DURING THE GRADIENT ALTHOUGH THIS PEAK APPEARED TO CONTAIN AT LEAST TWO COMPONENTS. THE CONDUCTIVITY AT THE PEAK WAS SLIGHTLY HIGHER (16 mMHOS VS. 12 mMHOS) THAN SEEN PREVIOUSLY, BUT THE SHOULDER OF THE PEAK CORRESPONDED WELL TO THE MAJOR REGION OF BIOLOGICAL ACTIVITY. A SECOND MINOR REGION OF BIOLOGICAL ACTIVITY ELUTED PRIOR TO THE PEAK WAS NOT INVESTIGATED FURTHER. IN RETROSPECT IT IS LIKELY THAT THIS REGION MAY HAVE REPRESENTED THE MINOR COMPONENT DESCRIBED BY KEUTMANN ET AL (65), AND MAY INDICATE THAT THE DIFFERENT SPECIES OF SALMON FROM WHICH THE GLANDS WERE COLLECTED DO, IN FACT, HAVE CALCITONINS OF SLIGHTLY DIFFERENT STRUCTURES. TO MINIMIZE CONTAMINATION A NARROW REGION OF MAXIMUM ACTIVITY WAS POOLED (FRACTIONS 84 TO 98) AND LYOPHILIZED. SPECIFIC ACTIVITY COULD NOT BE DETERMINED BECAUSE OF RESIDUAL SALT IN THE LYOPHILIZED PRODUCT, BUT THE DEGREE OF PURIFICATION DID NOT APPEAR TO BE GREAT SINCE ONLY MINOR CONTAMINANT PEAKS WERE REMOVED.

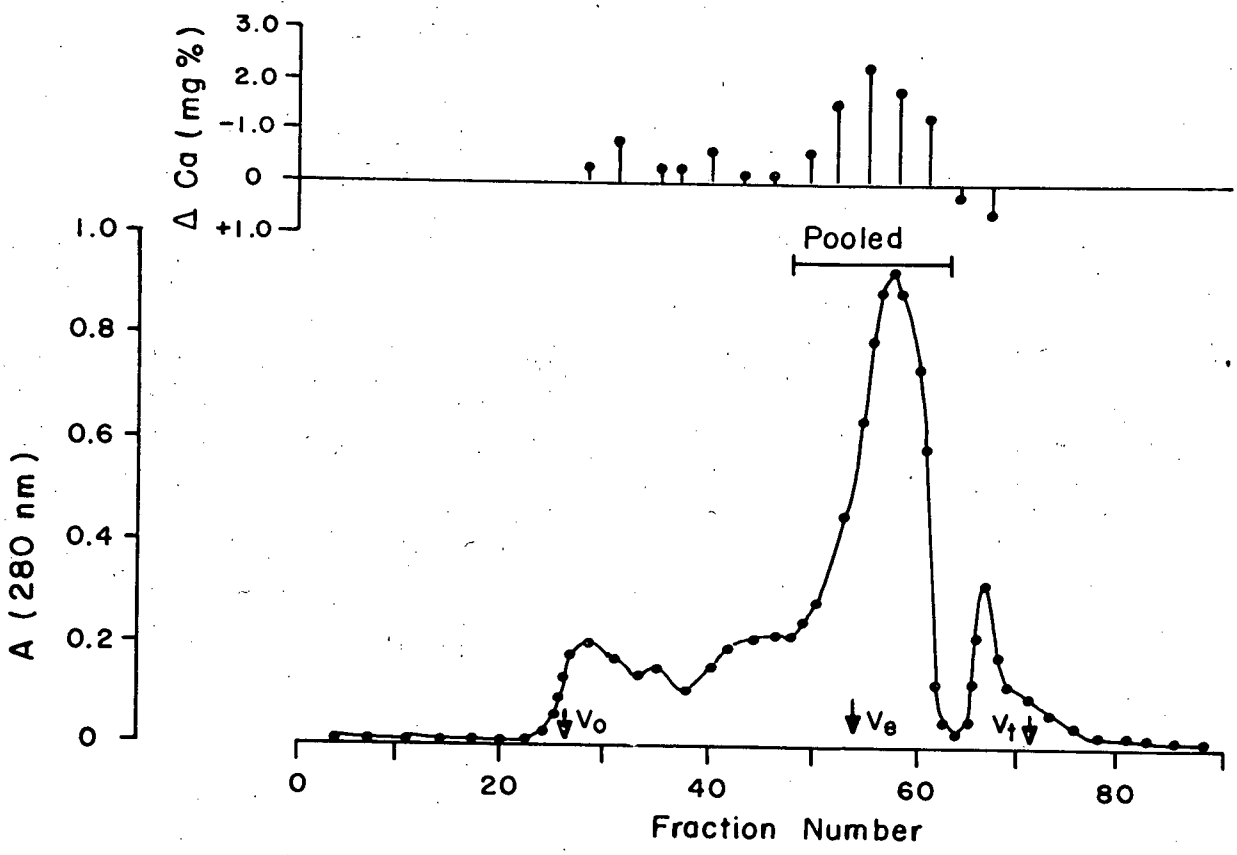


FIGURE 14. ELUTION PROFILE OF STAGE 2 PRODUCT ON SEPHADEX G-50: STAGE 3. COLUMN, 1.2 x 220 CM; ELUANT 0.1 M FORMIC ACID; TEMPERATURE, 4° C; FLOW RATE, 6 ML/HR; FRACTION SIZE, 3.4 ML.

4. Stage 3 chromatography.

This stage was essentially a de-salting step, but a long, high resolution column was used to remove contaminants left by the first large gel filtration column. A 1.2 x 220 cm column of Sephadex G-50 was prepared at 4° C in 0.1 M formic acid. The stage 2 product was dissolved in 7 ml of 8 M urea in 0.1 M formic acid and layered onto the top of the column. The eluate was collected in 3.4 ml fractions at a rate of 6 ml/hr.

As shown in Figure 14, the biological activity was confined to a single major peak with a K_D of 0.6. This peak was slightly skewed, but later results indicated that this asymmetry was a characteristic of the column used. Fractions 48 to 63 were pooled and after lyophilization the pool yielded 55 mg of material with a specific activity of about 1200 MRC U/mg (see Figure 18) for a total recovery of 65,000 MRC U.

5. Stage 4 Chromatography.

For the second ion exchange stage a 1.2 x 20 cm column of SE-Sephadex C-25 equilibrated at 4° C with 0.1 M ammonium acetate at pH 4.5 was prepared. The 55 mg sample of stage 3 material was dissolved in 3 ml of starting buffer and allowed to flow onto the column. After the column had been washed with 400 ml of 0.1 M ammonium acetate, a linear gradient of pH 4.5 ammonium formate buffer from 0.1 M (conductivity, 3.5 mMhos; volume, 350 ml) to 0.5 M (conductivity, 16.0 mMhos;

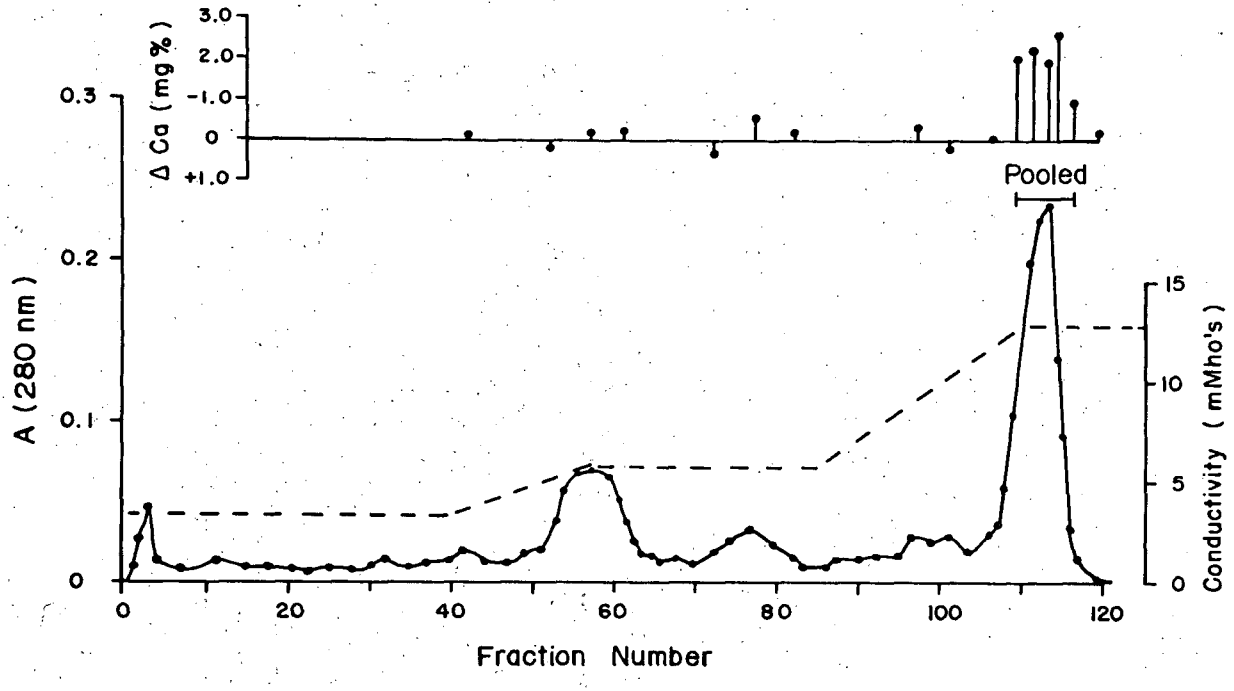


FIGURE 15. ELUTION PROFILE OF STAGE 3 PRODUCT ON SE-SEPHADEX C-25: STAGE 4. COLUMN, 1.2 X 20 CM; ELUANT, LINEAR GRADIENTS OF pH 4.5 AMMONIUM ACETATE FROM 0.1 M TO 0.2 M AND FROM 0.2 M TO 0.5 M; TEMPERATURE 4° C; FLOW RATE, 12 ML/HR; FRACTION SIZE, 9 ML.

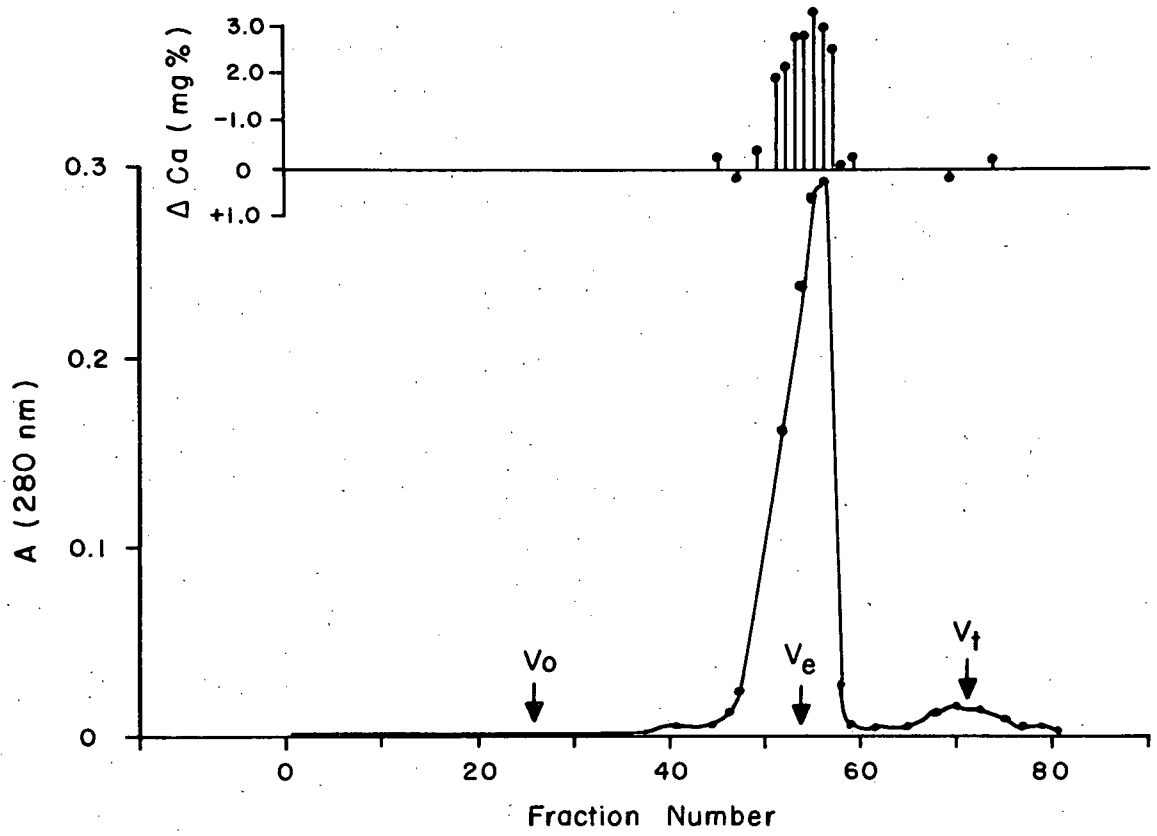


FIGURE 16; ELUTION PROFILE OF STAGE 4 PRODUCT ON SEPHADEX G-50: STAGE 5. CONDITIONS IDENTICAL TO FIGURE 14.

VOLUME, 350 ML) WAS BEGUN. WHEN THE FIRST PEAK BEGAN TO APPEAR IN THE ELUATE THE 0.5 M BUFFER WAS DISCONNECTED AND ELUTION CONTINUED WITH 250 ML OF BUFFER AT THE ESTABLISHED CONCENTRATION. WHEN THE ABSORBANCE HAD RETURNED TO ZERO A NEW GRADIENT WAS BEGUN WITH pH 4.5 AMMONIUM ACETATE BUFFER FROM 0.2 M (CONDUCTIVITY, 5.6 MMHOS; VOLUME, 300 ML) TO 0.5 M (CONDUCTIVITY, 16.0 MMHOS; VOLUME, 300 ML). THIS GRADIENT WAS USED UNTIL A NEW PEAK BEGAN, AT WHICH TIME THE PROCEDURE DESCRIBED ABOVE WAS REPEATED. THE ELUATE WAS COLLECTED IN 9 ML FRACTIONS AT A RATE OF 12 ML/HR.

FIGURE 15 SHOWS THE ELUTION PROFILE, CONDUCTIVITY GRADIENT AND BIOLOGICAL ACTIVITY SURVEY. THREE WELL DEFINED PEAKS WERE ELUTED FROM THE COLUMN, ONLY ONE OF WHICH WAS ASSOCIATED WITH ANY BIOLOGICAL ACTIVITY. THIS PEAK WAS SYMETRICAL AND SHOWED NO INDICATION OF CONTAMINATION. FRACTIONS 109 TO 121 WERE POOLED AND LYOPHILIZED.

6. STAGE 5 CHROMATOGRAPHY.

THE PROCEDURES AND EQUIPMENT USED IN THE STAGE 5 CHROMATOGRAPHY WERE IDENTICAL IN ALL RESPECTS TO THOSE DESCRIBED FOR STAGE 3. THE SAMPLE USED WAS THE LYOPHILIZED POOL FROM STAGE 4 DISSOLVED IN 1 ML OF 0.1 M FORMIC ACID. BIOLOGICAL ACTIVITY WAS MEASURED IN ASSAYS ON AN ALIQUOT FROM FRACTION 56 AS PREVIOUSLY DESCRIBED, AND SPECIFIC ACTIVITY CALCULATED FROM THE WEIGHT OF A LYOPHILIZED ALIQUOT OF THIS FRACTION.

THE ELUTION PROFILE SHOWN IN FIGURE 16 FOR THE FINAL PURIFICATION STAGE CONTAINED A SINGLE MAJOR PEAK AND A SMALL

FIGURE 17

SUMMARY OF PURIFICATION OF SALMON CT

PROCEDURE	WEIGHT	SPECIFIC ACTIVITY (MRC U/MG)	APPROX. ACTIVITY (MRC U)
SEPTAL TISSUE	50 KG	-	-
↓ ACID EXTRACTION BY ARMOUR CO.			
FINAL EXTRACT	4 G	150-230	750,000
↓ MOLECULAR SIEVING ON SEPHADEX G-50			
STAGE 1 ACTIVITY	350 MG	650	230,000
↓ ION EXCHANGE ON SE-SEPHADEX, PH 3.4			
STAGE 2 ACTIVITY	-	-	-
↓ MOLECULAR SIEVING ON SEPHADEX G-50			
STAGE 3 ACTIVITY	55 MG	1200	65,000
↓ ION EXCHANGE ON SE-SEPHADEX, PH 4.5			
STAGE 4 ACTIVITY	-	-	-
↓ MOLECULAR SIEVING ON SEPHADEX G-50			
STAGE 5 ACTIVITY	15 MG	4500	65,000

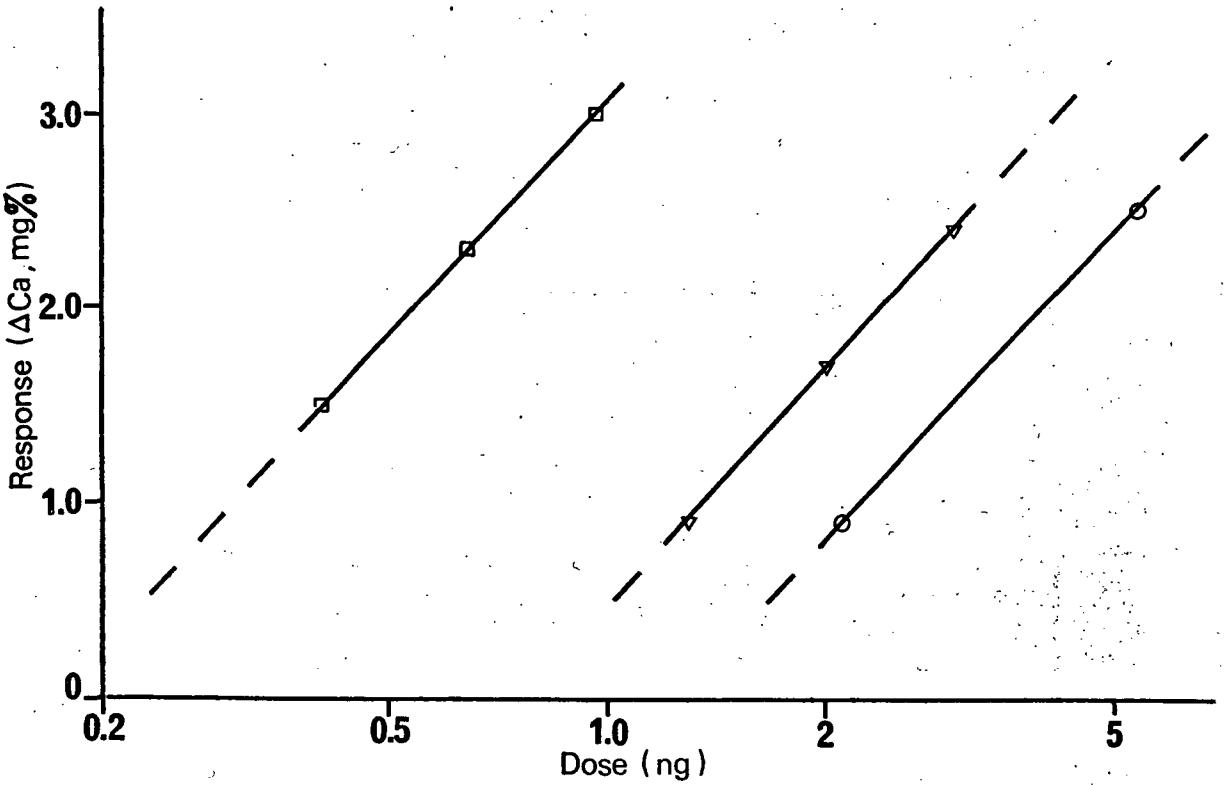


FIGURE 18. REGRESSIONS OF LOG DOSE ON RESPONSE FOR STAGE 1, 3 AND 5 PRODUCTS. CALCULATIONS AS DESCRIBED FOR FIGURE 3. ○ – STAGE 1, ▽ – STAGE 3, □ – STAGE 5.

PEAK AT V_T INDICATING THAT THE STAGE 5 MATERIAL WAS ESSENTIALLY FREE OF PROTEIN CONTAMINANTS. THE SLIGHT ASYMMETRY WAS A CHARACTERISTIC OF THE COLUMN USED, AS THE TESTS FOR HOMOGENEITY DESCRIBED IN SECTION G SHOWED THAT ONLY A SINGLE PEPTIDE WAS PRESENT IN THE PEAK. THE BIOLOGICAL ACTIVITY CORRESPONDED WELL TO THE PEAK AND VARIED WITH THE CONCENTRATION IN EACH FRACTION. THE RESULTS OF THE BIOASSAY ON THE MATERIAL IN FRACTION 56 ARE SHOWN IN FIGURE 18. ON THE BASIS OF THIS ASSAY AND THE PEPTIDE CONCENTRATION IN THE FRACTION, 0.69 MG/ML, THE ABSOLUTE SPECIFIC ACTIVITY OF PURE SALMON CT WAS CALCULATED TO BE CLOSE TO 4500 MRC U/MG--NEARLY 25 TIMES THE SPECIFIC ACTIVITY OF PURE PORCINE CT. THE YIELD AT THIS STAGE WAS 14.5 MG OF PURE HORMONE, A TOTAL OF 65,000 MRC U REPRESENTING ESSENTIALLY 100% OF THE MATERIAL APPLIED IN STAGE 4.

7. SUMMARY OF PURIFICATION SCHEDULE.

FIGURE 17 SUMMARIZES THE STAGES USED IN THE PREPARATION OF PURE SALMON CT. THE RESULTS OF BIOASSAYS AT STAGES 1,3 AND 5 ARE SHOWN IN FIGURE 18. THE RECOVERY AT EACH STAGE IS ALSO RECORDED IN FIGURE 17. ALTHOUGH HIGH LOSSES OCCURED DURING THE FIRST THREE STAGES, PROBABLY DUE IN PART TO AN ACTIVE MINOR COMPONENT WHICH WAS REMOVED, THE RECOVERIES IN THE TWO FINAL STAGES WERE ESSENTIALLY 100%. THE OVERALL RECOVERY WAS ABOUT 10%, BUT A 300,000 FOLD PURIFICATION WAS ACHIEVED FROM SEPTAL TISSUE WITH AN ESTIMATED SPECIFIC ACTIVITY OF 15 MRC U/G TO PURE HORMONE WITH AN ACTIVITY OF 4500 MRC U/MG.

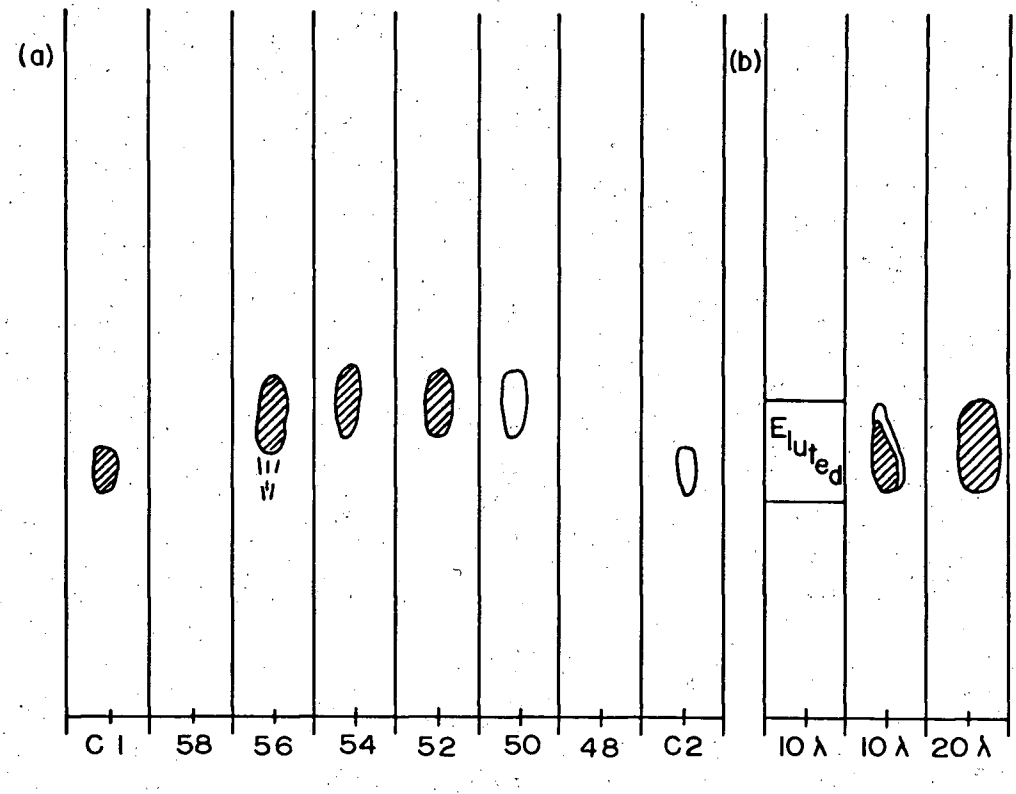


FIGURE 19. THIN LAYER CHROMATOGRAPHY OF STAGE 5 PRODUCT ON SILICA GEL. SOLVENT SYSTEM; BUTANOL, ACETIC ACID, WATER, PYRIDINE (15: 10: 12: 3). O-PAQ POSITIVE, ⊘ - PAQ AND NINHYDRIN POSITIVE.

(A) 10 μL SAMPLES OF ALTERNATE FRACTIONS FROM 48 TO 58. C-1, 1 NMOLE ARG. C-2, 0.1 NMOLE ARG.

(B) 10 AND 20 μL SAMPLES FROM FRACTION 54.

8. Criteria of Homogeneity.

Since all stages of purification had involved separations based on either charge or molecular size a separation based on differential solubilities seemed most likely to detect any contaminant. Thin layer chromatography in a system used on porcine CT (15) was chosen as the best procedure. Thin layer chromatograms were run in an ascending solvent system on Eastman precoated 20 x 20 cm, 6061, silica gel plates (Distillation Products Industries, Rochester N.Y.) activated at 110°C for 30 minutes. A butanol, pyridine, water, acetic acid (15:10:12:3) buffer system was used. 10 µL aliquots from fractions 50, 52, 54, 56, 58 were applied to the origin and allowed to migrate for a period of 5 hours. The plates were then sprayed with phenanthrenequinone (PAQ) reagent. After drying and observation under UV light to detect arginine containing spots the plates were sprayed a second time with a special ninhydrin reagent designed to follow the PAQ. Preparation of these reagents is described in Appendix C. In a second experiment aliquots of various sizes were taken from fraction 54 and treated in a similar manner. One channel was retained undeveloped, however, and the peptide containing region eluted with vehicle and bioassayed.

Tracings of the thin layer chromatograms are shown in Figure 19(a). Only fractions 52, 54, and 56 contained sufficient material for detection by both reagents. The PAQ reagent produced distinct flourescent spots in all but two fractions and on test samples containing 0.1 and 1.0 nmole

OF ARGININE. THE 1 NMOLE SAMPLE OF ARGININE WAS FAINTLY NINHYDRIN POSITIVE. IN THE FRACTIONS 50 TO 56 ONLY A SINGLE SPOT COULD BE DETECTED AND IN EACH CASE THE SPOT HAD AN R_F OF ABOUT 0.5 WITH THE MORE CONCENTRATED SAMPLES BEING SLIGHTLY RETARDED. THUS IT APPEARED THAT THE PEAK CONTAINED ONLY A SINGLE COMPONENT. TO CONFIRM THIS A SECOND EXPERIMENT WAS DONE (FIGURE 19B) USING TWICE AS MUCH SAMPLE WITH SIMILAR RESULTS. BIOASSAY OF AN UNDEVELOPED SAMPLE FROM THE SECOND CHROMATOGRAM CONFIRMED THAT THIS COMPONENT WAS BIOLOGICALLY ACTIVE.

AMINO ACID ANALYSES PERFORMED ON ALTERNATE FRACTIONS BETWEEN NUMBERS 50 AND 56 AS DESCRIBED IN SECTION E FURTHER CONFIRMED THE HOMOGENEITY OF THE ACTIVE PEAK. THE RESULTS OF THESE ANALYSES AS SHOWN IN TABLE IV INDICATE NO SIGNIFICANT DIFFERENCE BETWEEN THE MOLE RATIOS OF THE AMINO ACIDS IN THE VARIOUS FRACTIONS. ON THIS BASIS IT WAS CONCLUDED THAT THE MATERIAL IN FRACTIONS 49-57 REPRESENTED PURE SALMON CT IN SUFFICIENT QUANTITY TO ALLOW COMPLETE CHEMICAL CHARACTERIZATION.

D. Characterization of the Whole Molecule.

1. Methods.

a. Amino Acid Composition.

AMINO ACID ANALYSES WERE CARRIED OUT ON FRACTIONS 50, 52, 54, AND 56 OF THE STAGE 5 ELUATE. 100 µL ALIQUOTS OF THESE FRACTIONS WERE LYOPHILIZED AND HYDROLYSED IN 5.7 M HYDROCHLORIC ACID FOR 17 HOURS AT 110° C. THE HYDROLYSED

SAMPLES WERE DRIED *IN VACUO* AND AIR OXIDIZED TO INSURE COMPLETE RECOVERY OF ½-CYSTINE. THE DETAILED METHODOLOGY IS DESCRIBED IN APPENDIX D. SAMPLES EQUIVALENT TO 40 µL OF THE ORIGINAL ALIQUOTS WERE THEN SUBJECTED TO AUTOMATED AMINO ACID ANALYSIS BY THE METHOD OF SPACKMAN *ET AL* (119) ON A BIOCHROM MODEL 200 ANALYSER WHICH HAD BEEN MODIFIED WITH A WHEATSTONE BRIDGE-PHOTOTRANSISTOR DETECTION CIRCUIT AND SPECIAL LONG PATHLENGTH FLOW CELLS TO ALLOW QUANTITATION OF AMINO ACIDS AT LEVELS DOWN TO 2 NMOLES.* THESE PROCEDURES ALLOWED DETERMINATION OF ALL AMINO ACIDS EXCEPT GLUTAMINE AND ASPARAGINE WHICH ARE DEAMIDATED BY ACID HYDROLYSES (IDENTIFICATION OF THESE RESIDUES WILL BE DISCUSSED LATER) AND TRYPTOPHAN WHICH IS DESTROYED BY ACID HYDROLYSIS.

DETERMINATION OF TRYPTOPHAN WAS BASED ON MEASUREMENTS OF THE MOLAR EXTINCTION COEFFICIENTS OF THE WHOLE MOLECULE AT 280 AND 230 NM AND ON THE RESULTS ON A TEST WITH EHRLICH REAGENT WHICH IS SPECIFIC FOR TRYPTOPHAN. MILLIGRAM EXTINCTION COEFFICIENTS (ABSORBANCE IN A 1 CM PATHLENGTH OF A SOLUTION CONTAINING 1 MG/ML OF THE PROTEIN) WERE CALCULATED FROM THE MEASURED ABSORBANCE OF FRACTION 56 IN A 1 CM CELL AT 230 AND 280 NM IN A BAUSCH AND LOMB SPECTRONIC 600 SPECTROPHOTOMETER AT PH 2 IN 0.1 M FORMIC ACID AGAINST A BLANK OF THE SAME BUFFER. SAMPLES OF THE FRACTION WERE LATER LYO-

*EXTENSIVE MODIFICATION OF THIS MACHINE OCCURED DURING THE COURSE OF THESE STUDIES AND THE ANALYSES PRESENTED IN VARIOUS SECTIONS OF THIS THESIS WERE PERFORMED AT DIFFERENT STAGES OF THIS MODIFICATION. APPENDIX D CONTAINS A DESCRIPTION OF THE EVOLUTION OF THE AMINO ACID ANALYSER, AND THE DETAILS IN THE TEXT ARE PRESENTED ONLY TO ALLOW THE READER TO IDENTIFY THE STAGE AT WHICH A PARTICULAR ANALYSIS WAS PERFORMED.

TABLE IV

AMINO ACID ANALYSES OF FRACTIONS FROM STAGE 5 CHROMATOGRAPHY

AMINO ACIDS	50 NANO-MOLES	50 MOLE* RATIO	52. NANO-MOLES	52. MOLE RATIO	54 NANO-MOLES	54 MOLE RATIO	56 NANO-MOLES	56 MOLE RATIO
ASPARTIC ACID	5.1	2.0	8.7	2.0	15.1	2.0	18.0	2.0
THREONINE	12.8	5.1	22.4	5.1	37.8	5.0	45.0	5.0
SERINE	10.0	4.0	17.4	4.0	24.6	3.3	37.0	4.1
GLUTAMIC ACID	7.6	3.0	12.9	3.0	22.7	3.0	26.6	3.0
PROLINE	6.2	2.5	9.3	2.1	13.4	1.8	17.3	1.9
GLYCINE	8.8	3.5	14.1	3.2	23.2	3.0	28.8	3.2
ALANINE	0	0	0	0	0	0	0	0
½ CYSTINE	4.6	1.8	9.3	2.1	12.5	1.7	17.0	1.9
VALINE	2.7	1.1	4.8	1.1	6.5	0.9	8.0	0.9
METHIONINE	0	0	0	0	0	0	0	0
ISOLEUCINE	0	0	0	0	0	0	0	0
LEUCINE	13.0	5.2	20.2	4.7	38.5	5.1	48.7	5.4
TYROSINE	2.4	1.0	3.8	0.9	6.5	0.9	7.4	0.8
PHENYLALANINE	0	0	0	0	0	0	0	0
SEPARATE ANALYSES ON SHORT COLUMN								
LYSINE					7.0	2.0	16.3	2.0
HISTIDINE					3.7	1.0	9.1	1.1
ARGININE					4.0	1.1	8.1	1.0
AMMONIA					19.0	5.3	44.4	5.5

*WITH REFERENCE TO ASPARTIC ACID OR LYSINE.

PHILIZED AND WEIGHED TO DETERMINE PROTEIN CONCENTRATION. MOLAR EXTINCTION COEFFICIENTS WERE CALCULATED FROM THESE VALUES AND THE MOLECULAR WEIGHT OF THE POLYPEPTIDE AS DETERMINED FROM THE AMINO ACID ANALYSES. THE EHRLICH REAGENT USE WAS A 0.5% (w/v) SOLUTION OF P-DIMETHYLAMINOBENZALDEHYDE (FISHER SCIENTIFIC Co.) IN 95% ETHANOL CONTAINING 2% (v/v) CONCENTRATED HYDROCHLORIC ACID (68). THIS WAS SPRAYED ONTO A SILICA GEL THIN LAYER PLATE WHICH HAD BEEN SPOTTED WITH 10 µL OF FRACTION 56.

B. END GROUP ANALYSIS.

THE N-TERMINAL AMINO ACID IN THE NATIVE MOLECULE WAS DETERMINED BY THE DANSYL METHOD OF GRAY AND HARTLEY(47). A ONE NMOLE SAMPLE OF THE HORMONE WAS DISSOLVED IN 0.2 M SODIUM BICARBONATE AND TREATED WITH DANSYL CHLORIDE IN ACETONE. HYDROLYSIS OF THE SAMPLE IN 5.7 M HYDROCHLORIC ACID WAS USED TO RELEASE THE DANSYL DERIVATIVE OF THE N-TERMINAL AMINO ACID WHICH WAS THEN IDENTIFIED BY POLYAMIDE LAYER CHROMATOGRAPHY AS DESCRIBED BY WOODS AND WANG (132) AND MODIFIED BY HARTLEY (52). DETAILS OF THESE METHODS ARE INCLUDED IN APPENDIX E.

2. RESULTS AND DISCUSSION.

THE RESULTS OF THE AMINO ACID ANALYSES OF THE FOUR FRACTIONS ACROSS THE ACTIVE PEAK ARE SHOWN IN TABLE IV. NO SIGNIFICANT DIFFERENCE COULD BE SEEN IN THE MOLE RATIOS OF THE VARIOUS SAMPLES DESPITE THE OBVIOUS DIFFERENCES IN THE ABSOLUTE QUANTITIES ANALYSED. THE AVERAGE VALUES FOR THE

TABLE V

AMINO ACID COMPOSITIONS OF FIVE CALCITONINS

AMINO ACIDS	SALMON CT*	HUMAN CT (80)	PORCINE CT (96)	BOVINE CT (17)	OVINE CT (97)
ASPARTIC ACID	2	3	4	4	4
THREONINE	5	5	2	2	2
SERINE	4	1	4	4	4
GLUTAMIC ACID	3	2	1	1	1
PROLINE	2	2	2	2	2
GLYCINE	3	4	3	3	3
ALANINE	0	2	1	1	1
½ CYSTINE	2	2	2	2	2
VALINE	1	1	1	1	1
METHIONINE	0	1	1	1	1
ISOLEUCINE	0	1	0	0	0
LEUCINE	5	2	3	3	3
TYROSINE	1	1	1	2	3
PHENYLALANINE	0	3	3	2	1
LYSINE	2	1	0	1	1
HISTIDINE	1	1	1	1	1
ARGININE	1	0	2	1	1
AMMONIA	5	5	5	4	4
TRYPTOPHAN	0**	0	1	1	1

* MOLE INTEGERS BASED ON AVERAGES FROM EIGHT ANALYSES.
**FROM $A_{280\ NM}$ AND NEGATIVE EHRLICH'S TEST.

MOLE RATIOS BASED ON A TOTAL OF EIGHT ANALYSES AND ARE VERY CLOSE TO THE INTEGRAL VALUES AS SHOWN IN TABLE V. AS THE YIELDS OF ALL AMINO ACIDS WERE GOOD AND CONSISTENT FROM SAMPLE TO SAMPLE HYDROLYSES FOR VARYING TIME INTERVALS WERE NOT PERFORMED.

THE ABSORBANCES AT 280 NM (0.292) AND 230 NM (1.39) FOR A SOLUTION CONTAINING 0.69 MG OF PEPTIDE PER ML INDICATED MILLIGRAM EXTINCTION COEFFICENTS OF 0.45 AT 280 NM AND 2.0 AT 230 NM. BASED ON A MOLECULAR WEIGHT OF 3427 FROM AMINO ACID ANALYSIS THIS SOLUTION WAS 2.0×10^{-4} M IN HORMONE, AND THE MOLAR EXTINCTION COEFFICIENTS OF THE POLYPEPTIDE WERE 1500 AT 280 NM AND 7000 AT 230 NM. THIS MOLAR EXTINCTION COEFFICIENT AT 280 NM WAS APPROXIMATELY EQUAL TO THAT OF TYROSINE UNDER SIMILAR CONDITIONS INDICATING THAT THE SINGLE TYROSINE FOUND BY AMINO ACID ANALYSIS WAS THE ONLY AROMATIC RESIDUE PRESENT IN THE MOLECULE. THE TEST WITH EHRLICH REAGENT CONFIRMED THE ABSENCE OF TRYPTOPHAN.

TABLE V THEREFORE REPRESENTS THE COMPLETE COMPOSITION OF SALMON CT AS COMPARED TO THE COMPOSITIONS OF PORCINE, BOVINE, OVINE, AND HUMAN CALCITONINS. IDENTIFICATION OF THE N-TERMINAL RESIDUE OF THE SALMON MOLECULE AS CYSTINE CONFIRMED THAT ALL FIVE HORMONES CONSIST OF A 32 AMINO ACID CHAIN WITH A DISULFIDE RING AT THE N-TERMINUS AND TWO PROLINE RESIDUES, BUT DRAMATIC DIFFERENCES EXISTED FOR MOST OF THE OTHER RESIDUES. THE SALMON MOLECULE LACKED FIVE OF THE EIGHTEEN DETECTABLE RESIDUES, CONTAINING NO ALANINE, ISOLEUCINE, METHIONINE, PHENYLALANINE OR TRYPTOPHAN. ITS LOW CONTENT OF

AROMATIC AND HYDROPHOBIC RESIDUES AND THE PRESENCE OF THREE ADDITIONAL RESIDUES CONTAINING HYDROXYL GROUPS (NINE VERSUS SIX FOR PORCINE) PROBABLY ACCOUNTS FOR THE HIGHER APPARENT MOLECULAR WEIGHT OF SALMON CT OBSERVED ON SEPHADEX G-50. MOLECULES OF LOW AROMATIC AND HYDROPHOBIC CONTENT ARE RETARDED LESS ON SEPHADEX THAN THOSE WITH HIGH CONTENTS (60) AND THE LARGER HYDRATED RADIUS OF A HYDROPHILIC MOLECULE COULD REINFORCE THIS TENDENCY (17). A TOTAL OF FIVE ACIDIC RESIDUES WERE FOUND AS IN THE OTHER CALCITONINS, BUT THE FIVE AMMONIA RESIDUES INDICATED THAT MOST OF THESE WERE ORIGINALLY AMIDATED, ACCOUNTING FOR THE SMALL CHANGE IN CHARGE WHICH RESULTED FROM INCREASING THE BUFFER pH FROM 3.4 TO 4.5 ON THE SECOND SE-SEPHADEX COLUMN. THE BINDING OF THE HORMONE TO THIS RESIN WAS DUE TO THE NUMBER OF BASIC RESIDUES PRESENT (TWO LYSINES, ONE ARGININE AND ONE HISTIDINE) WHICH WAS GREATER THAN IN ANY OF THE OTHER CALCITONINS CHARACTERIZED.

E. Preparation and Isolation of Tryptic Peptides.

THE PRESENCE OF TWO LYSINES AND ONE ARGININE IN THE MOLECULE SUGGESTED THAT DIGESTION WITH TRYPSIN SHOULD PROVIDE FOUR PEPTIDES OF CONVENIENT SIZE FOR SEQUENCE DETERMINATION BY EDMAN DEGRADATIONS. THE MOLECULE WAS FIRST OXIDIZED WITH PERFORMIC ACID TO AVOID DISULFIDE INTERCHANGE AND OTHER PROBLEMS WITH CYSTEINE WHICH HAD BEEN ENCOUNTERED DURING SEQUENCE WORK ON PORCINE CT (8,9).

1. Methods.

a. Performic acid oxidation.

Performic acid was prepared by adding 0.5 ml of 30% hydrogen peroxide to 9.5 ml of 90% formic acid and allowing the mixture to stand at room temperature for four hours. It was then cooled to 0° C and 1 ml added to 10 mg of pooled stage 5 material in a vessel precooled to 0°C. The mixture was shaken, allowed to stand for 3 hours at 0° C. and lyophilized. The residue was redissolved in 2 ml distilled water and relyophilized. This last step was repeated and a 10 µL sample was taken for amino acid analysis.

B. Amino Acid Analysis.

Preparation for analysis of the oxidized material was carried out as previously described, but without the air oxidation step. The analyses were done as before, but using a single column, three buffer system (38) which allowed determination of all residues on a single sample. Details of this system are described in Appendix D.

C. Digestion with Trypsin.

Trypsin (Worthington, TR6EA) was dissolved to a concentration of 10 mg/ml in 1 mM hydrochloric acid which was 1 mM in calcium chloride. 20 µL of this stock solution was added to 10 mg of oxidized material in 1 ml of 0.5% (w/v) ammonium bicarbonate buffer at pH 8.0 to give an enzyme to substrate ratio of 1:50 (w/w). After incubation at 37° C for 4 hours the sample was lyophilized and redissolved in 3 ml of 0.1 M formic acid and stored frozen.

D. High Voltage Electrophoresis.

The initial separation of the peptides was by high voltage electrophoresis (HVE) at pH 6.5 and pH 1.9 in the system described in Appendix C. For experiments at pH 6.5, 300 µL samples of the tryptic digest solution containing approximately 0.3 µM of peptide were applied to 3 cm of the origin line 32 cm from the cathode end of a 57 cm sheet of Whatman 3 MM paper and flanked with 5 µL spots of two standard amino acid solutions to test developing reagents and allow calculation of relative mobilities (See Figure 21). Visible dye markers were also employed as described in Appendix C. After saturation with pH 6.5 buffer the sheet was placed in the electrophoresis tank and run at 4000 V and approximately 5 milliamperes per centimeter of paper width until the markers reached a predetermined point. The electrophoretogram was then removed and allowed to dry. A 5 x 57 cm strip was removed from the center of the sheet such that it contained approximately 75% of the applied sample, and the remainder of the sheet developed with either PAQ reagent, cadmium-ninhydrin or both as described in Appendix C. The segments of the undeveloped strip containing peptides were cut out and saved for further purification or elution.

Electrophoresis at pH 1.9 was performed on the excized, undeveloped strips from the pH 6.5 electrophoretograms and from paper chromatograms by sewing the strips onto new sheets of Whatman 3 MM on an origin line 10 cm from the anode and

REMOVING THE AREA OF THE NEW SHEET BENEATH THE SEWED IN STRIP. ELECTROPHORESIS WAS THEN PERFORMED AS PREVIOUSLY DESCRIBED AND THE EDGE STRIPS OF THE RESULTANT ELECTROPHORETOGRAMS DRIED AND DEVELOPED. SEGMENTS OF THE UNDEVELOPED CENTER STRIP WHICH CONTAINED PEPTIDES WERE CUT OUT AND ELUTED WITH 0.5 M ACETIC ACID (58).

E. PAPER CHROMATOGRAPHY.

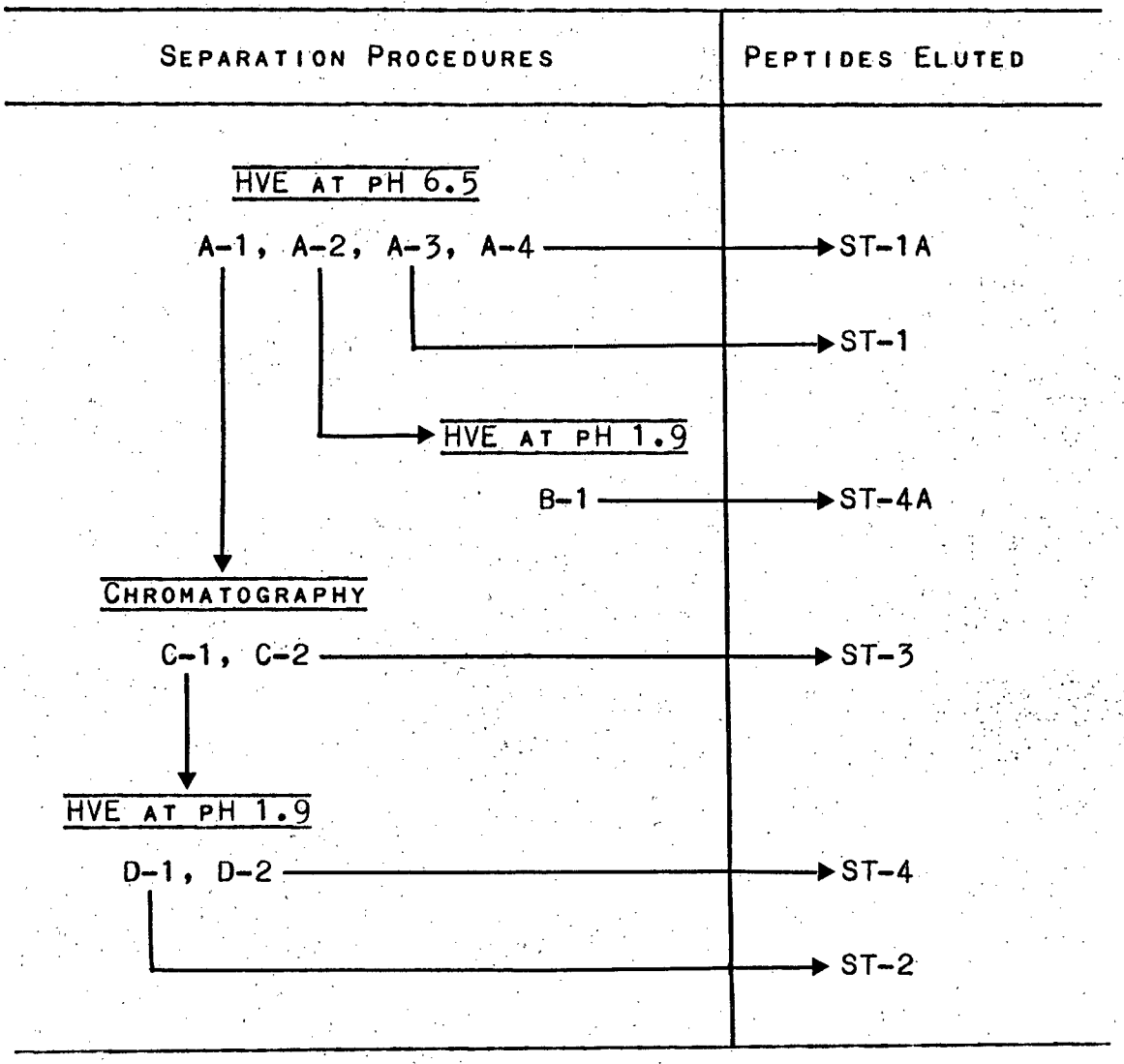
PEPTIDES WHICH COULD NOT BE SEPARATED BY ELECTROPHORESIS ALONE WERE SUBJECTED TO DECENDING PAPER CHROMATOGRAPHY IN A SINGLE PHASE SYSTEM CONSISTING OF BUTANOL, ACETIC ACID, PYRIDINE AND WATER (30:6:20:24) (131). UNDEVELOPED STRIPS FROM ELECTROPHORETOGRAMS WERE SEWED ON AT THE ORIGIN, 7 CM FROM THE TOP OF 15 x 46 CM SHEETS OF WHATMAN 3 MM PAPER. THESE SHEETS WERE CUT FROM STANDARD 46 x 57 CM SHEETS SUCH THAT THE SOLVENT FLOW WAS PERPENDICULAR TO THE GRAIN OF THE PAPER. UNDER THESE CONDITIONS THE SOLVENT FRONT REQUIRED ABOUT 14 HOURS TO REACH THE BOTTOM OF THE PAPER. AFTER DRYING THE CHROMATOGRAMS WERE DIVIDED AND THE EDGE STRIPS DEVELOPED WITH THE REAGENTS USED FOR THE ELECTROPHORETOGRAMS. THE UNDEVELOPED SEGMENTS CONTAINING PEPTIDES WERE CUT OUT FOR ELUTION OR FURTHER PURIFICATION.

2. RESULTS AND DISCUSSION.

THE AMINO ACID ANALYSIS OF THE OXIDIZED SAMPLE WAS SIMILAR TO THAT OF THE NATIVE HORMONE EXCEPT FOR THE LOSS OF THE TWO $\frac{1}{2}$-CYSTINE RESIDUES AND PARTIAL CONVERSION OF TYROSINE TO CHLOROTYROSINE (128). CYSTEIC ACID WAS PRESENT

FIGURE 20

OUTLINE OF PROCEDURES USED TO ISOLATE TRYPTIC PEPTIDES FROM SALMON CT.



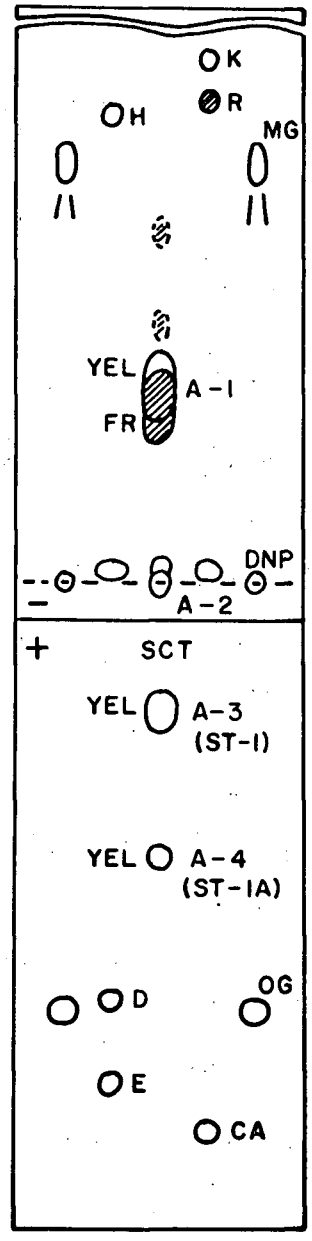


FIGURE 21. pH 6.5 ELECTROPHORETOGRAM OF SALMON CT TRYPTIC PEPTIDES. COMPOSITE OF SEVERAL RUNS. O- NINHYDRIN POSITIVE, ⊘-PAQ POSITIVE, ⊘-PAQ AND NINHYDRIN POSITIVE. SINGLE LETTER DESIGNATIONS USED FOR AMINO ACIDS. OTHER ABBREVIATIONS TA, TAURINE; CA, CYSTEIC ACID; MG, METHYL GREEN; DNP, ε-DNP-LYSINE; OG, ORANGE G; YEL, YELLOW NINHYDRIN COLOR; FR, FAST RED; YR, YELLOW CHANGING TO RED. SINGLE LETTER DESIGNATIONS (EG. A-3) INDICATE PEPTIDE CONTAINING REGIONS. TWO LETTER DESIGNATIONS (EG. ST-1) INDICATE ISOLATED PEPTIDES. DOTTED LINE IS NEUTRAL.

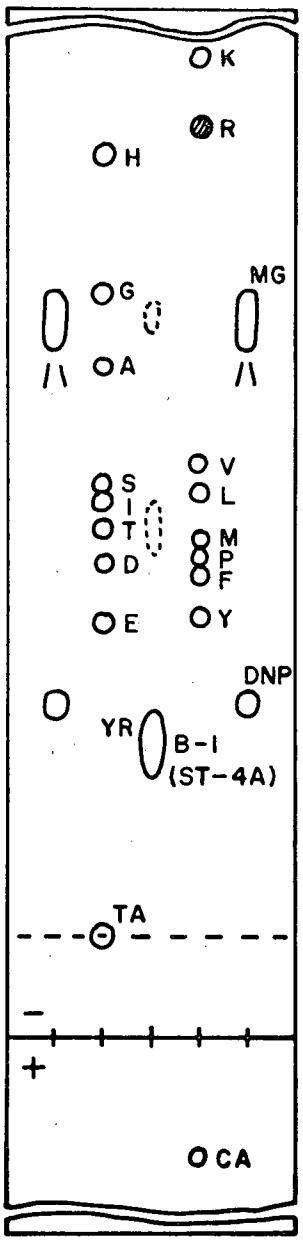


FIGURE 22. pH 1.9 ELECTROPHORETOGRAM OF REGION A-2. OTHER DATA AS DESCRIBED IN FIGURE 21.

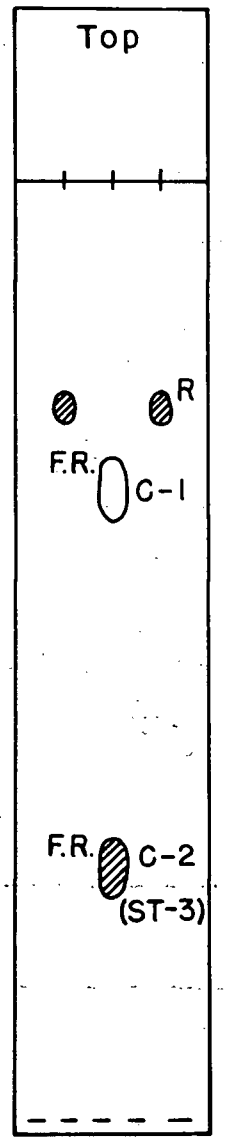


FIGURE 23. PAPER CHROMATOGRAM OF REGION A-1 IN BUTANOL, ACETIC ACID, PYRIDINE, WATER SYSTEM. (30: 6: 20: 24). OTHER DATA AS DESCRIBED IN FIGURE 21. DOTTED LINE IS SOLVENT FRONT.

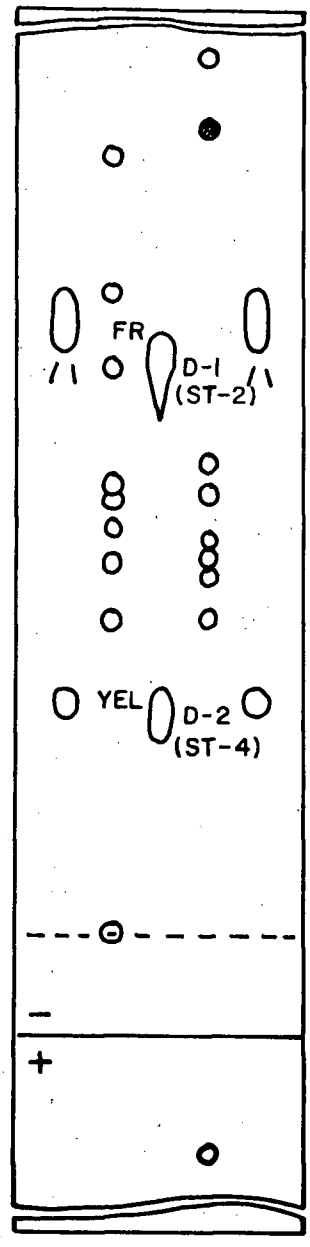


FIGURE 24. pH 1.9 ELECTROPHORETOGRAM OF REGION C-1. OTHER DATA AS DESCRIBED IN FIGURE 21.

WITH A MOLE RATIO OF 1.8 INDICATING THE EXPECTED OXIDATION HAD OCCURED AND THAT THE MATERIAL WAS SUITABLE FOR DIGESTION WITH TRYPSIN.

THE PEPTIDES PRODUCED BY THIS DIGESTION WERE SEPARATED BY A SERIES OF ELECTROPHORETIC AND CHROMATOGRAPHIC STAGES AS SHOWN IN FIGURES 21 THRU 24. THE PEPTIDE MAPS SHOWN IN THESE FIGURES ARE COMPOSITES BASED ON MORE THAN ONE EXPERIMENT SINCE ALL OF THE DATA PRESENTED COULD NOT BE OBTAINED FROM A SINGLE SAMPLE. THE SINGLE LETTER, SINGLE-NUMBER DESIGNATIONS REFER TO PEPTIDE CONTAINING REGIONS WHILE THE DESIGNATIONS IN PARENTHESIS ARE USED ONLY FOR REGIONS CONTAINING A SINGLE COMPONENT. THE INITIAL SEPARATION BY HVE AT PH 6.5 SHOWN IN FIGURE 21 YIELDED FOUR NINHYDRIN POSITIVE REGIONS AND TWO MINOR SPOTS SEEN ONLY WITH PAQ WHICH WERE NOT INVESTIGATED FURTHER. TWO OF THE FOUR MAJOR REGIONS WERE SHOWN TO BE ISOLATED PEPTIDES (ST-1 AND ST-1A), BUT THE OTHER TWO CONTAINED MORE THAN ONE COMPONENT AND WERE SUBJECTED TO FURTHER PURIFICATION AS OUTLINED IN FIGURE 20. TWO MINOR CONTAMINANTS WERE SEPARATED FROM A SINGLE MAJOR PEPTIDE (ST-4A) IN REGION A-2 BY HVE AT PH 1.9 AS SHOWN IN FIGURE 22, AND REGION A-1 WAS SUBJECTED TO PAPER CHROMATOGRAPHY FOR FURTHER PURIFICATION. THIS CHROMATOGRAPHY YIELDED TWO NINHYDRIN POSITIVE REGIONS AS SHOWN IN FIGURE 23, BUT ONLY ONE OF THESE WAS HOMOGENEOUS (ST-3). THE SECOND REGION (C-1) WAS FURTHER PURIFIED BY HVE AT PH 1.9 AS SHOWN IN FIGURE 24 TO YIELD TWO ISOLATED PEPTIDES, ST-2 AND ST-4. THUS THE TRYPTIC DIGEST WAS SHOWN TO CONTAIN SIX MAJOR PEPTIDES RATHER

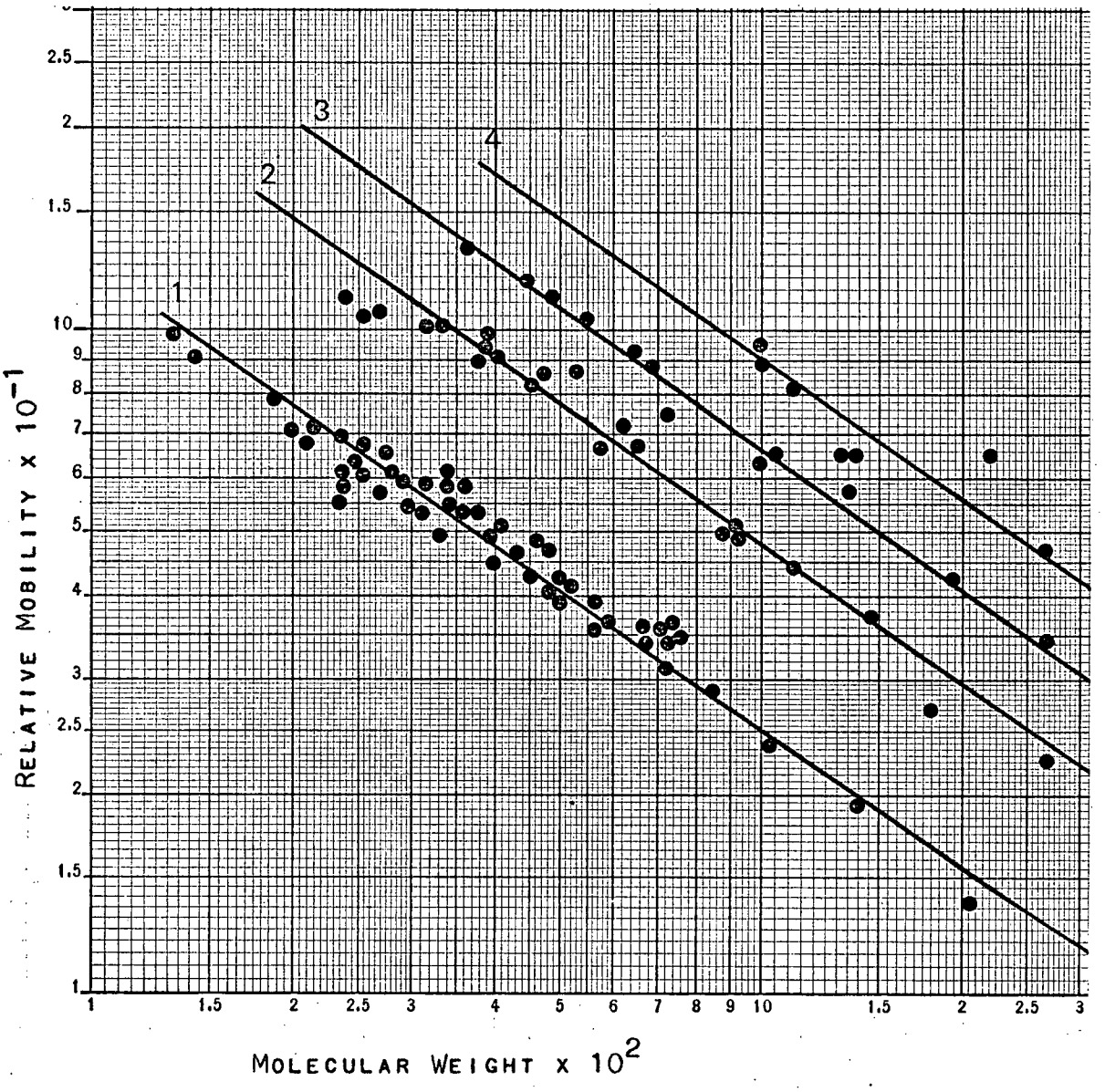


FIGURE 25. LOGARITHMIC PLOT OF ELECTROPHORETIC MOBILITY (AT pH 6.5 RELATIVE TO ASPARTIC ACID) VERSUS MOLECULAR WEIGHT FOR PEPTIDES. ALIGNMENTS CORRESPOND TO UNIT CHARGES AS INDICATED. (88)

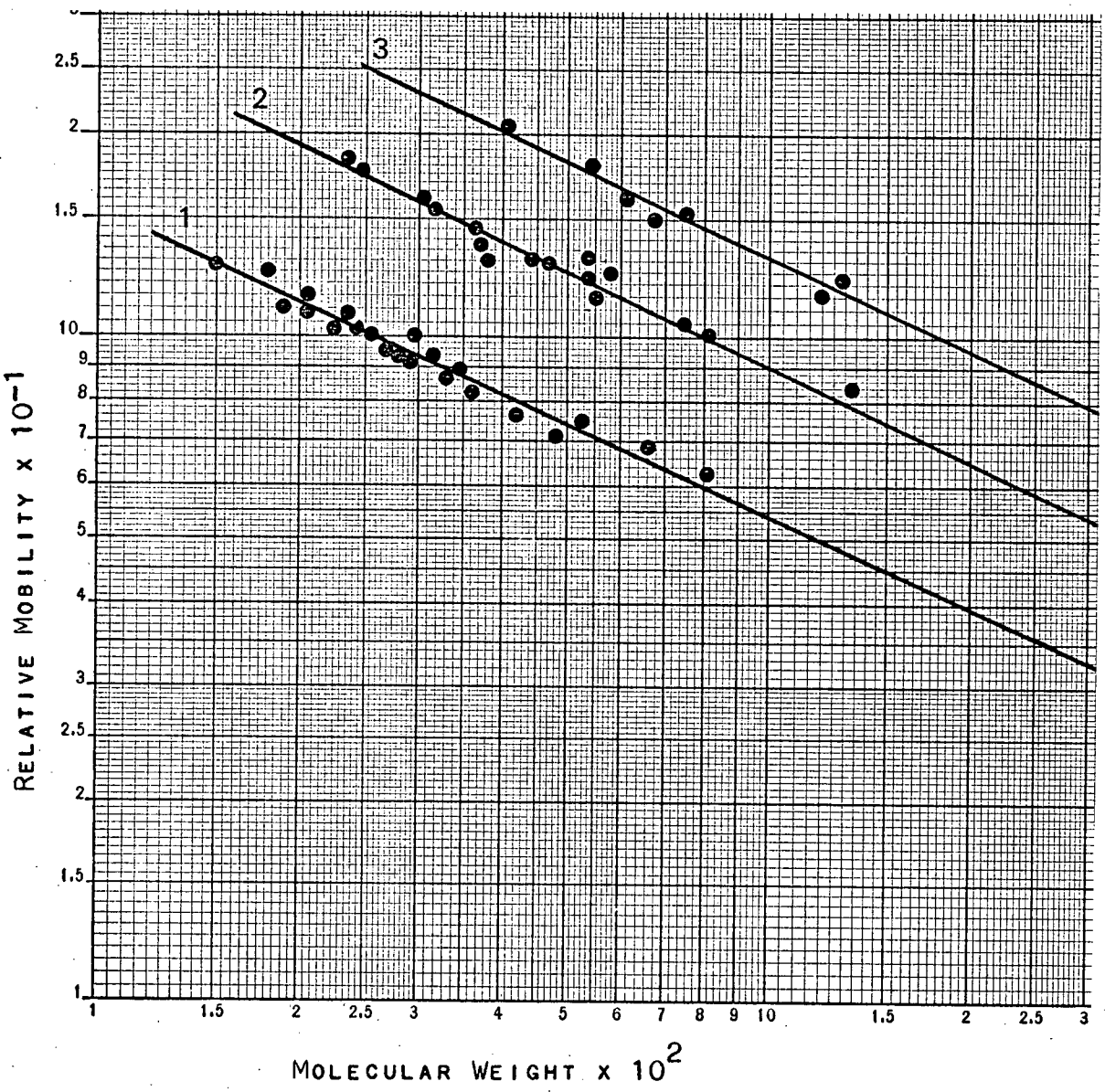


FIGURE 26. LOGARITHMIC PLOT OF ELECTROPHORETIC MOBILITY (AT PH 1.9 RELATIVE TO SERINE) VERSUS MOLECULAR WEIGHT FOR PEPTIDES. ALIGNMENTS CORRESPOND TO UNIT CHARGES AS INDICATED. (88)

THAN THE EXPECTED FOUR. THESE WERE RECOVERED BY ELUTION FROM THE PAPER STRIPS IN QUANTITIES ADEQUATE TO ALLOW CHARACTERIZATION AS DESCRIBED IN THE NEXT SECTION.

F. Characterization of Tryptic Peptides.

ALIQUOTS OF THE ELUTED PEPTIDES WERE TAKEN FOR AMINO ACID ANALYSIS TO DETERMINE THEIR COMPOSITION AND THE MOBILITIES OF THE PEPTIDES DURING HVE WERE COMPARED TO THESE COMPOSITIONS TO CALCULATE NET CHARGE AND DETERMINE WHETHER OR NOT THE ACIDIC RESIDUES WERE AMIDATED. N-TERMINAL ANALYSES WERE CARRIED OUT TO OBTAIN DATA ON THE ORDER OF THE PEPTIDES IN THE WHOLE MOLECULE.

1. Methods.

a. Amino Acid Analysis.

PREPARATION FOR ANALYSIS WAS CARRIED OUT AS DESCRIBED PREVIOUSLY, BUT WITHOUT AIR OXIDATION. THE ANALYSES WERE PERFORMED USING A SINGLE COLUMN-TWO BUFFER SYSTEM AND A 6 MM DIAMETER COLUMN TO INCREASE SENSITIVITY. THE DETECTION SYSTEM USED WAS A LOG AMPLIFIER-PHOTOTRANSISTOR CIRCUIT AS DESCRIBED IN APPENDIX D.

b. Determination of Amide Groups.

OFFORD (88) HAS SHOWN THAT PEPTIDES OF KNOWN ELECTROPHORETIC MOBILITY RELATIVE TO ASPARTIC ACID (μ_{ASP}) AT PH 6.5 OR TO SERINE (μ_{SER}) AT PH 1.9 AND KNOWN MOLECULAR WEIGHT FALL INTO CERTAIN POPULATIONS WHEN PLOTTED GRAPHICALLY AS SHOWN IN FIGURES 25 AND 26. PEPTIDES WHICH LIE NEAR A GIVEN

TABLE VI

AMINO ACID ANALYSES OF TRYPTIC PEPTIDES FROM SALMON CT

AMINO ACIDS	MOLE RATIOS ST-1	ST-1A	ST-2	ST-3	ST-4	ST-4A
CYSTEIC ACID	1.9	1.7	0	0	0	0
ASPARTIC ACID	1.0	1.0	0	0	1.0	1.0
THREONINE	0.8	0.8	0	0.8	2.9	2.8
SERINE	1.8	1.7	0.8	0	1.1	0.8
GLUTAMIC ACID	0	0	2.1	1.2	0	0
PROLINE	0	0	0	1.1	1.1	1.0
GLYCINE	1.1	1.0	0	0	2.0	2.1
ALANINE	0	0	0	0	0	0
VALINE	1.0	1.1	0	0	0	0
METHIONINE	0	0	0	0	0	0
ISOLEUCINE	0	0	0	0	0	0
LEUCINE	1.7	1.7	1.8	1.0	0	0
TYROSINE	0	0	0	1*	0	0
PHENYLALANINE	0	0	0	0	0	0
LYSINE	0.9	0.8	1.0	0	0	0
HISTIDINE	0	0	0.8	0	0	0
ARGININE	0	0	0	1.0	0	0
% YIELD **	5.5%	10%	75%	35%	70%	10%

* DETERMINED AS CHLOROTYROSINE
** APPROXIMATIONS

LINE ON THESE GRAPHS HAVE BEEN SHOWN TO HAVE THE NET CHARGE INDICATED FOR THAT LINE AT THE GIVEN pH. THE RELATIVE MOBILITIES OF THE TRYPTIC PEPTIDES WERE CALCULATED FROM THE AVAILABLE DATA IN THE PRECEDING SECTION AND COMPARED TO THE MOLECULAR WEIGHTS CALCULATED FROM THE AMINO ACID COMPOSITION DATA TO GIVE THE NET CHARGE OF THE PEPTIDE. KNOWING THE NET CHARGE AND THE COMPOSITION AS DETERMINED BY AMINO ACID ANALYSIS OF HYDROLYSED SAMPLES IT WAS GENERALLY POSSIBLE TO DETERMINE WHETHER OR NOT THE CARBOXYL GROUPS PRESENT HAD BEEN IN THE AMIDE FORM PRIOR TO HYDROLYSIS.

c. End Group Analysis.

The N-terminal residues of the peptides were determined by dansylation of 0.7 nmole samples of the peptides and subsequent identification as described in Appendix E. The C-terminal residues of 4 of the six major peptides could be determined from their compositions and known specificity of trypsin for the carboxyl side of lysine and arginine residues.

2. Results and Discussion.

Table VI shows the amino acid analyses of the six tryptic peptides and the approximate yields of each peptide. The yield figures are only rough estimates since the percentage lost on the devloped edge strips varies in an indeterminant manner, but they were useful in determining the source of the two extra peptides. As shown in the table the yields of peptides ST-1A and ST-4A were quite low and their amino acid analyses were identical to those of ST-1

TABLE VII

NET CHARGES OF SALMON CT TRYPTIC PEPTIDES FROM ELECTROPHORETIC MOBILITIES AND MOLECULAR WEIGHTS (88)

PEPTIDES	ASP (pH 6.5)	SER (pH 1.9)	MOLECULAR WEIGHT	NET CHARGE
ST-1	0.30		1220	-1
ST-1A	0.59		1220	-2
ST-2	-0.41		854	+1.5
		1.31		+3
ST-3	-0.41		777	+1
ST-4	-0.44		734	+1
		0.52		+1
ST-4A	0		734	0
		0.47		+1

AND ST-4. THE MOBILITIES OF THE MINOR PEPTIDES WERE QUITE DIFFERENT FROM THOSE OF THE MAJOR PEPTIDES, HOWEVER, AND AS SHOWN IN TABLE VII, THEY EACH APPEARED TO HAVE ONE MORE NEGATIVE CHARGE THAN THE CORRESPONDING MAJOR PEPTIDE. THE CALCULATED NET CHARGES OF THE MAJOR PEPTIDES INDICATED THAT THEY EACH CONTAINED ASPARAGINE AND THE ADDITIONAL NEGATIVE CHARGE ON THE MINOR PEPTIDES SUGGESTED THAT THEY CONTAINED ASPARTIC ACID. IT THUS APPEARED LIKELY THAT A PARTIAL DEAMIDATION HAD OCCURED DURING THE PERFORMIC ACID OXIDATION AND THAT THE NATIVE MOLECULE HAD CONTAINED ASPARAGINE RATHER THAN ASPARTIC ACID.

THE COMPOSITION OF ST-1 INDICATED THAT IT WAS THE N-TERMINAL PEPTIDE SINCE IT CONTAINED BOTH CYSTEIC ACID RESIDUES AND IDENTIFICATION OF DANSYL-CYSTEIC ACID AFTER DANSYLATION CONFIRMED THIS. ST-4 CONTAINED NEITHER LYSINE NOR ARGININE AND THEREFORE COULD ONLY HAVE BEEN THE C-TERMINAL PEPTIDE. THE FACT THAT ITS MOBILITY INDICATED THAT IT CARRIED A NET +1 CHARGE IN SPITE OF THE ABSENCE OF BASIC RESIDUES SUGGESTED THAT ITS C-TERMINUS WAS BLOCKED--A FURTHER INDICATION THAT IT WAS THE C-TERMINAL PEPTIDE. THE PRESENCE OF PROLINE IN THIS PEPTIDE SUGGESTED THAT THIS BLOCKED C-TERMINAL RESIDUE WAS PROBABLY PROLYLAMIDE, AS SEEN IN THE OTHER CALCITONINS, BUT THIS WAS NOT CONFIRMED. THE N-TERMINAL RESIDUE OF THIS PEPTIDE WAS THREONINE. PEPTIDE ST-3 HAD AN N-TERMINAL LEUCINE AND A C-TERMINAL ARGININE, AND ITS NET CHARGE AT pH 6.5 WAS +1 INDICATING THAT IT CONTAINED GLUTAMINE RATHER THAN GLUTAMIC ACID. PEPTIDE

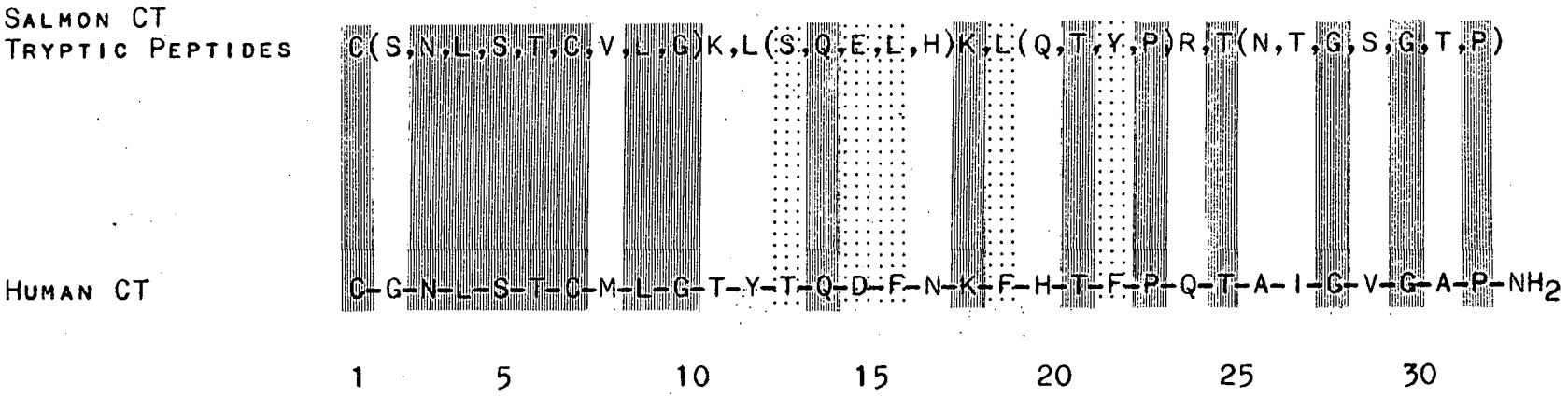


FIGURE 27. SALMON CT TRYPTIC PEPTIDES ALIGNED WITH HUMAN CT. ▒ POSSIBLE HOMOLOGIES. ⁙ POSSIBLE CONSERVATIVE SUBSTITUTIONS. SINGLE LETTER NOTATION USED FOR AMINO ACIDS.

ST-2 HAD AN N-TERMINAL LEUCINE AS WELL AND A C-TERMINAL LYSINE, BUT BECAUSE OF THE HISTIDINE PRESENT THE MOBILITY DATA AT pH 6.5 DID NOT FIT ANY OF THE LINES WELL (88). IT DID HOWEVER CARRY A NET POSITIVE CHARGE INDICATING THAT AT LEAST ONE OF THE TWO GLUTAMIC ACID RESIDUES HAD BEEN AMIDATED, AND THE FACT THAT ONLY FIVE AMMONIA RESIDUES WERE SEEN IN THE ANALYSIS OF THE WHOLE MOLECULE SUGGESTED THAT ONLY ONE WAS AMIDATED SINCE TWO ASPARAGINES, TWO GLUTAMINES AND A C-TERMINAL AMIDE WOULD ACCOUNT FOR ALL OBSERVED AMMONIA. THUS ALL OF THE AVAILABLE DATA WAS CONSISTENT WITH THE STRUCTURE INDICATED IN FIGURE 27 WHICH SUMMARIZES THE INFORMATION OBTAINED FROM TRYPTIC DIGESTION OF SALMON CALCITONIN.

THIS INFORMATION DOES NOT ALLOW ORDERING OF THE TWO CENTRAL PEPTIDES, BUT WHEN COMPARED TO THE KNOWN SEQUENCES OF HUMAN CT ONLY THE ORDER SHOWN IN FIGURE 28 ALLOWS ANY SIGNIFICANT HOMOLOGY BETWEEN THE MOLECULES IN THIS REGION. EVEN IN THIS ARRANGEMENT, HOWEVER, HOMOLOGY WAS POSSIBLE AT ONLY FOUR POINTS BETWEEN THE HUMAN AND SALMON MOLECULES IN THE CENTRAL REGION AND NO SIGNIFICANT HOMOLOGY BETWEEN SALMON CT AND PORCINE CT WAS POSSIBLE IN EITHER ARRANGEMENT. IT SEEMED CLEAR, EVEN WITHOUT FURTHER SEQUENCE DATA, THAT THE TWO ENDS OF THE SALMON MOLECULE WERE SIMILAR TO THOSE OF THE OTHER CALCITONINS, BUT THAT THE CENTRAL REGIONS DIFFERED EXTENSIVELY.

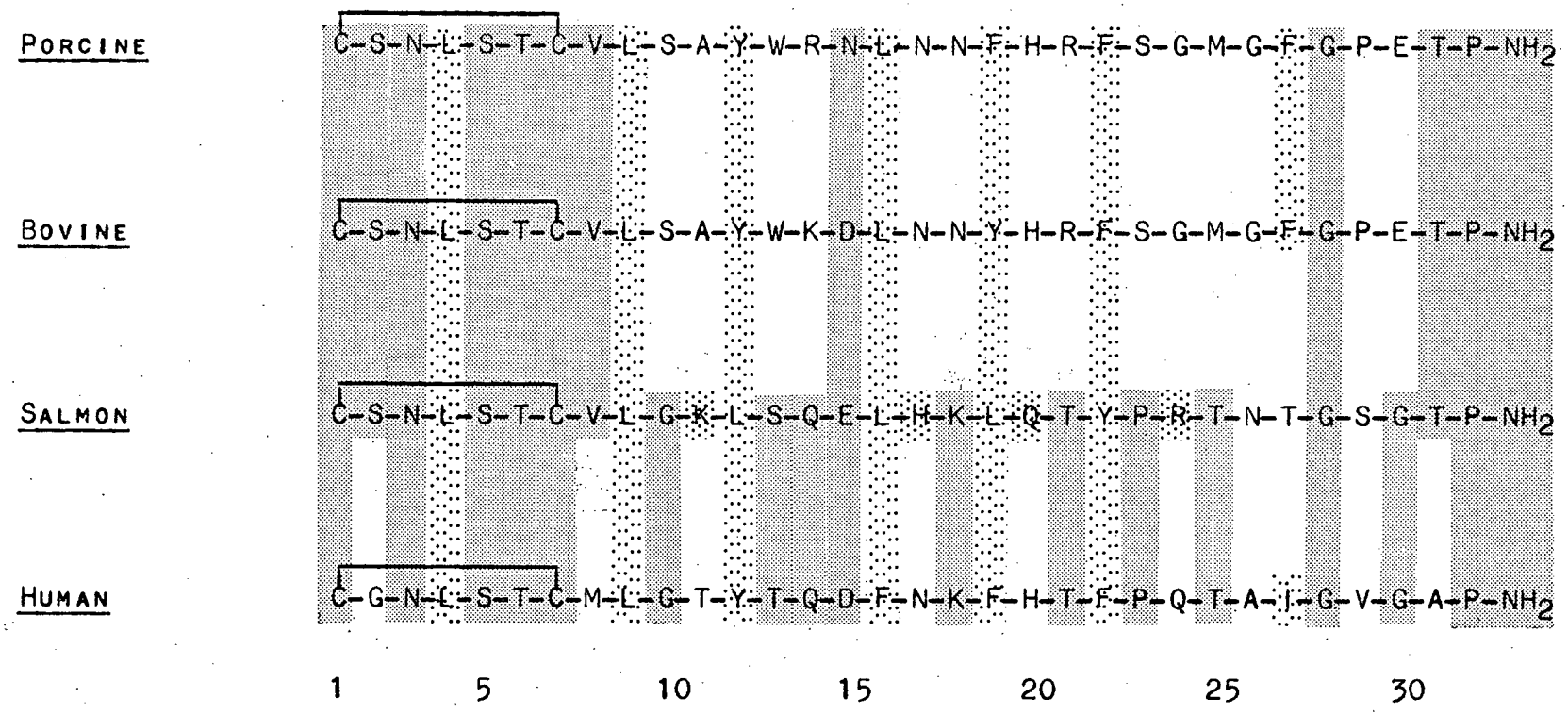


FIGURE 28. ALIGNMENT OF SALMON CT (84) WITH PORCINE CT (96), BOVINE CT (17), AND HUMAN CT (80). CONSISTENT HYDROPHOBIC RESIDUES, OTHER HOMOLOGIES AND CONSERVATIVE SUBSTITUTIONS, UNIQUE CHARGE PLACEMENT IN SALMON CT. SINGLE LETTER NOTATION USED FOR AMINO ACIDS.

G. Sequence of Salmon Calcitonin Compared to Known Sequences of Mammalian Calcitonins.

While the work on the tryptic peptides was being carried out the complete sequence of the salmon hormone was announced by Niall et al (84) based on automated sequential Edman degradations. It was thus futile to continue independent sequence work, particularly since both starting materials had been obtained from the same source and since the work completed confirmed their report. The sequence of the salmon hormone is shown in Figure 28 aligned with the mammalian hormones of known structure. Considerable information on the relationship of structure to biological activity can be obtained by analysis of these sequences. The N-terminal and C-terminal ends of the molecules are very similar. Three of the four hormones are identical for the first 9 residues and the human hormone varies only at positions 2 and 8. The 1-7 disulfide bridge is common to all four, and most evidence indicates that it is essential for biological activity (16, 123). Bell et al (10) have reported, however, that when reduced with mercaptoethanol the hormone retains biological activity, but they offer no evidence that reduction actually occured, and it is possible that the bridge was reformed during the bioassay. The C-terminal residues of the four molecules are also identical (prolylamide), and evidence from the synthetic work of Guttmann et al (50) and Sieber et al (115) indicates that even removal of the C-terminal amide results in a 97% loss of biological activity. The residues in positions 28, 30

AND 31 ALSO SHOW CONSIDERABLE HOMOLOGY BETWEEN THE FOUR MOLECULES.

IN THE CENTRAL REGION OF THE MOLECULES THE SIMILARITIES ARE MORE SUBTLE. ONLY ONE HOMOLOGY EXISTS HERE BETWEEN THE SALMON AND UNGULATE HORMONES--THE LEUCINE IN POSITION 16. POSITIONS 10, 14, 18, 21, 23, AND 25 ARE THE SAME IN THE SALMON AND HUMAN MOLECULES, HOWEVER. MORE INTERESTING THAN THE HOMOLOGIES HOWEVER, ARE THE CONSERVATIVE SUBSTITUTIONS. THE HYDROPHOBIC RESIDUES SUCH AS PHENYLALANINE, TYROSINE, AND LEUCINE OCCUR WITH MARKED REGULARITY IN POSITIONS 4, 9, 12, 16, 19 AND 22. THESE HYDROPHOBIC RESIDUES MAY CONTRIBUTE TO THE STABLILIZATION OF A TERTIARY STRUCTURE OF THE TYPE FREQUENTLY SEEN IN LARGER GLOBULAR PROTEINS (72). THIS UNIMOLECULAR MICELLULAR CONFORMATION IN DILUTE AQUEOUS SOLUTIONS WOULD BE MAINTAINED BY VAN DER WAALS FORCES BETWEEN THE HYDROPHOBIC GROUPS INTERNALLY AND BY HYDROGEN BONDING TO THE HYDRATION SPHERE EXTERNALLY. THE WORK OF BREWER AND EDELHOCH (14) ON THE PORCINE MOLECULE SUPPORTS SUCH A CONCLUSION SINCE HE SHOWS THAT THE RANDOM COIL FORMATION PREDOMINATES IN AQUEOUS SOLUTION, AND THAT THIS RANDOMNESS IS REARRANGED INTO AN α-HELICAL CONFORMATION IN 2-CHLOROETHANOL. HE SUGGESTS THAT THIS TYPE OF REARRANGEMENT MAY BE IMPORTANT AT THE RECEPTOR SITE ON THE CELLULAR MEMBRANE WHERE THE WATER CONCENTRATION IS SIGNIFICANTLY REDUCED. IT IS LIKELY THAT THE REGULARLY PLACED HYDROPHOBIC RESIDUES WOULD PLAY A MAJOR ROLE IN INDUCING SUCH A CONFORMATIONAL CHANGE. THE ONLY EVIDENCE CONFIRMING THE IMPORTANCE

OF THESE RESIDUES FOR BIOLOGICAL ACTIVITY IS FURTHER WORK BY BREWER (13) WHICH SHOWS THAT OXIDATION OF THE TYROSINE IN PORCINE CALCITONIN WITH TYROSINASE RESULTS IN A 90% LOSS OF ACTIVITY. OXIDATION OF THE METHIONINE IN POSITION 8 OF HUMAN CT SIGNIFICANTLY REDUCES BIOLOGICAL ACTIVITY (80), AND THIS POSITION IS ALSO HYDROPHOBIC IN ALL FOUR MOLECULES SINCE BOVINE, PORCINE AND SALMON CALCITONINS HAVE VALINE RESIDUES HERE. IN CONTRAST THE METHIONINE IN POSITION 25 IN THE UNGULATE CALCITONINS MAY BE OXIDIZED WITHOUT ACTIVITY LOSS (16) AND CORRESPONDS TO HYDROPHILIC THREONINE RESIDUES IN THE SALMON AND HUMAN MOLECULES. THE ONLY OTHER SIGNIFICANT CONSERVATIVE SUBSTITUTIONS ARE THE THREE ACIDIC RESIDUES IN HUMAN, SALMON AND BOVINE CALCITONINS AT POSITION 15 BUT THE PORCINE MOLECULE CONTAINS AN ASPARAGINE HERE INDICATING THAT A CHARGED GROUP IS NOT ESSENTIAL FOR ACTIVITY. AS THE OTHER CHARGED RESIDUES ARE SCATTERED THROUGHOUT THE CENTRAL PORTION OF THE MOLECULES IT IS UNLIKELY THAT THEIR POSITIONS ARE CRITICAL FOR ACTIVITY.

THE SIMILARITIES BETWEEN THE FOUR MOLECULES DISCUSSED GIVE AN INDICATION OF WHAT STRUCTURAL CHARACERISTICS ARE REQUIRED FOR BIOLOGICAL ACTIVITY, BUT OFFER LITTLE INFORMATION ON THE REASONS FOR THE MUCH HIGHER BIOLOGICAL ACTIVITY OF SALMON CT. PORCINE CT (15), BOVINE CT (17) AND HUMAN CT (80) ALL HAVE ACTIVITIES BETWEEN 100 AND 300 MRC U/MG WHEN ASSAYED IN THE RAT WHILE SALMON CT HAS A CORRESPONDING ACTIVITY BETWEEN 2500 AND 4500 MRC U/MG (65, 87), AND WHEN ASSAYED IN THE MOUSE (89) THE SALMON

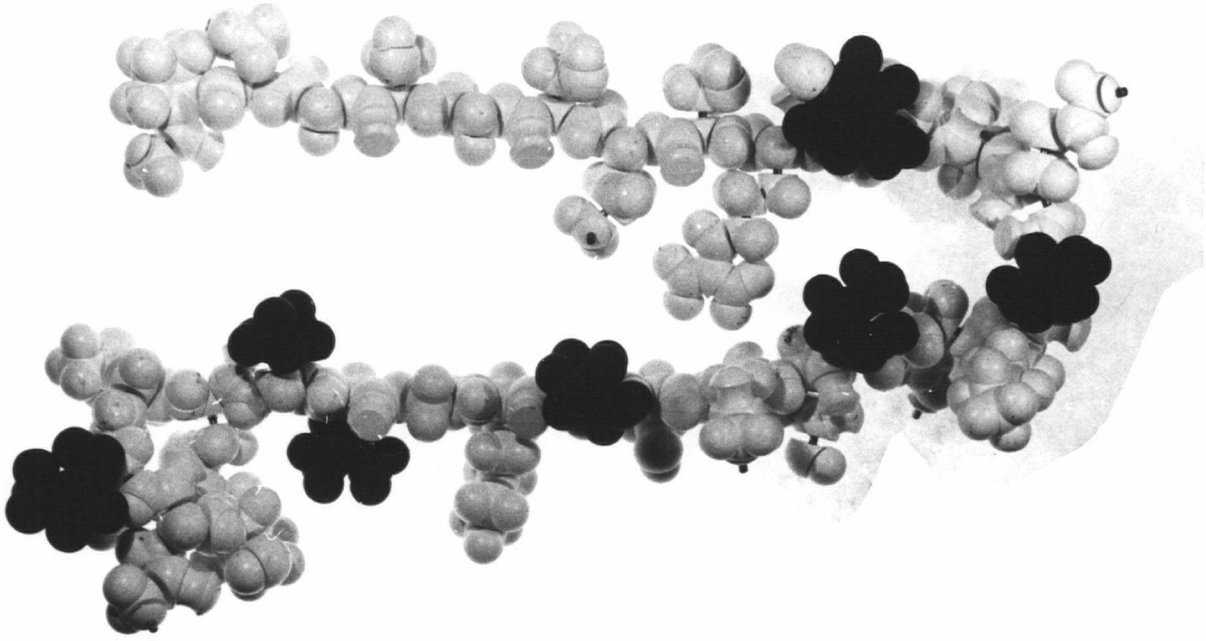
HORMONE HAS MORE THAN 250 TIMES THE ACTIVITY OF PORCINE CT (65). AN EXAMINATION OF THE DIFFERENCES BETWEEN SALMON CT AND THE MAMMALIAN HORMONES COULD ESTABLISH SOME STRUCTURAL BASIS FOR ITS HIGHER BIOLOGICAL ACTIVITY. ON THIS BASIS POSITIONS WHICH SHOW SIMILARITIES CAN BE ELIMINATED AND ONLY THOSE POSITIONS ON THE SALMON MOLECULE WHICH ARE NEITHER HOMOLOGOUS NOR CONSERVATIVELY SUBSTITUTED ARE OF INTEREST. THIS LEAVES ONLY SEVEN POSITIONS: 10, 17, 20, 24, 26, 27, AND 29. FOUR OF THESE SUBSTITUTIONS INVOLVE CHARGED OF POTENTIALLY CHARGED RESIDUES AND THE EQUALLY SPACED LYSINE, HISTIDINE AND ARGININE RESIDUES IN POSITIONS 10,17, AND 24 COULD BE HIGHLY SIGNIFICANT IN BINDING THE MOLECULE TO ITS RECEPTOR SITE. THE FOURTH CHARGE DIFFERENCE IS IN POSITION 20 WHERE THE GLUTAMINE IN THE SALMON MOLECULE IS REPLACED BY A HISTIDINE IN THE MAMMALIAN MOLECULES.

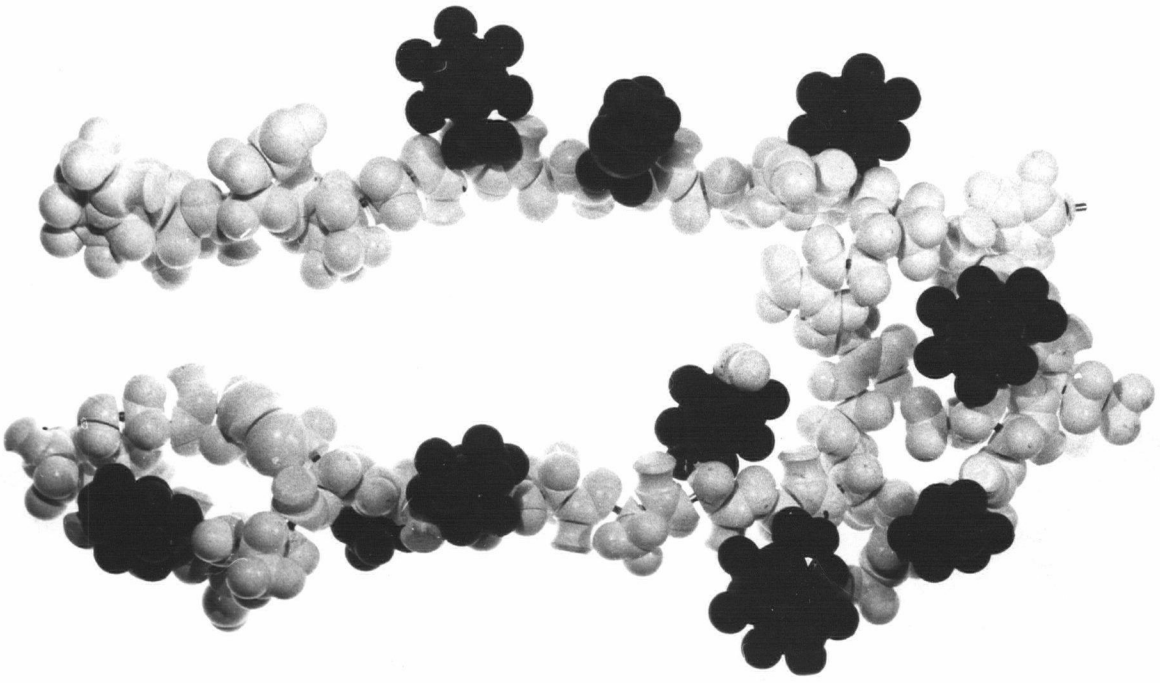
THE LAST THREE POSITIONS OF DIFFERENCE OCCUR NEAR THE C-TERMINAL END IN A REGION WHERE THE SALMON MOLECULE IS HIGHLY HYDROPHILIC. IN CONTRAST THE THREE MAMMALIAN MOLECULES CONTAIN HYDROPHOBIC RESIDUES IN POSITION 27. IF THE MICELLULAR STRUCTURE DISCUSSED EARLIER DOES EXIST THIS HYDROPHOBIC POSITION WOULD BE EXPECTED TO BE INVOLVED IN THE MAMMALIAN HORMONES, BUT THE C-TERMINAL END OF THE SALMON MOLECULE WOULD BE FREE RESULTING IN A MARKED DIFFERENCE IN CONFORMATION. THUS THE REGION BETWEEN THE PROLINE AT POSITION 23 AND THE C-TERMINUS OF THE SALMON MOLECULE WOULD SEEM WELL SUITED TO FOR AN α-HELICAL STRUCTURE IN AQUEOUS SOLUTION. BREWER ET AL (17) HAVE IN FACT SUGGESTED THAT

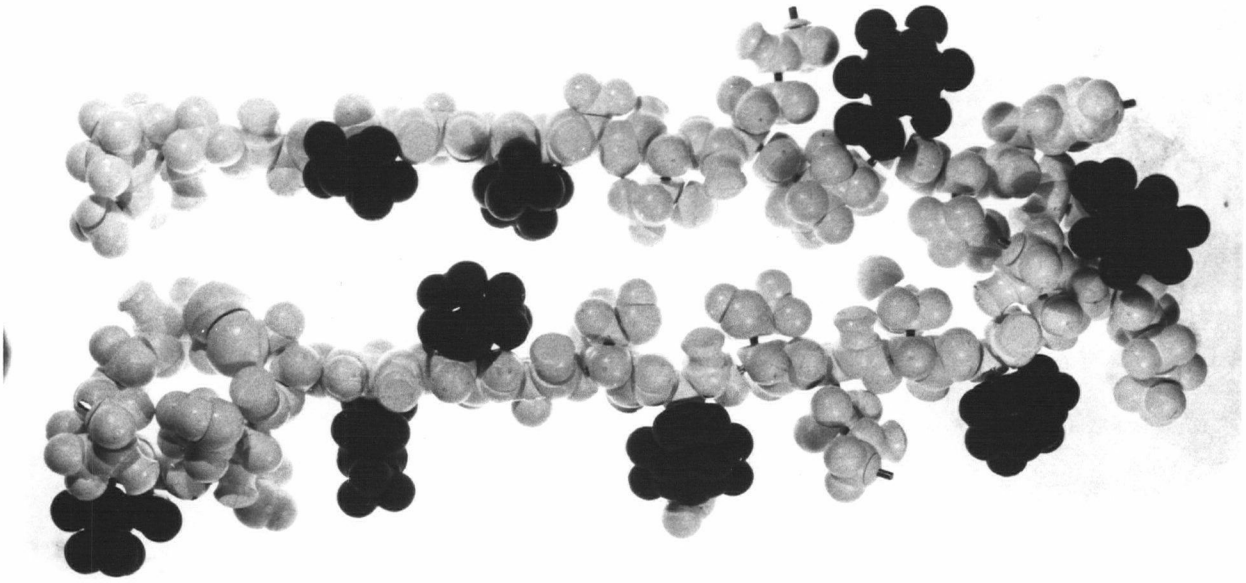
FIGURE 29. A POSSIBLE CONFIGURATION OF SALMON CT AS DETERMINED FROM A MOLECULAR MODEL. UPPER PHOTOGRAPH, OPEN CHAIN. LOWER PHOTOGRAPH, POSSIBLE CLOSED CONFORMATION EMPHASIZING HYDROPHOBIC BONDING. HYDROPHOBIC RESIDUES ARE SHOWN IN BLACK.

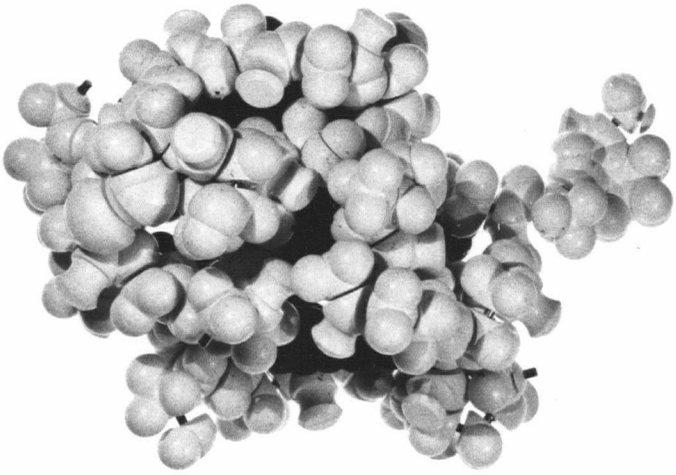
FIGURE 30. A POSSIBLE CONFIGURATION OF PORCINE CT AS DETERMINED FROM A MOLECULAR MODEL. OTHER DATA AS ABOVE.

FIGURE 31. A POSSIBLE CONFIGURATION OF HUMAN CT AS DETERMINED FROM A MOLECULAR MODEL. OTHER DATA AS ABOVE.









THE APPARENTLY HIGH MOLECULAR WEIGHT OF THE SALMON HORMONE DURING GEL FILTRATION MAY RESULT FROM SUCH DIFFERENCES IN TERTIARY STRUCTURE. TO TEST THE VALIDITY OF THIS CONJECTURE ON CONFORMATIONAL DIFFERENCES THE PORCINE, HUMAN AND SALMON MOLECULES WERE CONSTRUCTED USING SPACE FILLING MODELS (LANDE-EDMUND MOLECULAR MODELS, EDMUND SCIENTIFIC CO., BARRINGTON, N.J.). THESE MODELS ARE SHOWN IN FIGURES 29, 30, AND 31 IN OPEN CONFIGURATIONS (A) AND IN POSSIBLE RANDOM COIL CONFORMATIONS (B) WHICH EMPHASIZE HYDROPHOBIC BONDING. THE HYDROPHOBIC GROUPS ARE BLACK FOR CONTRAST AND IT CAN BE SEEN THAT EVEN IN THESE SMALL MOLECULES IT IS POSSIBLE TO FOLD THE HYDROPHOBIC SIDE CHAINS INTO COMPACT APOLAR REGIONS. ACTUALLY MANY CONFORMATIONS ARE POSSIBLE, BUT THE ONES SHOWN ALLOW A HIGH DEGREE OF SIDE CHAIN HYDROGEN BONDING AND IONIC BONDING BETWEEN CHARGED GROUPS. THE ABSENCE OF HYDROPHOBIC GROUPS NEAR THE C-TERMINUS OF SALMON CT IS CLEARLY SHOWN AND THE LONG HYDROPHILIC TAIL IS CLEARLY DIFFERENT FROM PORCINE CT AND HUMAN CT. THE TAIL IS NOT SHOWN IN ITS POSSIBLE α-HELICAL STRUCTURE SINCE ONLY EIGHT RESIDUES ARE PRESENT BETWEEN PROLINES SUGGESTING THAT SUCH A STRUCTURE WOULD HAVE RELATIVELY LITTLE STABILITY (19). CHARGE AND CONFORMATIONAL DIFFERENCES SUCH AS THESE WOULD PROBABLY INFLUENCE THE INTERACTION BETWEEN THE HORMONES AND THEIR RECEPTORS, BUT AS IT SEEMED LIKELY THAT A NUMBER OF OTHER FACTORS WERE INVOLVED DETERMINING THE RELATIVE BIOLOGICAL ACTIVITIES OF THE HORMONES FURTHER INVESTIGATIONS WERE UNDERTAKEN TO CLARIFY THE STRUCTURE-FUNCTION RELATIONSHIPS.

IV. STRUCTURE-FUNCTION RELATIONSHIPS IN CALCITONINS.

A. Introduction

In the preceding chapter a number of possible conformational variations in the calcitonins were indicated, and it was suggested that these differences played a role in the actions of the hormones at the receptors in the target organs. While such action on the target organs is the ultimate requirement for a hormone, it is only one of many functions a hormone molecule must perform. All definitions of hormones include the concept of release at a distal site followed by transport within the circulation to the target organs. Investigation of the behavior of the hormone during this transport stage involves the definition of the dynamics of secretion and removal whereby the circulating level of hormone is controlled in the normal animal and in the response of the animal to exogenous hormone. This chapter will be confined to the factors involved in the removal of CT from circulation rather than the dynamics of its secretion. Understanding of these factors requires information on both the circulating life of the hormone and the routes by which it is removed from the circulation. Such removal should be especially critical for a hormone like CT which in mammals, is fast acting (31) and responds to rapid changes in plasma calcium levels (46). The maintenance of a low circulating hormone level would be essential to effective endocrine function because the release of new hormone would have little effect on an already high

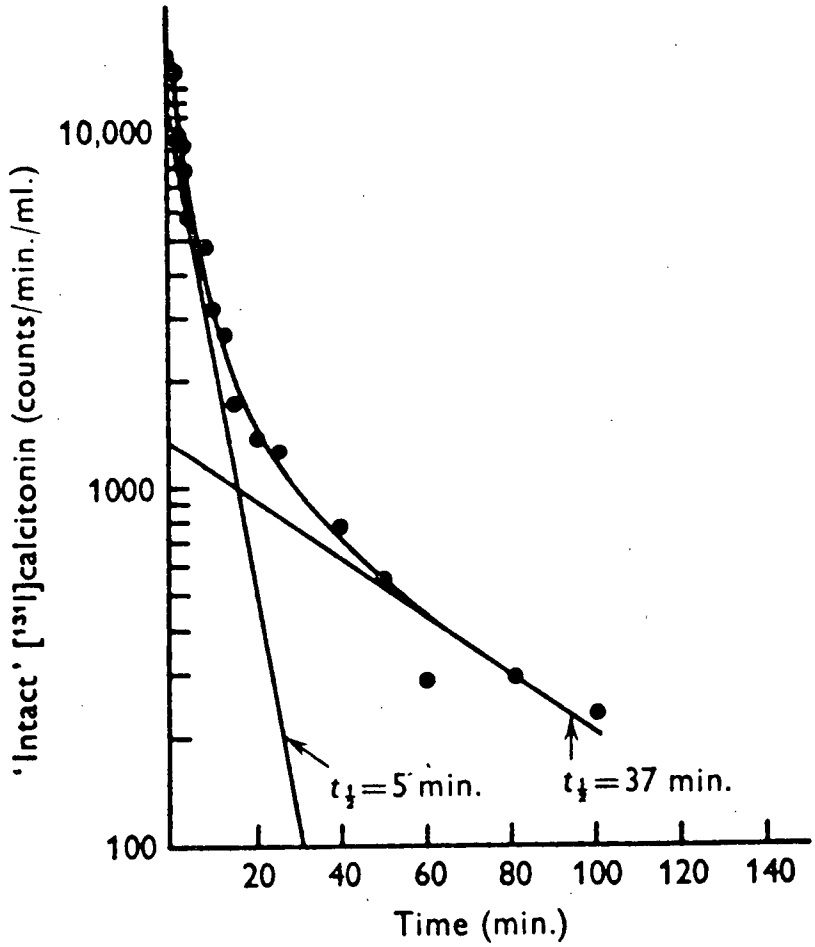
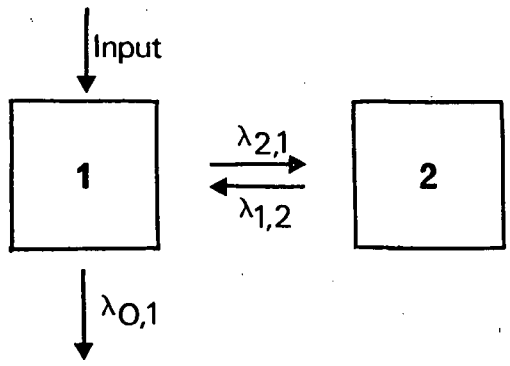


FIGURE 32. DISAPPEARANCE OF "INTACT" CALCITONIN AFTER INJECTION OF (^{131}I)-PORCINE CT (130).

CIRCULATING LEVEL AND THEREFORE EXERT LITTLE CONTROL. THIS IS CERTAINLY TRUE IN MAMMALS WHERE CT LEVELS ARE USUALLY LOW AND THE RESPONSE TO A CHALLENGE IS A RAPID RELEASE OF A PULSE OF HORMONE FOLLOWED BY A STEADY RELEASE AT A LOWER RATE (4). EARLY STUDIES ON SALMON CT SHOWED THAT IT HAD A PROLONGED ACTION IN MAMMALS AND WAS INACTIVATED AT A MUCH LOWER RATE IN IN VITRO INCUBATIONS WITH PLASMA (26). THESE RESULTS SUGGESTED THAT THE ENDOCRINE SYSTEM MIGHT FUNCTION DIFFERENTLY IN THE SALMON AND THAT THE SALMON HORMONE MIGHT BE USEFUL CLINICALLY. THEY ALSO SUGGESTED A POSSIBLE EXPLANATION FOR THE HIGH SPECIFIC ACTIVITY OF SALMON CT, AND THIS STUDY WAS UNDERTAKEN TO ELUCIDATE THE RELATIONSHIPS BETWEEN THE KNOWN STRUCTURAL DIFFERENCES OF THE CALCITONINS AND THEIR BIOLOGICAL ACTIVITIES.

THE BEST DATA ON THE CIRCULATING HALF-LIFE OF CT COMES FROM THE STUDIES OF WEST ET AL (130) ON THE CLEARANCE OF EXOGENOUS PORCINE CT FROM THE PIG. THE HORMONE CONCENTRATION DECREASES AS SHOWN IN FIGURE 32, WHICH IS A TWO-COMPONENT, EXPONENTIAL CURVE SHOWING THE DECLINE IN RADIOACTIVITY ASSOCIATED WITH (^{131}I)-PORCINE CT IN PLASMA TAKEN AT INTERVALS AFTER AN INJECTION OF LABELLED CT. A SIMILAR DECLINE WAS SEEN IN BIOLOGICAL ACTIVITY IN EXPERIMENTS USING LARGE DOSES OF UNLABELLED PORCINE CT. THE AUTHORS USED THE MODEL SHOWN BELOW TO ANALYSE THE KINETICS OF THE DISAPPEARANCE IN THE EXPERIMENT SHOWN IN FIGURE 32, AND SUGGESTED THAT THE RAPID COMPONENT ($T_{\frac{1}{2}}$ = 5 MIN) REPRESENTS THE EQUILIBRATION OF THE TWO COMPARTMENTS WHILE THE SLOWER COMPONENT

($T_{\frac{1}{2}}$ = 37 MIN) REPRESENTS THE ACTUAL RATE OF DEGRADATION OF THE HORMONE.



THEIR ANALYSIS ALSO INDICATED VOLUMES OF 9% AND 16% OF BODY WEIGHT FOR COMPARTMENTS 1 AND 2 RESPECTIVELY. WHILE THIS MODEL SYSTEM CAN PRODUCE THE PATTERN SEEN IN FIGURE 32, THERE ARE A NUMBER OF OTHER MODELS WHICH COULD EQUALLY WELL PRODUCE THIS RELATIVELY COMMON TYPE OF CURVE (109, 117), AND A VARIETY OF DATA IMPLY THAT THIS MODEL IS AN OVER-SIMPLIFICATION. THE AUTHORS THEMSELVES SUGGEST THAT TISSUE INACTIVATION IS A MAJOR FACTOR IN THE DECLINE OF ACTIVITY, BUT THIS MODEL FAILS TO ALLOW FOR INACTIVATION OUTSIDE OF COMPARTMENT 1.

A COMPLETE MODEL MUST ALSO INDICATE THE ROUTES OF DISAPPEARANCE AND ALLOW ANALYSIS OF THEIR RELATIVE IMPORTANCE. THE CURRENTLY AVAILABLE DATA FOR CT DO NOT ALLOW SUCH AN ANALYSIS, BUT THEY DO AT LEAST PROVIDE A BASIS FOR SUGGESTING A MORE COMPREHENSIVE MODEL. STUDIES OF OTHER POLYPEPTIDE HORMONES SUCH AS OXYTOCIN (53, 55), ADH (39, 129) AND INSULIN (63, 106) HAVE PROVIDED A FAIRLY CLEAR PICTURE OF THE FACTORS INVOLVED IN THE REMOVAL OF THIS TYPE OF HORMONE FROM

THE CIRCULATION, AND THE ROLE OF MANY OF THESE FACTORS IN REMOVING CT HAS BEEN INVESTIGATE. THE OVERALL PICTURE IS QUITE COMPLEX, BUT IT CAN BE BROKEN DOWN INTO THE FIVE MAJOR FACTORS LISTED BELOW.

1. EQUILIBRATION OF FREE HORMONE IN THE PLASMA WITH HORMONE BOUND TO PLASMA PROTEINS.
2. RENAL EXCRETION OF HORMONE.
3. UPTAKE OF HORMONE BY TARGET ORGANS AND OTHER ORGANS.
4. INACTIVATION OF HORMONE BY TARGET ORGANS AND OTHER ORGANS.
5. INACTIVATION OF HORMONE IN PLASMA.

THE FIRST FACTOR WAS NOT CONSIDERED IN THE MODEL OF WEST ET AL ALTHOUGH CONSIDERABLE EVIDENCE EXISTS FOR THE BINDING OF CT TO PLASMA PROTEINS (67, 121). THERE IS, HOWEVER, NO CLEAR EVIDENCE THAT THESE PROTEINS ARE SPECIFIC CARRIERS. HORMONE BOUND TO LARGE PROTEINS WOULD BEHAVE QUITE DIFFERENTLY FROM FREE HORMONE SINCE IT WOULD PROBABLY BE PROTECTED FROM DIFFUSION OUT OF THE PLASMA, RENAL EXCRETION AND INACTIVATION BY PLASMA ENZYMES. THE PRESENCE OF SUCH PROTEINS COULD IN FACT EXPLAIN CERTAIN ANOMALIES IN THE RESULTS OF WEST ET AL WHICH INDICATED THAT THE VOLUME OF COMPARTMENT 1 WAS NEARLY DOUBLE THE PLASMA VOLUME AS DETERMINED FROM THE VOLUME OF DISTRIBUTION FOR (^{131}I)-PORCINE ALBUMIN (5% BODY WEIGHT). THEIR STUDY ALSO SHOWED THAT THE APPARENT VOLUME OF THIS COMPARTMENT INCREASED AS THE DOSE OF INJECTED CALCITONIN INCREASED. THESE RESULTS WOULD BE

EXPECTED IF COMPARTMENT 1 CONTAINED TWO COMPONENTS, ONE BOUND AND ONE FREE, AND IF PLASMA SAMPLING ACTUALLY MEASURED BOTH COMPARTMENTS. WHILE THERE IS NO CLEAR EVIDENCE FOR THIS FROM TRACER STUDIES, WORK BY LEGGATE ET AL (67) INDICATES THAT BOTH BOUND AND FREE CT CAN BE DETECTED BY BIOASSAY. IF THIS SITUATION DOES EXIST HIGHER DOSES OF CT WHICH INCREASE THE LEVEL OF FREE HORMONE WOULD SHIFT INCREASING AMOUNTS OF HORMONE ONTO BINDING PROTEINS AND THE APPARENT SIZE OF THE COMPARTMENT WOULD INCREASE. THESE BINDING PROTEINS WOULD CERTAINLY PLAY A MAJOR ROLE IN RETAINING CT IN THE PLASMA, AND STRUCTUAL DIFFERENCES BETWEEN CALCITONINS COULD INFLUENCE THE DEGREE OF BINDING.

DETAILED INFORMATION OF THE ROLE OF RENAL EXCRETION IN THE REMOVAL OF CT FROM PLASMA IS NOT AVAILABLE, HOWEVER, SEVERAL REPORTS SUGGEST THAT IT IS RELATIVELY MINOR. NEER ET AL (79) HAVE REPORTED THAT THE RADIO-IMMUNOASSAYABLE CT IN THE URINE FOLLOWING AN INFUSION OF PORCINE CT INTO A PATIENT WITH PAGET'S DISEASE ACCOUNTED FOR ONLY 0.1% OF THE TOTAL INFUSED. WEST ET AL (130) REPORTED SOMEWHAT HIGHER VALUES FOR TCA PRECIPITABLE LABELLED CT IN URINE AFTER INJECTIONS OF (^{131}I)-PORCINE CT RANGING FROM 2.5 TO 14% OF THE TOTAL INJECTION. THEIR RESULTS INDICATED THAT AT LEAST PART OF THIS MATERIAL WAS STILL BIOLOGICALLY ACTIVE. THUS EXCRETION IS A ROUTE OF REMOVAL, BUT ITS RELATIVE IMPORTANCE IS UNCLEAR.

THE QUESTION OF UPTAKE BY VARIOUS ORGANS APPEARS TO BE CONSIDERABLY MORE IMPORTANT, AND MORE COMPLEX. UPTAKE BY TARGET ORGANS MUST OCCUR IF THE HORMONE IS TO CAUSE A RESPONSE,

TABLE VIII

DISTRIBUTION OF ^{125}I-CT AND ^{125}I-PTH IN RAT TISSUES AFTER INTERVENOUS INJECTION. (36)

TISSUE	CT % PER G TISSUE	PTH % PER G TISSUE	CT % IN TOTAL TISSUE MASS	PTH % IN TOTAL TISSUE MASS
LIVER	6.4	3.2	13.9	7.0
SKELETAL MUSCLE	*	*	13.0	14.7
BLOOD	1.0	1.5	6.5	10.1
BONE	0.3	0.6	4.4	7.5
KIDNEY	3.2	22.6	2.6	18.0

* NOT REPORTED.

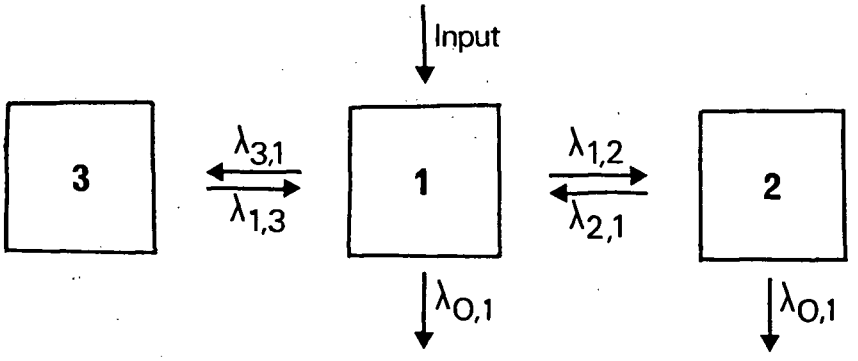
AND THE CONSEQUENCES OF THIS UPTAKE ARE QUITE DIFFERENT FROM THOSE OF UPTAKE BY OTHER ORGANS. THE HORMONE TAKEN UP BY THE TARGET ORGANS CAUSES THE PHYSIOLOGICAL RESPONSE WHILE THAT TAKEN UP BY OTHER ORGANS IS AT BEST STORED AND IS MOST LIKELY COMPLETELY REMOVED FROM ANY POSSIBLE EFFECT ON THE RESPONSE. THE RESULTS OF DE LUISE ET AL (36) SHOWN IN TABLE VIII INDICATE THE DISTRIBUTION OF RADIOACTIVITY IN VARIOUS TISSUES 10 MINUTES AFTER IV INJECTION OF (^{125}I)-PORCINE CT AND (^{125}I)-PTH INTO RATS. THESE RESULTS CONFIRM THE RAPID DISAPPEARANCE OF CT FROM THE PLASMA AND INDICATE THAT MUCH OF THE HORMONE MOVES INTO VARIOUS TISSUES. THE AMOUNT PRESENT IN BONE, THOUGHT TO BE THE PRIMARY TARGET ORGAN FOR CT, SEEMS SMALL; BUT IN TERMS OF THE AMOUNT OF LIVING TISSUE IT IS ACTUALLY QUITE A LARGE PROPORTION OF THE TOTAL. THE LIVER ALSO CONTAINS A DISPROPORTIONATELY LARGE AMOUNT OF CT AND A SMALL AMOUNT OF PTH SUGGESTING SOME DEGREE OF SELECTIVE UPTAKE BY THIS ORGAN.

ALTHOUGH THIS WORK ESTABLISHES THAT CT IS RAPIDLY TAKEN UP BY VARIOUS TISSUES, THERE IS NO INDICATION WHETHER THE HORMONE'S BIOLOGICAL ACTIVITY IS THEN ENDED OR IF IT IS MERELY STORED PENDING LATER RELEASE. IF THE CT IN THESE TISSUES IS RAPIDLY INACTIVATED THE MOVEMENT BETWEEN PLASMA AND TISSUE WILL BE ESSENTIALLY UNIDIRECTIONAL, SERVING ONLY TO MAINTAIN LOW CIRCULATING LEVELS. IF, ON THE OTHER HAND, IT IS NOT DESTROYED, THE CT IN THE TISSUES COULD RETURN TO THE PLASMA WHEN THE CIRCULATING LEVEL WAS REDUCED AND THE RESPONSE TO A SINGLE DOSE OR PULSE WOULD BE PROLONGED.

FURTHER WORK BY DE LUISE ET AL (37) INDICATES THE INACTIVATION OF PORCINE CT BY VARIOUS TISSUE SLICES AND HOMOGENATES FROM RATS, PARTICULARLY FROM THE LIVER, IS RAPID. OTHER WORKERS HAVE ALSO SEEN SIMILAR RAPID INACTIVATION OF PORCINE CT IN TISSUE HOMOGENATES (44, 75) WHICH SUPPORT THE IDEA THAT THIS TYPE OF INACTIVATION IS IMPORTANT IN THE DISTRUCTION OF CT.

A SECOND TYPE OF INACTIVATION OCCURS IN THE PLASMA. TASHJIAN AND MUNSON (121) FIST SHOWED THIS DURING INCUBATIONS OF PORCINE CT IN HUMAN SERUM. WEST ET AL (130) HAVE REPORTED A $T_{\frac{1}{2}}$ FOR PORCINE CT IN PORCINE PLASMA IN VITRO AT 37^{0} C OF 1.5 - 2.0 HOUR. IF A SIMILAR RATE OF INACTIVATION OCCURS IN VIVO IT COULD ACCOUNT FOR ONLY ABOUT 10% OF THE TOTAL INACTIVATION SEEN IN THE SLOW COMPONENT OF FIGURE 32. WHILE THIS IS QUANTITATIVELY ONLY A RELATIVELY SMALL FRACTION OF TOTAL INACTIVATION IT MAY HAVE A DISPROPORTIONATE EFFECT ON THE RESPONSE SINCE ITS ACTION EXTENDS FROM THE INSTANT OF RELEASE OR INJECTION TO THE TIME OF UPTAKE BY THE TARGET ORGANS AND ALL OF THE HORMONE IT EFFECTS IS POTENTIALLY ACTIVE. HORMONE TAKEN UP BY OTHER ORGANS MAY NO LONGER BE ACTIVE EVEN THOUGH IT HAS NOT YET BEEN DESTROYED.

THE MODEL SHOWN ON THE NEXT PAGE PROVIDES FOR THE EQUILIBRATION AND INACTIVATION DISCUSSED AND CAN STILL FIT THE TYPE OF DOUBLE EXPONENTIAL CURVE SEEN IN FIGURE 32. IN THIS MODEL COMPARTMENT 1 REPRESENTS FREE HORMONE IN THE PLASMA, WHILE COMPARTMENT 3 REPRESENTS HORMONE BOUND TO PLASMA PROTEINS. $\lambda_{3,1}$ AND $\lambda_{1,3}$ REPRESENT THE EQUILIBRIUM BETWEEN



BOUND AND FREE HORMONE, AND HORMONE IN COMPARTMENT 3 IS ASSUMED TO BE IMMUNE TO DIRECT INACTIVATION OR EXCRETION. COMPARTMENT 2 INCLUDES ALL EXTRAPLASMA SPACES AND $\lambda_{2,1}$ AND $\lambda_{1,2}$ ARE THE FRACTIONAL TURNOVER RATES BETWEEN THESE COMPARTMENTS. $\lambda_{0,1}$ INCLUDES DISTRUCTION IN PLASMA AND EXCRETION, WHILE $\lambda_{0,2}$ REPRESENTS THE COMBINED DISTRUCTION RATES FOR ALL OF THE EXTRAPLASMA TISSUES. CLEARLY THE DATA AVAILABLE DOES NOT PERMIT ANALYSIS OF ALL OF THESE COMPONENTS, BUT THE MODEL IS USEFUL IN DETERMINING THE IMPORTANCE OF THE VARIOUS FACTORS INVOLVED. THIS IS PARTICULARLY VALUABLE SINCE THERE IS CONSIDERABLE EVIDENCE THAT THE RELATIVE IMPORTANCE OF THESE FACTORS VARIES FROM SPECIES TO SPECIES AND MUST BE ALLOWED FOR IN COMPARATIVE STUDIES. THE VARIATIONS IN THE PERCENT OF A TOTAL DOSE EXCRETED MENTIONED EARLIER IS AN EXAMPLE OF THIS.

A NUMBER OF SUCH COMPARATIVE INACTIVATION STUDIES HAVE BEEN DONE IN THIS DEPARTMENT INDICATING THAT STRUCTURAL DIFFERENCES MAY BE CORREALATED WITH VARIATIONS IN INACTIVATION RATE. EARLY WORK (26) SUGGESTED A RELATIONSHIP BETWEEN THE MAGNITUDE OF THE AREA RESPONSES (31) AND THE

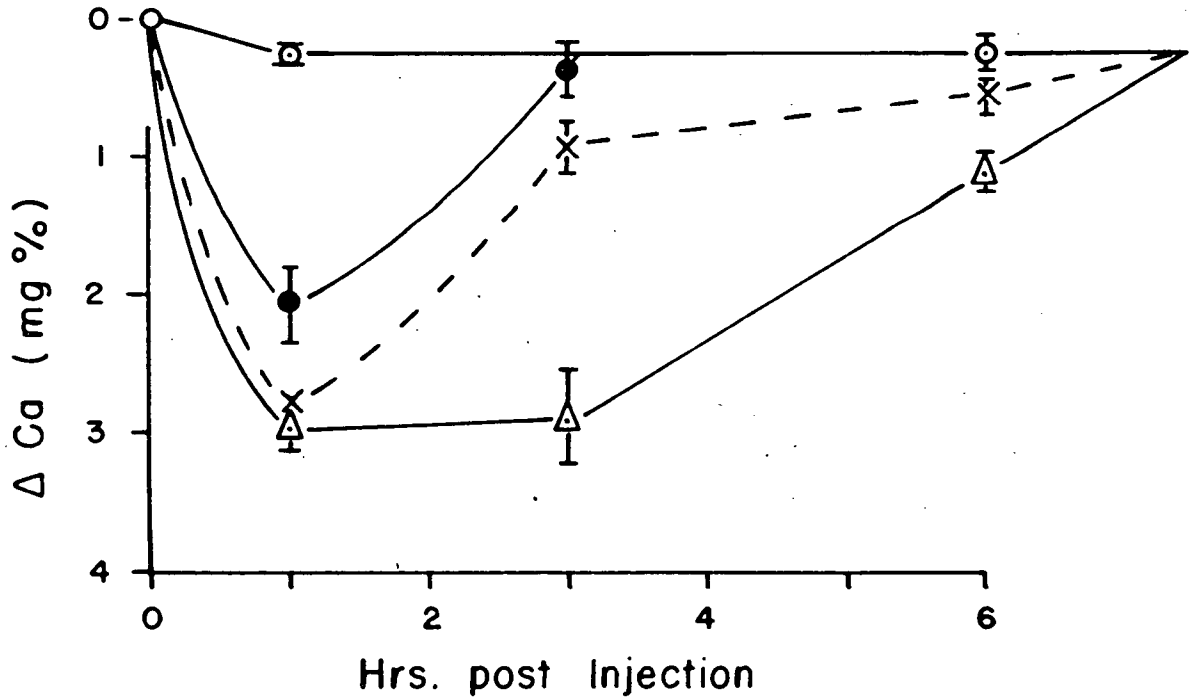


FIGURE 33. AREA RESPONSE CURVES FOR VARYING DOSES OF HUMAN CT IN RATS (85). O- VEHICLE ONLY, ● - 20 MRC U, X - 100 MRC U, Δ - 400 MRC U.

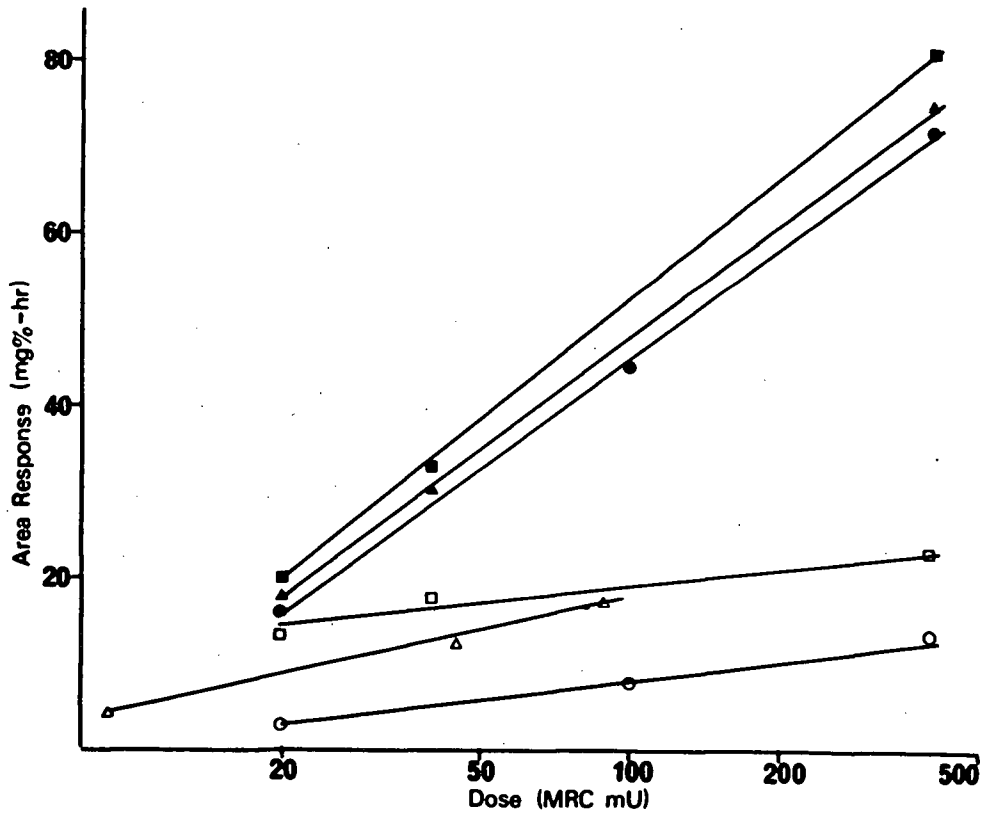


FIGURE 34. PLOTS OF LOG DOSE VERSUS AREA RESPONSE FOR CALCITONINS FROM SEVERAL SPECIES (85). ○- HUMAN CT, △- BOVINE CT (31), □- PORCINE CT, ●-COD CT, ▲-SALMON CT, ■- CHICKEN CT.

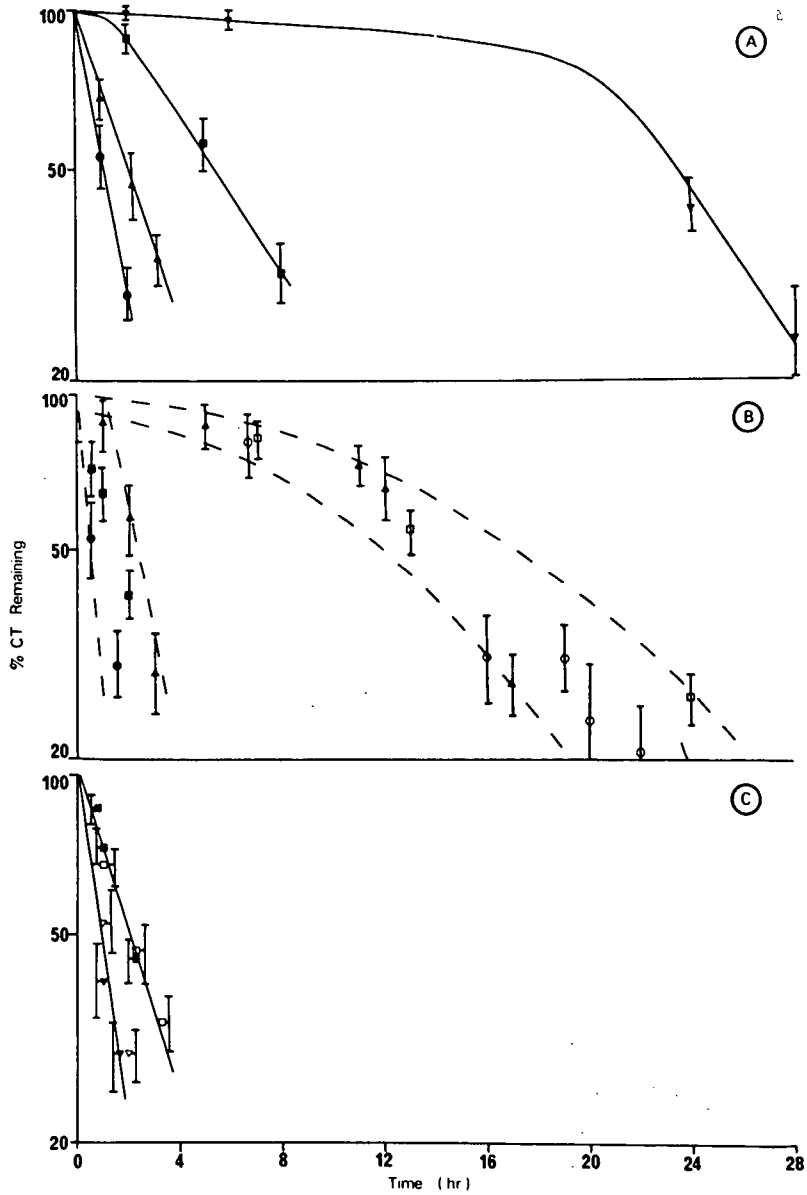


FIGURE 35. INACTIVATION OF CALCITONINS FROM SEVERAL SPECIES DURING INCUBATION IN SERUMS FROM SEVERAL SPECIES (85).

(A) RAT SERUM. ● – PORCINE CT, ▲ – HUMAN CT, ■ – SALMON CT, ▼ – CHICKEN CT.

(B) PORCINE CT IN: ▲ – PORCINE SERUM, ● – CHICKEN SERUM, ■ – SALMON SERUM.
SALMON CT IN: △ – PORCINE SERUM, ○ – CHICKEN SERUM, □ – SALMON SERUM.

(C) HUMAN SERUM. ■ – HUMAN CT, ▼ – PORCINE CT.
RAT SERUM. □ – HUMAN CT, ▽ – PORCINE CT.

IN VITRO INACTIVATION RATES OF THE HORMONES INCUBATED IN PLASMA. MORE RECENT EXPERIMENTS (85) HAVE CONFIRMED AND EXTENDED THESE FINDING. THE TABLE BELOW PRESENTS A TYPICAL SET OF AREA RESPONSES FOR HUMAN CT IN THE RAT CALCULATED FROM THE DATA IN FIGURE 33.

DOSE (MRC MU)	AREA (MG% - HOUR)
20	3.5
100	6.8
400	13.3

WHEN THE AREA RESPONSES ARE PLOTTED AGAINST THE LOG OF THE DOSE A LINEAR RELATIONSHIP IS SEEN AS SHOWN IN FIGURE 34. THIS FIGURE ALSO SHOWS THE PLOTS FOR SIMILAR DATA FOR TWO OTHER THYROID CALCITONINS AND FOR THREE ULTIMOBRANCHIAL CALCITONINS. THE SLOPES OF THESE LINES INDICATED THAT A CLEAR DICHOTOMY EXISTED BETWEEN THESE TWO TYPES OF CT AND THAT THE RESPONSE TO THE ULTIMOBRANCHIAL CALCITONINS, HAD A MARKEDLY DIFFERENT TIME COURSE.

WHEN THESE CALCITONINS WERE INCUBATED WITH SERUM FROM VARIOUS SPECIES AT 37^{0} AND THE RATE OF INACTIVATION DETERMINED BY BIOASSAY, A FAIRLY CLEAR PATTERN EMERGED WITH THE MAMMALIAN CALCITONINS AS A GROUP BEING MORE RAPIDLY INACTIVATED THAN THE ULTIMOBRANCHIAL CALCITONINS AS SHOWN IN FIGURE 35 (A). AS SHOWN IN FIGURE 35 (B) AND (C) THIS RELATIONSHIP WAS RELATIVELY CONSTANT REGARDLESS OF THE PLASMA USED AS THE INACTIVATING MEDIUM, SUGGESTING THAT SOME FUNDAMENTAL STRUCTURAL DIFFERENCE BETWEEN THE TWO TYPES OF CALCITONINS WAS RESPONSIBLE FOR DIFFERENTIAL INACTIVATION

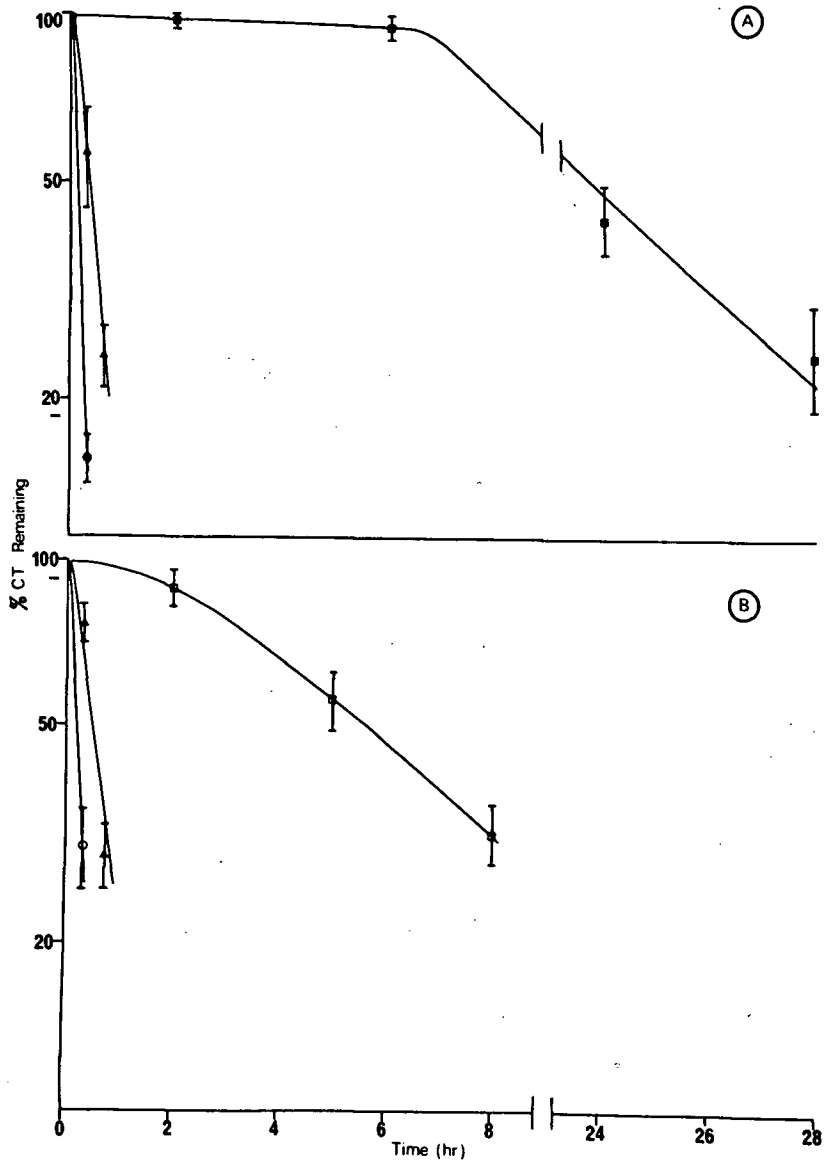


FIGURE 36. COMPARISON OF IN VITRO INACTIVATION OF SALMON AND CHICKEN CT IN RAT SERUM TO THEIR DISAPPEARANCE RATES IN NORMAL AND NEPHRECTOMIZED RATS (85).

(A) CHICKEN CT ■- RAT SERUM, ▲- NEPHRECTOMIZED RATS, ●- NORMAL RATS.

(B) SALMON CT □- RAT SERUM, △- NEPHRECTOMIZED RATS, ○- NORMAL RATS.

RATES IN PLASMA. THUS THE STABILITY OF A CT *IN VITRO* IN SERUM WAS A FAIRLY GOOD INDEX OF THE EXPECTED TIME COURSE FOR THE *IN VIVO* RESPONSE TO THAT CT. THIS DID NOT MEAN THAT THERE WAS A DIRECT CAUSE AND EFFECT RELATIONSHIP, HOWEVER. THE RESULTS OF DE LUISE *ET AL* (37) INDICATED THAT THE INACTIVATION OF SALMON CT BY LIVER HOMOGENATES WAS ALSO MUCH SLOWER THAN THE INACTIVATION OF PORCINE CT, SUGGESTING THAT THE MECHANISM FOR INACTIVATION IN THE LIVER MIGHT BE SIMILAR TO THAT IN PLASMA. PERHAPS THE SAME ENZYMES WERE RESPONSIBLE IN BOTH CASES SINCE THE LIVER IS KNOWN TO BE THE SOURCE OF MANY PLASMA ENZYMES.

AN ADDITIONAL POSSIBILITY WHICH HAD TO BE CONSIDERED WAS THAT THE DECREASED INACTIVATION OF THE ULTIMOBRANCHIAL CALCITONINS RESULTED FROM INCREASED BINDING TO PROTECTING PROTEINS RATHER THAN FROM A DECREASED RATE OF ENZYMATIC DEGRADATION. THE DISAPPEARANCE RATES OF BIOLOGICAL ACTIVITY FOR TWO CALCITONINS IN *IN VIVO* EXPERIMENTS IN NORMAL AND NEPHRECTOMIZED RATS ARE SHOWN IN FIGURE 36 COMPARED TO THE DISAPPEARANCE RATES RESULTING FROM *IN VITRO* INCUBATIONS IN RAT SERUM. THE VARIATIONS IN THE *IN VIVO* DISAPPEARANCE RATES WERE NOT SIGNIFICANT AND SHOWED NO CORRELATION WITH THE AREA RESPONSE DATA. IT SHOULD BE NOTED THAT THE DOSES GIVEN WERE NOT LARGE ENOUGH TO ALLOW MEASUREMENT OF THE SECOND EXPONENTIAL COMPONENT, SINCE CIRCULATING HORMONE LEVELS DROPPED TO UNMEASURABLE LEVELS BEFORE THIS PART OF THE CURVE WOULD BE REACHED. WEST *ET AL* (130) REPORTED SIMILAR RESULTS IN EXPERIMENTS USING RELATIVELY LOW DOSES

WHERE HORMONE LEVELS WERE DETERMINED BY BIOASSAY. THESE CURVES THEREFORE GIVE NO INFORMATION ABOUT INACTIVATION RATES, BUT RATHER INDICATE THAT NEITHER THE DIFFUSION RATES FROM COMPARTMENT 1 TO COMPARTMENT 2 NOR RENAL EXCRETION RATES FOR THESE CALCITONINS DIFFER SIGNIFICANTLY. THIS SUGGESTS THAT THE BINDING OF THE CALCITONINS TO PLASMA PROTEINS IS NOT A MAJOR FACTOR IN DETERMINING THE AREA RESPONSES.

ALL OF THE DATA DISCUSSED ABOVE POINTED TOWARDS THE PROTEOLYTIC ENZYMES IN PLASMA AS A MAJOR FACTOR IN DETERMINING THE TIME COURSE OF THE PHYSIOLOGICAL RESPONSE TO STRUCTUALLY DIFFERENT CALCITONINS. THE POSSIBILITY STILL EXISTED THAT THE PLASMA ENZYMES WERE NOT DIRECTLY RESPONSIBLE, BUT IT SEEMED CLEAR THAT THE RATE OF INACTIVATION BY THESE ENZYMES WAS INDICATIVE OF THE OVERALL RATE OF INACTIVATION. THE ROLE OF CARRIER PROTEINS WAS ALSO UNCLEAR, BUT IT APPEARED THAT A BETTER KNOWLEDGE OF THE CHARACTERISTICS OF THE DEGRADING ENZYMES WOULD BE REQUIRED BEFORE THIS PROBLEM COULD BE STUDIED. IT WAS THEREFORE ESSENTIAL TO STUDY THESE ENZYMES IN GREATER DETAIL TO OBTAIN A CLEARER PICTURE OF HOW VARIATIONS IN HORMONE STRUCTURE INFLUENCED THE RESPONSE TO THE HORMONES.

THIS TYPE OF INFORMATION IS PARTICULARLY IMPORTANT SINCE WORK BY NEER ET AL (79) HAS SHOWN THAT THE RESPONSE DIFFERENCES OCCURED IN MAN AS WELL AS THE RAT AND THAT THE MORE STABLE SALMON CT IS POTENTIALLY MUCH MORE VALUABLE THERAPEUTICALLY THAN IS PORCINE CT. SINCE ONE OF THE MAJOR

VALUES OF COMPARATIVE DATA IS ITS APPLICATION TO FINDING OR DESIGNING MORE STABLE AND MORE ACTIVE HORMONES FOR CLINICAL APPLICATION, HUMAN PLASMA WAS USED IN THESE STUDIES. THE WORK OF TASHJIAN AND VOELKEL (122) ON THE HUMAN PLASMA INACTIVATORS PROVED VALUABLE IN DESIGNING EARLY EXPERIMENTS.

A LARGE NUMBER OF PROTEOLYTIC ENZYMES ARE KNOWN TO EXIST IN HUMAN PLASMA AND SERUM, AND A NUMBER OF THEM COULD ACT ON THE CALCITONINS. THE CHARACTERIZATION OF THE ROLES OF ALL THESE ENZYMES IN THE INACTIVATION OF THE CALCITONINS WAS BEYOND THE SCOPE OF THIS STUDY AND WORK WAS CONFINED TO ENZYMES WHICH ACTED AS MAJOR PATHWAYS FOR DEGRADATION AND WHICH APPEARED TO DIFFERENTIATE BETWEEN CALCITONINS WITH KNOWN STRUCTURAL DIFFERENCES. AMONG THE ENZYMES OF KNOWN SPECIFICITIES WHICH MIGHT BE EXPECTED TO FIT THIS DESCRIPTION WERE THOSE WHICH CLEAVED AT AROMATIC RESIDUES SUCH AS PEPSIN, CONVERTING ENZYME AND POSSIBLY CHYMOTRYPSIN; ALL OF WHICH ARE KNOWN TO OCCUR IN PLASMA TO AT LEAST A LIMITED EXTENT AND WOULD BE MUCH MORE DAMAGING TO THE MORE AROMATIC MAMMALIAN CALCITONINS THAN TO THE SALMON HORMONE. A SECOND GROUP OF ENZYMES LIKELY TO BE INVOLVED IN DEGRADING THE CALCITONINS WERE THOSE WITH SPECIFICITIES FOR BASIC RESIDUES SUCH AS TRYPSIN, THE PLASMA KALLIKREINS, THE ENZYMES FROM PROTHROMBIN (AUTOPROTHROMBIN C AND THROMBIN) AND THE PLASMIN TYPE ENZYMES. ANOTHER REACTION WHICH COULD OCCUR, ADDING EVEN MORE CONFUSION TO THE PICTURE, IS REDUCTION, BY GLUTATHIONE AND OTHER REDUCTIVE AGENTS AND DISULFIDE INTERCHANGE WITH OTHER PLASMA PROTEINS. THE REDUCTIVE INACTIVATION REACTIONS

MAY OCCUR AT DIFFERENT RATE *IN VIVO* AND *IN VITRO* SINCE CONTINUAL RENEWAL OF THE REDUCTION AGENT WOULD BE POSSIBLE *IN VIVO*. THE PROBLEM OF DISULFIDE INTERCHANGE HAS BEEN PARTICULARLY PREVALENT WITH HUMAN CT WHICH APPEARS TO DIMERIZE READILY (80).

THE WORK DESCRIBED IN THIS CHAPTER PARTIALLY CHARACTERIZES THE ROLES OF SOME OF THESE FACTORS IN THE DIFFERENTIAL INACTIVATION OF THE VARIOUS CALCITONINS, AND PROVIDES A STARTING POINT FOR FURTHER STUDIES ON THE DETAILS OF THIS UNDOUBTABLY COMPLEX PROBLEM. THE FIRST STEP IN THIS TYPE OF STUDY IS TO ESTABLISH PROCEDURES FOR ASSAYING THE INACTIVATING PROPERTIES OF THE VARIOUS PLASMA COMPONENTS. THE PROBLEMS INVOLVED IN SUCH AN ASSAY AND THE PROCEDURES WHICH WERE TRIED AND EVENTUALLY ADOPTED ARE DESCRIBED IN THE NEXT SECTION.

B. Assay Procedures.

IT WAS DECIDED THAT ONLY THE BIOASSAY COULD PROVIDE UNEQUIVOCAL DATA ON INACTIVATION SINCE PROCEDURES SUCH AS TCA PRECIPITATION OF ^{131}I LABELLED CT AND RADIOIMMUNOASSAYS CANNOT BE EXPECTED TO CORRESPOND TO THE BIOASSAY UNDER THE CONDITIONS OF THE EXPERIMENTS. FOR EXAMPLE THE SPECIFICITIES OF THE RADIOIMMUNOASSAYS FOR CALCITONIN STUDIED HAVE BEEN SHOWN TO BE FOR ONLY ONE PORTION OF THE MOLECULE (18, 34, 40). CLEARLY THIS PROCEDURE COULD NOT DISTINGUISH BETWEEN INACTIVE FRAGMENTS AND THE ACTIVE WHOLE MOLECULE. THUS THE PROBLEMS OF THE INACTIVATION ASSAY WERE COMPOUNDED BY THE INHERENT VARIABILITY OF THE BIOASSAY.

IN THE EARLY STAGES OF THE EXPERIMENTS ATTEMPTS WERE

MADE TO DEVELOP QUANTITATIVE ASSAY PROCEDURES WHICH WOULD ALLOW DETERMINATION OF THE TOTAL INACTIVATOR PRESENT IN A SAMPLE AND PERMIT ESTIMATION OF RECOVERY IN SUBSEQUENT PURIFICATION STAGES. THESE MET WITH SOME SUCCESS AND IT WAS POSSIBLE TO MEASURE RATE CONSTANTS AND ESTIMATE MAXIMUM VELOCITIES FOR SOME OF THE STARTING MATERIALS. THE PROCEDURES INVOLVED WERE QUITE COMPLEX HOWEVER, AND REQUIRED VERY EXTENSIVE USE OF BIOASSAYS SINCE NUMEROUS INCUBATIONS WERE NECESSARY WITH FREQUENT SAMPLES FOR BIOASSAY. IT BECAME EVIDENT THAT THIS PROCEDURE WAS NOT SUITABLE FOR SURVEYING COLUMN ELUATES BECAUSE OF ITS COMPLEXITY AND BECAUSE OF THE INSTABILITY OF THE CALCITONINS AT LOW CONCENTRATION IN BUFFER SOLUTIONS. ALDRED AND SCHLUETER (1) HAVE CHARACTERIZED THIS TYPE OF INACTIVATION IN A NUMBER OF SITUATIONS. BRIEFLY, THEIR RESULTS INDICATED THAT INACTIVATION OCCURRED RAPIDLY ABOVE pH 5 IN BUFFER SOLUTIONS AND THAT THE INACTIVATION COULD BE RETARDED BY THE PRESENCE OF A VARIETY OF OTHER PROTEINS IN THE SOLUTION. IT WAS FOUND, FOR EXAMPLE, THAT WHILE INCUBATION OF CT IN SERUM OR IN SODIUM ACETATE BUFFER CAUSED COMPLETE INACTIVATION IN 4 HOURS AT 37^{0} C, THE INACTIVATION WAS INSIGNIFICANT AT SERUM DILUTIONS OF 1:100 TO 1:1000. THUS SMALL QUANTITIES OF PROTEINS WERE PROTECTIVE. THIS TYPE OF PHENOMENON OCCURED IN THE PRESENT STUDY AND LED TO SITUATIONS WHERE COLUMN ELUATE FRACTIONS CONTAINING LITTLE OR NO PROTEIN APPEARED TO CONTAIN MORE INACTIVATOR THAN THE STARTING MATERIAL--A CLEARLY UNSATISFACTORY RESULT. EVENTUALLY A SEMI-QUANTITATIVE ASSAY FOR INACTIVATOR WAS

DEVELOPED WHICH WAS NOT SENSITIVE TO VARIATIONS IN PROTEIN CONCENTRATION. IT SEEMED LIKELY THAT A MAJOR FACTOR IN THIS BUFFER INACTIVATION WAS ADSORPTION OF THE CT TO THE WALLS OF THE INCUBATION VESSELS, AND A SERIES OF TESTS WERE CARRIED OUT TO SEE IF THIS ADSORPTION COULD BE BLOCKED BY MODIFYING THE VESSEL WALLS. SINCE ADDITION OF EXTRANEOUS PROTECTIVE PROTEINS SEEMED LIKELY TO INTERFER WITH THE ENZYMATIC INACTIVATION WHICH WAS BEING MEASURED THIS APPROACH WAS NOT SUITABLE FOR THE PRESENT PURPOSES. SILICONIZED GLASS AND PLASTIC VESSELS PROVED TO CAUSE A MORE RAPID INACTIVATION THAN GLASS, AND ACID WASHED GLASS WAS EQUALLY UNSUITABLE. IN FACT, ACID WASHED GLASS INACTIVATED AT A GREATER RATE THAN DETERGENT WASHED GLASS SUGGESTING THAT THE GLASS SURFACE MIGHT BE ACTING AS A CATION EXCHANGER, AND GLASS VESSELS WASHED IN 2 M SODIUM HYDROXIDE PRIOR TO A DISTILLED WATER RINSE WERE TRIED. CT COULD BE INCUBATED AT 37^{0} C IN pH 7.4 TRIS-HCL BUFFER FOR PERIODS UP TO TWO HOURS IN THESE VESSELS WITH LITTLE OR NO INACTIVATION. IF THE INCUBATIONS WERE EXTENDED FOR LONGER PERIODS A SLOW INACTIVATION OCCURED POSSIBLY RESULTING FROM DISULFIDE INTERCHANGE AT THIS HIGH pH.

SINCE INCUBATION OF CT WITH THE MAJOR INACTIVATOR FRACTIONS RESULTED IN MARKED REDUCTION IN ACTIVITY IN A 30 MINUTE PERIOD AT 37^{0} C AND pH 7.4, IT WAS POSSIBLE TO USE THIS RELATIVELY SIMPLE PROCEDURE TO INDICATE WHICH FRACTIONS CONTAINED SIGNIFICANT INACTIVATOR ALTHOUGH NO DETAILED RECORD OF QUANTITATIVE YIELDS WAS POSSIBLE. IN GENERAL,

ALIQUOTS OF A SOLUTION CONTAINING 2.5 MRC U/ML OF PORCINE CT (ARMOUR PHARMACEUTICAL CO. AL0831) IN pH 7.4, 0.1 M TRIS-HCL WERE MIXED WITH AN EQUAL VOLUME OF VARIOUS COLUMN ELUATE FRACTIONS ADJUSTED TO pH 7.4 WITH TRIS BASE AND INCUBATED FOR ONE HOUR AT 37°C. SAMPLES, TAKEN AT ZERO TIME AND ONE HOUR WERE SUITABLY DILUTED AND BIOASSAYED AS DESCRIBED IN CHAPTER II. TWO DILUTIONS OF EACH SAMPLE WERE ASSAYED WITH THE SECOND USUALLY A TWO FOLD DILUTION OF THE FIRST. FOUR RATS WERE USED FOR EACH DILUTION AND THE REMAINING BIOLOGICAL ACTIVITY CALCULATED FROM THE AVERAGE RESPONSES OF EACH GROUP. THE ZERO TIME ACTIVITY WAS TAKEN AS 100% AND THE PERCENTAGE OF BIOLOGICAL ACTIVITY REMOVED IN ONE HOUR USED AS AN INDEX OF THE INACTIVATOR PRESENT. SLIGHT VARIATIONS OF THIS PROCEDURE WERE USED FOR VARIOUS ASSAYS AND THE DETAILS WILL BE INCLUDED IN THE DESCRIPTIONS OF THE PURIFICATION STAGES.

C. Partial Purification of a Selective "Calcitoninase".

1. Selection of starting material.

THE WORK OF TASHJIAN AND VOELKEL (122) SHOWED THE PRESENCE OF PORCINE CT INACTIVATING FACTORS IN HUMAN AND RAT SERUMS AND PARTIALLY CHARACTERIZED THESE FACTORS. THE WORK DESCRIBED IN THE INTRODUCTION TO THIS CHAPTER INDICATED THAT THESE FACTORS WERE NOT PARTICULARLY EFFECTIVE IN INACTIVATING SALMON CT AND THAT THIS FAILURE TO INACTIVATE THE SALMON HORMONE PROBABLY CONTRIBUTED SIGNIFICANTLY TO THE ENHANCED PHYSIOLOGICAL EFFECT OF THIS HORMONE. FURTHER CHARACTERIZATION OF THESE FACTORS COULD AID IN DEFINING THE STRUCTURAL DIFFERENCES IN THE SALMON MOLECULE RESPONSIBLE FOR THIS

RESISTANCE TO INACTIVATION. SUCH CHARACTERIZATION REQUIRED THAT THE INACTIVATING ENZYMES BE OBTAINED IN RELATIVELY PURE FORM SO THAT ONLY ONE TYPE OF INACTIVATION OCCURED IN A GIVEN INCUBATION ALLOWING IDENTIFICATION OF THE REACTION PRODUCTS TO DETERMINE THE SPECIFICITY OF THE ENZYME. BECAUSE OF THE VAST NUMBER OF COMPONENTS PRESENT IN SERUM AND THE RELATIVELY LARGE QUANTITY OF STARTING MATERIAL WHICH WOULD BE NEEDED TO YIELD SIGNIFICANT PURIFIED PRODUCT COMMERCIALLY PREPARED COHN PLASMA FRACTIONS WERE USED AS STARTING MATERIAL.

IT WAS FIRST NECESSARY, HOWEVER, TO DEMONSTRATE THAT UNCLOTTED PLASMA AND SERUM HAD A SIMILAR INACTIVATING ABILITIES SINCE THE POSSIBILITY EXISTED THAT THE CLOTTING ENZYMES WERE RESPONSIBLE FOR THE INACTIVATION. TO TEST THIS, SAMPLES OF RABBIT SERUM AND HEPARINIZED RABBIT PLASMA WERE PREPARED CONTAINING 20 MRC U/ML OF PORCINE CT AND INCUBATED AT 37^{0}C FOR 75 MIN. ALIQUOTS WERE TAKEN FROM THE INCUBATION SAMPLES AT ZERO TIME AND 75 MIN., AND WERE BIOASSAYED AS PREVIOUSLY DESCRIBED. THE MEAN PLASMA CALCIUM LEVEL OF THE TEN RATS INJECTED WITH BOTH ZERO TIME SAMPLES WAS 8.5 ± 0.1 MG% (±S.E.M.). AFTER THE 75 MIN INCUBATION THE MEAN PLASMA CALCIUM LEVEL OF FIVE RATS INJECTED WITH THE SERUM SAMPLE WAS 9.2 ± 0.1 MG%. THE CORRESPONDING VALUE FOR THE PLASMA SAMPLE WAS 9.3 ± 0.1 MG%. BOTH VALUES WERE SIGNIFICANTLY LOWER THAN THE ZERO TIME LEVEL ($P<.005$), BUT THERE WAS NO SIGNIFICANT DIFFERENCE BETWEEN THE TWO VALUES, INDICATING THAT THERE WAS LITTLE DIFFERENCE IN THE AMOUNT OF INACTIVATOR PRESENT.

TABLE IX

INACTIVATION OF PORCINE CT DURING INCUBATIONS WITH HUMAN PLASMA FRACTIONS

INCUBATION SOLUTION	PLASMA CALCIUM LEVELS ±S.E.M. (MG%) ZERO TIME	3 HOURS
BUFFER ONLY	10.2 ± 0.1 (5)*	-
LYOPHILIZED PLASMA	8.4 ± 0.1 (5)	9.1 ± 0.1 (2)
COHN I	"	8.3 ± 0.1 (2)
COHN II	"	8.3 ± 0.1 (2)
COHN III-O	"	10.1 ± 0.1 (2)
COHN III	"	8.5 ± 0.1 (2)
COHN IV-1	"	10.4 ± 0.1 (2)
COHN IV-4	"	10.7 ± 0.1 (2)
COHN V	"	8.8 ± 0.1 (2)

* NUMBER OF ANIMALS INJECTED.

Cohn fractions from human plasma (Nutritional Biochemical Corp., Cleveland, Ohio) as listed in Table IX were added to pH 7.4, 0.1 M Tris-HCl buffer in concentrations approximating those which exist in plasma (see 92), and shaken for 30 min. Any insoluble residue was removed by centrifugation and the supernates used for incubations with porcine CT. The samples were made up to a concentration of 20 MRC mU/ml, and incubated at 37°C. Bioassays were carried out at zero time and after 3 hours of incubation. As the plasma calciums of the rats injected at zero time did not vary significantly the data was pooled. The amount of CT in the redissolved lyophilized plasma sample after 3 hours was reduced markedly and no measurable CT remained in three of the seven Cohn fractions, indicating that inactivators were present in more than one fraction either because more than one enzyme was involved or because a single enzyme was present in more than one fraction. Further tests on diluted samples of these three fractions indicated that fraction IV-1 was slightly more potent in inactivating porcine CT and this fraction was chosen for further work.

2. Preliminary Purification Experiments.

During the experiments in the previous section it was observed that a large percentage of the material in fraction IV-1 was insoluble in the pH 7.4 buffer. Thus a considerable purification could be achieved by extracting the fraction with this buffer. In an initial experiment 1 g of fraction IV-1 was extracted four times with 10 ml aliquots of buffer.

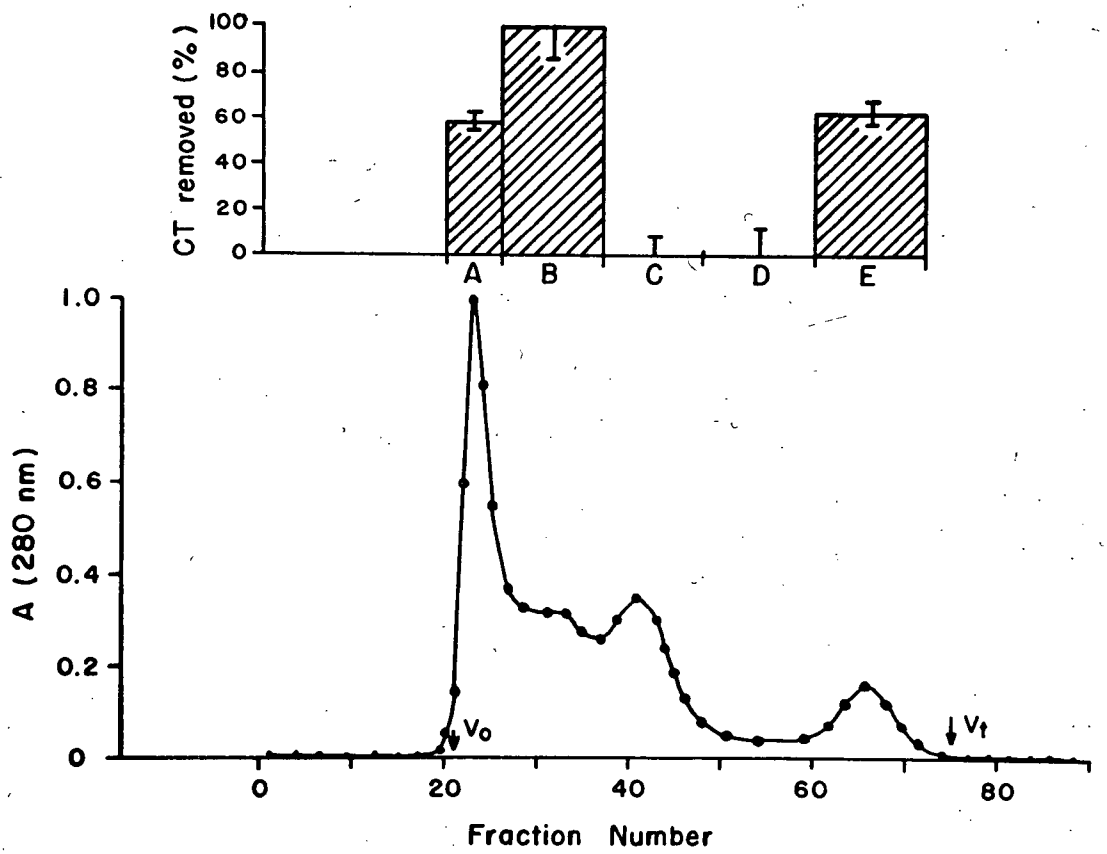


FIGURE 37. ELUTION PROFILE OF AN EXTRACT OF HUMAN COHN FRACTION IV-1 ON SEPHADEX G-200. COLUMN, 2.5 x 90 CM; ELUANT, pH 7.4, 0.1 M TRIS-HCL BUFFER; TEMPERATURE, 4° C; FLOW RATE, 20 CM/HR; FRACTION SIZE, 6.8 ML. %CT REMOVED DETERMINED IN INACTIVATION ASSAY AS DESCRIBED IN TEXT.

After addition of each 10 ml aliquot, the mixture was shaken for 30 min and then centrifuged and the supernates saved. An aliquot of each supernate was then incubated with porcine CT as previously described to determine its inactivation activity. The inactivation rate dropped markedly in the last two samples and it was decided that only the first two samples should be pooled for further purification.

A 3 ml sample of the pooled material was taken for chromatography at 4^{0} C on a 2.5 x 90 cm Sephadex adjustable column containing Sephadex G-200 equilibrated in 7.4, 0.1 M Tris-HCl buffer. The eluate was collected in 6.8 ml fractions at a rate of 20 ml/hr and the absorbance at 280 nm followed as an index of protein. The sample was applied to the bottom of the column through a three-way valve and eluant flow was upwards to prevent the Sephadex from packing. The elution profile for the column is shown in Figure 37. The column eluate was divided into five pools as indicated in the figure and each pool assayed for inactivator as described in Section B except that the CT solution contained only 40 MRC mU/ml so that the final incubation mixture contained 20 MRC mU/ml. The percent of CT activity removed in one hour is shown in the figure above the elution profile.

The results of this experiment were in many ways similar to those of Tashjian and Voelkel (122) on whole human serum. Two regions were observed to contain inactivators in each case and the molecular weights of the inactivators were similar. The larger component had a K_D less than 0.3 in

EACH CASE WHILE THE SMALLER COMPONENTS HAD K_D'S GREATER THAN 0.7. THESE K_D'S INDICATE MOLECULAR WEIGHTS GREATER THAN 100,000 AND LESS THAN 30,000 RESPECTIVELY. THIS DATA CANNOT BE TAKEN TO INDICATE THAT THE SAME COMPONENTS WERE RESPONSIBLE FOR INACTIVATION IN BOTH CASES, BUT THIS POSSIBILITY CANNOT BE RULED OUT. POOL B WAS INVESTIGATED FIRST SINCE IT APPEARED TO CONTAIN MORE INACTIVATING ACTIVITY THAN THE OTHER POOLS.

THE ENTIRE REMAINING VOLUME OF POOL B WAS ALLOWED TO FLOW ONTO A 2 x 20 CM COLUMN OF DEAE-SEPHADEX WHICH HAD BEEN EQUILIBRATED WITH pH 7.4, 0.1 M TRIS-HCL BUFFER AND THE ELUATE COLLECTED IN 6.8 ML FRACTIONS AT A RATE OF 10 ML/HR. A LINEAR GRADIENT OF TRIS-HCL BUFFER FROM 0.1 M (pH 7.4; CONDUCTIVITY, 3.5 mMHOS; VOLUME, 50 ML) TO 0.5 M (pH 7.4; CONDUCTIVITY, 16.0 mMHOS; VOLUME, 50 ML) WAS THEN APPLIED TO THE COLUMN. UNDER THE CONDITIONS OF THIS COLUMN NONE OF THE INACTIVATING ACTIVITY REMAINED ON THE RESIN IN THE INITIAL BUFFER, BUT ABOUT 50% OF THE MATERIAL ABSORBING AT 280 NM WAS ADSORBED AND SUBSEQUENTLY REMOVED AS A BROAD PEAK BY THE GRADIENT. THIS RESULT SUGGESTED THAT DEAE-SEPHADEX COULD BE USED IN A BULK ADSORPTION STEP FOLLOWING THE EXTRACTION TO REMOVE A NUMBER OF CONTAMINANTS.

THE INACTIVATING MATERIAL FROM THE INITIAL ELUATE FRACTIONS OF THIS COLUMN WERE POOLED AND USED IN A SERIES OF EXPERIMENTS TO DEFINE CONDITIONS FOR ADSORPTION OF THE ACTIVE COMPONENT TO CM-SEPHADEX FOR FURTHER PURIFICATION. THE CONDITIONS WHICH PROVED MOST SUCCESSFUL WERE USED IN

SUBSEQUENT EXPERIMENTS AND ARE DESCRIBED IN THE PURIFICATION SCHEDULE IN THE NEXT SECTION.

3. PREPARATION OF HIGHLY PURIFIED "CALCITONINASE".

A 5 G SAMPLE OF COHN FRACTION IV-1 WAS MIXED WITH 30 ML OF PH 7.4, 0.1 M TRIS-HCL BUFFER AND SHAKEN FOR ONE HOUR AT ROOM TEMPERATURE. IT WAS THEN CENTRIFUGED TO YIELD A CLEAR SUPERNATE WHICH WAS REMOVED AND SAVED. THE RESIDUE WAS RESUSPENDED IN ANOTHER 30 ML OF BUFFER AND SHAKEN AGAIN. THIS PROCESS OF RESUSPENSION AND CENTRIFUGATION WAS REPEATED A TOTAL OF THREE TIMES YIELDING 80 ML OF POOLED SUPERNATES. THE POOLED SUPERNATES WERE ADDED TO 20 G OF DEAE-SEPHADEX WHICH HAD BEEN PREVIOUSLY EQUILIBRATED WITH PH 7.4, 0.1 M TRIS-HCL BUFFER AND THE MIXTURE WAS STIRRED FOR TWO HOURS AT ROOM TEMPERATURE. THE MIXTURE WAS THEN FILTERED AND THE SEPHADEX WASHED TWICE WITH 50 ML OF PH 7.4, 0.1 M TRIS-HCL BUFFER. THIS WAS FOLLOWED BY TWO WASHES WITH 50 ML OF DISTILLED WATER, AND ALL OF THE FILTRATES WERE POOLED. THE TOTAL FILTRATE VOLUME OF ABOUT 300 ML WAS REDUCED TO 10 ML BY LYOPHILIZATION, AND THIS 10 ML EXTRACT USED FOR FURTHER PURIFICATION BY COLUMN CHROMATOGRAPHY.

THE ENTIRE 10 ML SAMPLE WAS APPLIED TO A SEPHADEX G-200 COLUMN IDENTICAL TO THAT DESCRIBED IN THE PRECEDING SECTION EXCEPT THAT 0.1 M FORMIC ACID WAS USED AS AN ELUANT. THE HIGH MOLECULAR WEIGHT COMPONENTS CAME OFF IN A SINGLE BROAD PEAK WHICH WAS WELL SEPARATED FROM THE SMALLER COMPONENTS AND THE SALT, AND THE COLUMN FRACTIONS WERE DIVIDED INTO TWO

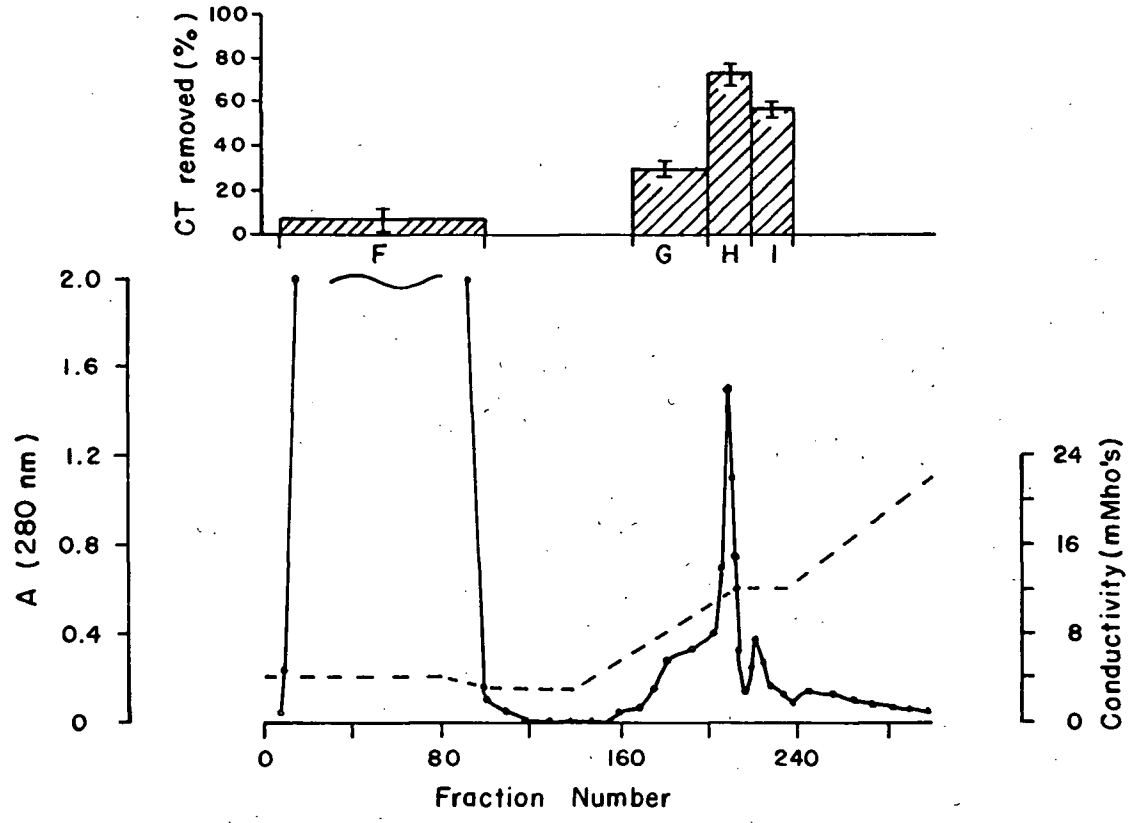


FIGURE 38. ELUTION PROFILE OF SEPHADEX G-200 PRODUCT ON CM-SEPHADEX C-25. COLUMN, 2 X 20 CM; ELUANT, LINEAR GRADIENTS OF pH 5.0 AMMONIUM ACETATE FROM 0.1 M TO 0.5 M AND FROM 0.5 M TO 1.0 M; TEMPERATURE, 4° C; FLOW RATE, 20 ML/HR; FRACTION SIZE, 6.8 ML.

POOLS.

THE POOL CONTAINING THE LARGE COMPONENTS (FRACTIONS 20 THROUGH 45, SEE FIGURE 37) HAD A TOTAL VOLUME OF 170 ML. WHEN THIS SOLUTION WAS ADJUSTED TO pH 5.0 WITH AMMONIUM HYDROXIDE FOR APPLICATION TO THE NEXT COLUMN THE SOLUTION CLOUDED. THE SUSPENDED COLLOID WAS NOT REMOVED BUT THE ENTIRE SAMPLE INCLUDING THE SUSPENDED COLLOID WAS APPLIED TO A 2 X 20 CM COLUMN OF CM-SEPHADEX, C-25 EQUILIBRATED WITH 0.1 M AMMONIUM ACETATE BUFFER AT pH 5.0. THE COLUMN WAS WASHED WITH 250 ML OF 0.1 M AMMONIUM ACETATE BUFFER AT pH 5.0 AND THE MATERIAL WHICH REMAINED ADSORBED ON THE COLUMN WAS ELUTED WITH A LINEAR GRADIENT OF AMMONIUM ACETATE BUFFER FROM 0.1 M (pH 5.0; CONDUCTIVITY, 3.5 mMHOS; VOLUME, 250 ML) TO 0.5 M (pH5.0; CONDUCTIVITY, 16.0 mMHOS; VOLUME, 250 ML). A SECOND LINEAR GRADIENT OF AMMONIUM ACETATE BUFFER FROM 0.5 M (pH 5.0; CONDUCTIVITY, 16.0 mMHOS; VOLUME, 250 ML) TO 1.0 M (pH 5.0; CONDUCTIVITY, 26.0 mMHOS; VOLUME, 250 ML) WAS EMPLOYED TO INSURE THAT ALL COMPONENTS WERE REMOVED. ELUATE WAS COLLECTED IN 6.8 ML FRACTIONS AT A RATE OF 20 ML/HR.

THE RESULTS FROM THIS COLUMN ARE SHOWN IN FIGURE 38. THE ELUATE FRACTIONS WERE POOLED IN THE SIX REGIONS INDICATED AND THE CT INACTIVATING ACTIVITY ASSESSED AS PREVIOUSLY DESCRIBED. 2.0 MRC U OF PORCINE CT WERE ADDED TO EACH SAMPLE TO A FINAL CONCENTRATION OF 1.0 MRC U/ML, AND THE BIOLOGICAL ACTIVITY REMAINING AFTER 30 MIN OF INCUBATION AT 37^{0} C DETERMINED. THE PERCENTAGE ACTIVITY REMOVED IS SHOWN IN THE FIGURE FOR EACH POOL. POOL H CONTAINED THE MAJOR

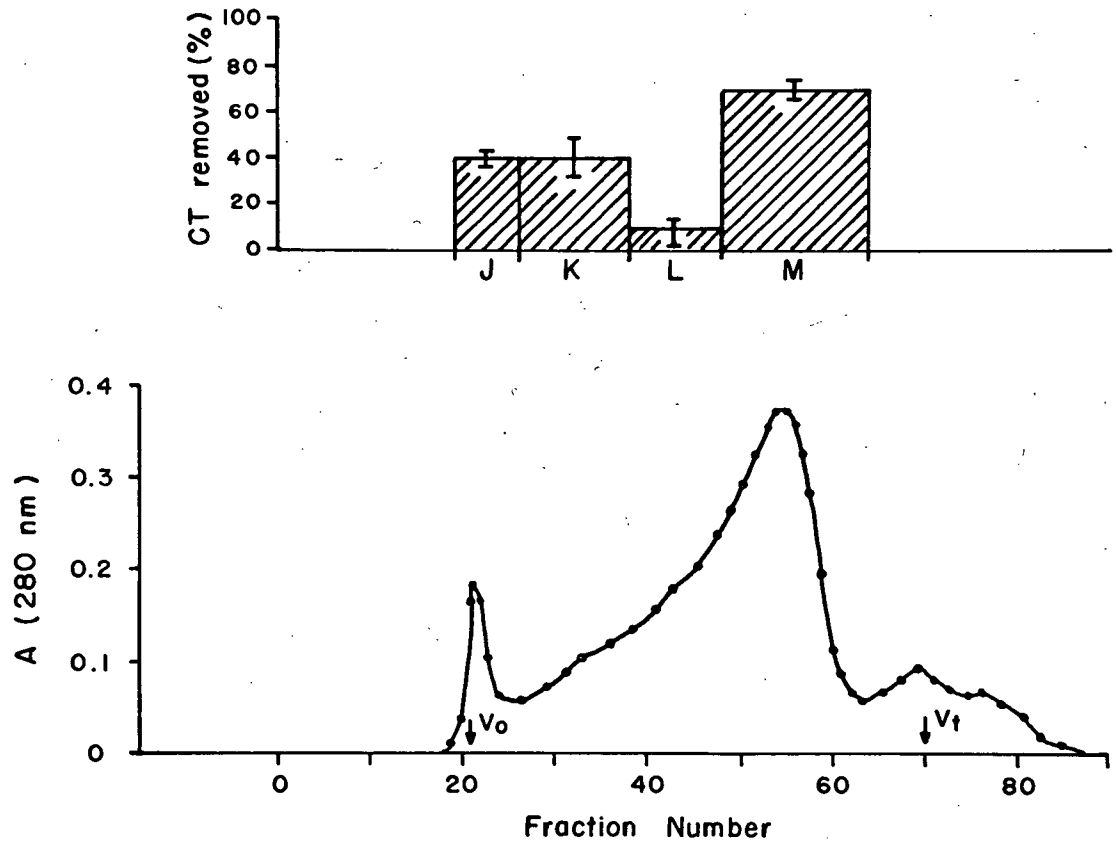


FIGURE 39. ELUTION PROFILE OF CM-SEPHADEX PRODUCT ON SEPHADEX G-200. ELUANT, 0.05 M ACETIC ACID; OTHER CONDITIONS AS INDICATED IN FIGURE 37.

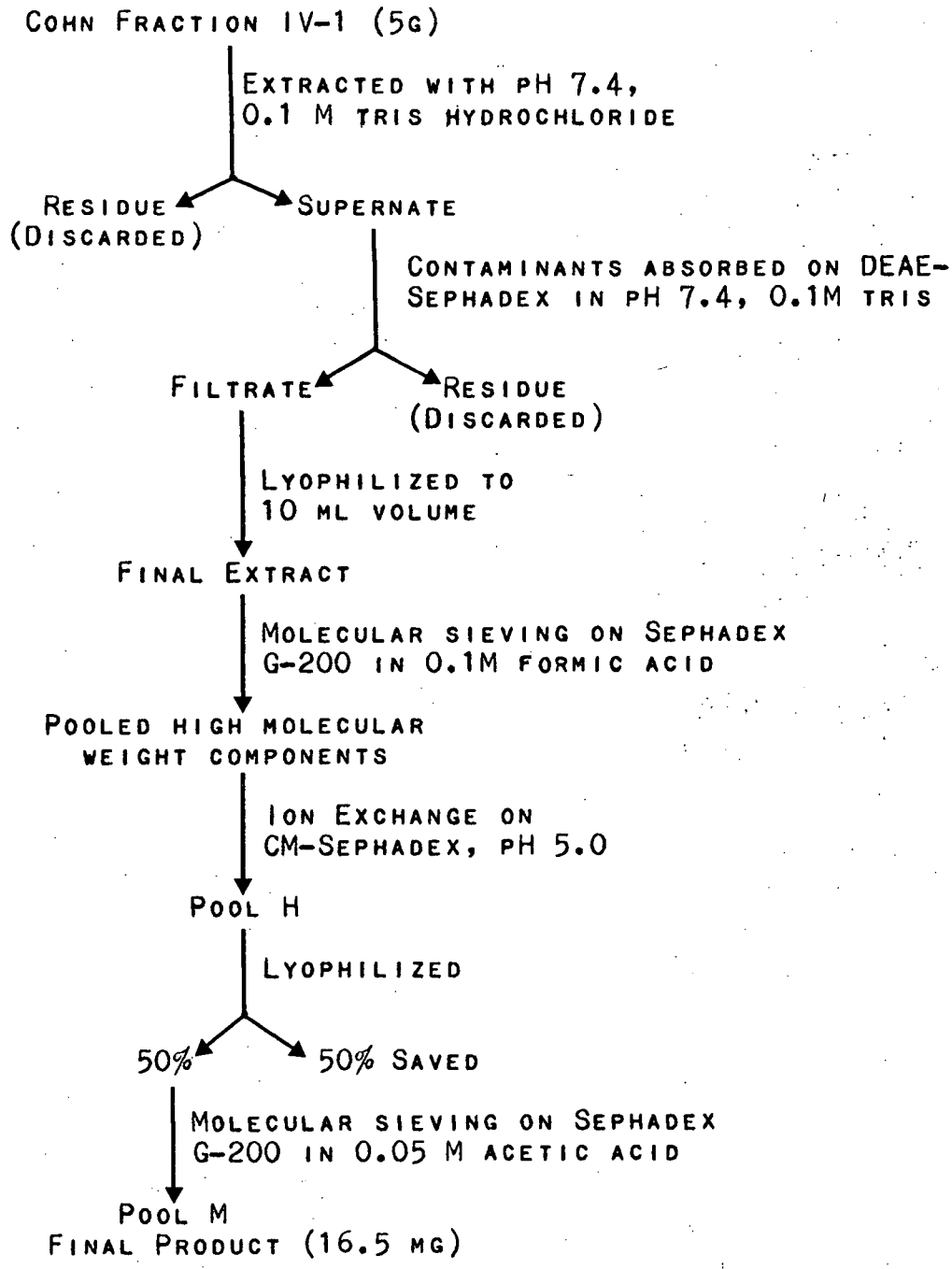
INACTIVATOR ACITIVITY AND WAS SELECTED FOR FURTHER PURIFICATION. THE ADJACENT POOLS G AND I ALSO CONTAINED SOME INACTIVATOR, BUT IT SEEMED LIKELY THAT THIS WAS DUE PRIMARILY TO OVERLAP OF THE CENTRAL PEAK INTO THE ADJACENT POOLS.

POOL H CONTAINED 120 ML OF ELUATE AND WAS LYOPHILIZED TO DRYNESS TO REMOVE EXCESS WATER AND SALT. THE DRY SAMPLE WAS REDISSOLVED IN 10 ML OF DISTILLED WATER AND 5 ML OF THIS WAS APPLIED TO A COLUMN OF SEPHADEX G-200 AS PREVIOUSLY DESCRIBED BUT USING 0.05 M ACETIC ACID AS AN ELUANT. THE ELUATE WAS COLLECTED IN 6.8 ML FRACTIONS AT A RATE OF 20 ML/HR. THE ELUATE FRACTIONS WERE DIVIDED INTO FOUR POOLS AND THEIR INACTIVATOR ACTIVITY ASSESSED AS DESCRIBED FOR THE PREVIOUS COLUMN.

AS SHOWN IN FIGURE 39 THE COLUMN PROFILE AND INACTIVATOR ASSAY RESULTS WERE RATHER UNEXPECTED. THE SAMPLE, WHICH HAD ORIGINALLY CONTAINED ONLY MATERIAL OF RELATIVELY HIGH MOLECULAR WEIGHT WAS COMPRISED PRIMARILY OF MATERIAL OF RELATIVELY LOW MOLECULAR WEIGHT, AND POOL M CONTAINED MOST OF THE INACTIVATOR. THE TWO POOLS, J AND K, WHERE INACTIVATOR WAS EXPECTED ACTUALLY CONTAINED LITTLE PROTEIN AND ONLY A SMALL FRACTION OF THE INACTIVATOR, SUGGESTING THAT THE LOW MOLECULAR WEIGHT INACTIVATOR WAS DERIVED FROM THE LARGER COMPONENT AND PERHAPS REPRESENTED THE ONLY ACTIVE ENZYME INVOLVED IN INACTIVATION. AN OBVIOUS ANALOGY FOR THIS TYPE OF RELATIONSHIP EXISTS IN THE PROTHROMBIN-THROMBIN SYSTEM WHERE IT HAS BEEN SHOWN THAT THE ACTIVE ENZYME CAN BE RELEASED

FIGURE 40

SUMMARY OF PURIFICATION PROCEDURE FOR "CALCITONINASE".



FROM THE PRECURSOR BY INCUBATION IN A 25% (w/v) SODIUM CITRATE SOLUTION (111). PERHAPS A SIMILAR TYPE RELEASE OCCURED IN THE CONCENTRATED AMMONIUM ACETATE SOLUTION WHICH RESULTED FROM LYOPHILIZATION OF POOL H. ELUCIDATION OF THE RELATIONSHIPS INVOLVED IN THIS TYPE OF SYSTEM WAS BEYOND THE SCOPE OF THIS STUDY AND IT WAS LIKELY THAT FURTHER PURIFICATION WOULD PROVE DIFFICULT SINCE A NUMBER OF SPONTANEOUSLY GENERATED INACTIVE FRAGMENTS COULD BE PRODUCED. IT WAS DECIDED THEREFORE THAT POOL M REPRESENTED MATERIAL OF SUFFICIENT PURITY FOR USE IN COMPARATIVE STUDIES. THE DEGREE OF PURITY AND SOME CHARACTERISTICS OF THE ENZYME WERE EXAMINED BUT NO FURTHER PURIFICATION WAS ATTEMPTED.

POOL M CONTAINED 110 ML AND LYOPHILIZED SAMPLES OF THIS POOL YIELDED 0.15 MG OF PROTEIN PER ML. THIS POOL THUS CONTAINED 16.5 MG OF MATERIAL PREPARED FROM THE EQUIVALENT OF 2.5 G OF COHN FRACTION IV-1.

4. SUMMARY OF PURIFICATION.

FIGURE 40 OUTLINES THE STAGES OF PURIFICATION USED TO PREPARE THE MATERIAL IN POOL M WHICH WAS USED FOR ALL FURTHER STUDIES. THE 16.5 MG OF MATERIAL FROM 2.5 G OF COHN FRACTION IV-1 REPRESENTS 150 FOLD REDUCTION IN WEIGHT OVER THE COHN FRACTION USED AS A STARTING MATERIAL, BUT SINCE THIS FRACTION REPRESENTS ONLY 6% OF THE TOTAL PLASMA PROTEIN THE FINAL PRODUCT REPRESENTS A 2500 FOLD REDUCTION IN WEIGHT FROM TOTAL PLASMA PROTEIN. THIS IS NOT NECESSARILY EQUIVALENT TO THE DEGREE OF PURIFICAION, BUT IT IS THE BEST

ESTIMATE AVAILABLE SINCE NO QUANTITATIVE MEASUREMENT OF INACTIVATOR WAS POSSIBLE.

5. TESTS OF PURITY.

SAMPLES OF POOL M WERE SUBJECTED TO ELECTROPHORESIS ON CELLULOSE ACETATE STRIPS AND ON POLYACRILAMIDE GELS TO DETERMINE THE NUMBER OF COMPONENTS PRESENT. ELECTROPHORESIS ON CELLULOSE ACETATE STRIPS WAS CARRIED OUT USING A GELMAN #51170 CHAMBER AND SERAPHORE III STRIPS FROM THE GELMAN INSTRUMENT CO., ANN ARBOR, MICHIGAN. THE BUFFER WAS STANDARD HIGH RESOLUTION BARBITONE BUFFER AT pH 8.6 FROM THE SAME SOURCE. SAMPLES CONTAINING 25 µG OF PROTEIN IN 5 µL WERE APPLIED NEAR THE CATHODE AND ALLOWED TO MIGRATE FOR 75 MIN AT 360 VOLTS. THE STRIPS WERE THEN DEVELOPED IN A SOLUTION CONTAINING 0.2% (w/v) PONCEAU S STAIN IN 5% (w/v) TCA AND DESTAINED WITH 3 WASHES IN 5% ACETIC ACID. HUMAN PLASMA SAMPLES WERE RUN SIMULTANEOUSLY AS CONTROLS.

POLYACRILAMIDE GEL ELCTROPHORESIS WAS CARRIED OUT AS DESCRIBED IN APPENDIX F. AFTER THE GELS HAD BEEN EQUILIBRATED WITH 0.01 M ACETIC ACID A 10 µL SAMPLE CONTAINING 2 NG/µL OF POOL M MATERIAL IN 1 M SUCROSE IN 0.002 M ACETIC ACID WAS APPLIED TO THE TOP OF THE GEL AND THE APPLICATION ZONE SHARPENED FOR 25 MIN IN A 32 VOLT FIELD. ELECTROPHORESES WAS THEN CARRIED OUT AT 320 VOLTS FOR 70 MINUTES AND THE GELS WERE REMOVED FROM THE TUBES FOR STAINING AS DESCRIBED IN THE APPENDIX.

TWO DISTINCT COMPONENTS WERE SEPARATED BY ELECTRO-

PHORESIS ON CELLULOSE ACETATE STRIPS. THE MAJOR BAND MIGRATED WITH THE α_2-GLOBULINS WHILE THE MINOR BAND HAD A HIGHER MOBILITY APPROXIMATELY EQUAL TO ALBUMIN. THE SEPARATION ON THE POLYACRILAMIDE GELS REVEALED 5 MINOR BANDS OF HIGH MOBILITY AND A MAJOR BAND OF RELATIVELY LOW MOBILITY. THIS CONFIRMED THAT POOL M CONTAINED A NUMBER OF COMPONENTS AND THAT ISOLATION OF THE INACTIVATING ENZYME WOULD REQUIRE FURTHER PURIFICATION STAGES. THE PURIFICATION ACHIEVED WAS ADEQUATE, HOWEVER, TO ALLOW ANALYSIS OF SEVERAL CHARACTERISTICS OF THE ENZYME.

D. Selective Inactivation of Porcine Calcitonin by "Calcitoninase".

INCUBATION OF THE THREE CALCITONINS WITH THE POOL M MATERIAL WAS CARRIED OUT IN THE MANNER SIMILAR TO THAT DESCRIBED FOR ASSAYING INACTIVATOR. THE INCUBATION MIXTURES CONTAINED 75 µG/ML OF POOL M MATERIAL AND APPROXIMATELY 10 µG/ML OF CT IN 0.1 M TRIS-ACETATE BUFFER AT PH 7.4. THE MATERIALS USED WERE PURE NATIVE SALMON CT AS DESCRIBED IN CHAPTER III, SYNTHETIC HUMAN CT FROM CIBA LTD., BASLE, AND PORCINE CT (AL0831) FROM ARMOUR PHARMACEUTICAL CO. KANKAKEE, ILLINOIS. THE TWO PURE MATERIALS WERE WEIGHED DIRECTLY, BUT THE WEIGHT OF PORCINE CT WAS BASED ON A BIOLOGICAL ACTIVITY OF 250 U/MG. ALIQUOTS OF EACH OF THE THREE MIXTURES WERE SUITABLY DILUTED AND BIOASSAYED PRIOR TO INCUBATION. AFTER INCUBATION AT 37° C FOR ONE HOUR ALIQUOTS WERE SIMILARLY DILUTED AND BIOASSAYED.

THE PLASMA CALCIUM LEVELS AFTER INJECTION OF PORCINE CT

WERE 7.96 ± 0.04 MG% AT ZERO TIME AND 9.11 ± 0.08 MG% AFTER ONE HOUR OF INCUBATION. THIS WAS A SIGNIFICANT DIFFERENCE ($P < 0.001$) AND REPRESENTED A LOSS OF 54% OF THE CT. THE HUMAN AND SALMON CALCITONINS SHOWED NO SIGNIFICANT LOSS OF BIOLOGICAL ACTIVITY AFTER INCUBATION THUS CONFIRMING THAT THE ENZYME ACTED SELECTIVELY ON PORCINE CT.

E. Peptides from Calcitonins Digested with "Calcitoninase."

1. Methods.

a. Digestion with Enzyme.

TWO PROCEDURES WERE USED FOR DIGESTIONS. THE FIRST WAS A MODIFICATION OF THE INACTIVATION ASSAY PROCEDURE, AND CONSISTED OF DISSOLVING THE CT SUBSTRATE IN THE POOL M SOLUTION (0.15 MG PROTEIN PER ML IN 0.05 M ACETIC ACID) TO A CONCENTRATION OF 5 MG/ML. THE pH OF THE SOLUTION WAS THEN ADJUSTED TO pH 7.5 BY ADDING 0.1 ML OF 2.0 M TRIS BASE SOLUTION PER 1 ML OF ENZYME SOLUTION. THE RESULTANT TRIS-ACETATE BUFFER SOLUTION WAS INCUBATED OVER-NIGHT (17 HR) AT 37° C AND ANALYSED FOR PEPTIDES. THIS PROCEDURE WAS USED FOR DIGESTION OF SYNTHETIC PORCINE CT SUPPLIED BY CIBA LTD., BASLE AND FOR PURE NATIVE SALMON CT.

THE SECOND DIGESTION PROCEDURE WAS A MODIFICATION OF THAT USED BY MUTT *ET AL* (78) FOR DIGESTION OF SECRETIN WITH THROMBIN. IN THIS PROCEDURE 1 MG OF CT WAS DISSOLVED IN 0.5 ML OF 1.0% AQUEOUS AMMONIUM BICARBONATE AND 10 µL OF AN ENZYME STOCK SOLUTION ADDED EVERY 2 HOURS. THE STOCK SOLUTION CONTAINED 2 MG/ML OF POOL M MATERIAL IN 1% AQUEOUS AMMONIUM

BICARBONATE. AFTER INCUBATION FOR 6 HOURS AT ROOM TEMPERATURE (21° C) THE SOLUTION WAS LYOPHILIZED AND REDISSOLVED IN DISTILLED WATER. THE SOLUTION WAS THEN PLACED IN BOILING WATER FOR 6 MINUTES AND RELYOPHILIZED. THIS PROCEDURE WAS USED FOR A SECOND DIGESTION OF PURE NATIVE SALMON CT.

B. HIGH VOLTAGE ELECTROPHORESIS.

THE DIGEST PRODUCTS WERE SPOTTED ON 3 CM OF THE ORIGIN LINE ON SHEETS OF WHATMAN 3 MM PAPER AND SUBJECTED TO ELECTROPHORESIS AT PH 1.9 AS DESCRIBED IN CHAPTER III. EDGE STRIPS WERE DEVELOPED WITH PAQ AND ACID NINHYDRIN REAGENTS. UNDEVELOPED AREAS CONTAINING PEPTIDES WERE CUT OUT AND ELUTED AS PREVIOUSLY DESCRIBED.

C. AMINO ACID ANALYSIS.

ALIQUOTS OF THE ELUTED PEPTIDES WERE HYDROLYSED AND AMINO ACID ANALYSES CARRIED OUT IN THE SAME MANNER USED FOR THE TRYPTIC PEPTIDES IN CHAPTER III.

D. END GROUP ANALYSIS.

N-TERMINAL RESIDUES OF THE PEPTIDES WERE DETERMINED BY DANSYLATION AS DESCRIBED FOR THE TRYPTIC PEPTIDES IN CHAPTER III.

2. RESULTS AND DISCUSSION.

DURING THE INCUBATIONS A CONSIDERABLE PROPORTION OF THE MATERIAL IN SOLUTION PRECIPITATED OUT. THIS PRECIPITATION BEGAN AS SOON AS THE PH WAS RAISED AND INCREASED DURING THE INCUBATION PERIOD. SINCE ANY FRAGMENTS PRODUCED WOULD

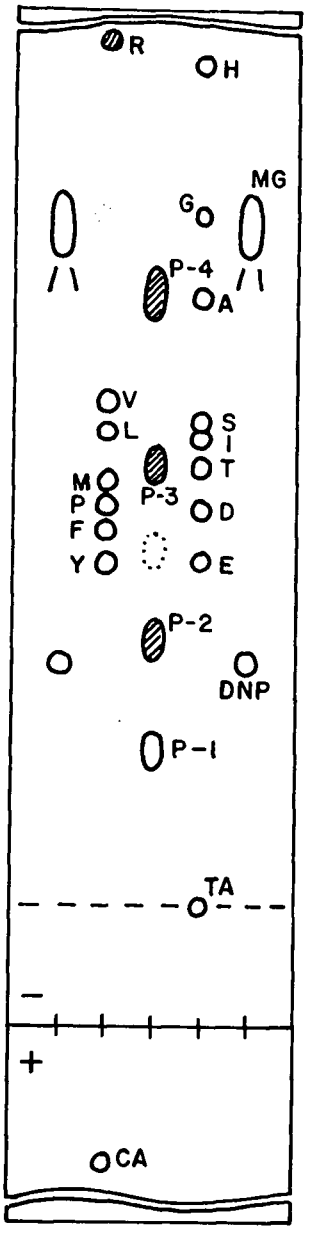


FIGURE 41. pH 1.9 ELECTROPHORETOGRAM OF PEPTIDES FROM PORCINE CT PRODUCED BY "CALCITONINASE". OTHER DATA AS INDICATED IN FIGURE 21.

TEND TO BE MORE SOLUBLE THAN THE WHOLE MOLECULE THE DIGESTS WERE CENTRIFUGED TO REMOVE THE PRECIPITATE AND THE SUPERNATES ONLY WERE ANALYSED FOR PEPTIDES. ATTEMPTS TO REDISSOLVE THE PRECIPITATES IN DILUTE ACID MET WITH ONLY LIMITED SUCCESS INDICATING THAT THE PRECIPITATION PROCESS WAS NOT COMPLETELY REVERSIBLE, AND SUGGESTING THAT THE HORMONES WERE UNDERGOING DISULFIDE INTERCHANGE IN THESE CONCENTRATED SOLUTIONS RESULTING IN POLYMERIZATION.

THE SUPERNATES OF THE PORCINE CT DIGESTS CONTAINED ANALYSABLE QUANTITIES OF PEPTIDE FRAGMENTS, INDICATING THAT THE INACTIVATING ENZYME IN POOL M WAS IN FACT A PEPTIDASE. FIGURE 41 SHOWS THE PATTERN OF PEPTIDES PRODUCED BY ELECTROPHORESIS OF THE SUPERNATE AT pH 1.9. FIVE WELL ISOLATED PEPTIDES WERE DETECTED, BUT ONLY FOUR WERE PRESENT IN SUFFICIENT QUANTITY FOR FURTHER ANALYSIS. AMINO ACID ANALYSIS OF PEPTIDE P-1 INDICATED ITS COMPOSITION WAS THR, SER, GLU, PRO_2, GLY_3, MET, PHE_2 AND ITS N-TERMINAL GROUP WAS PHENYLALANINE. PEPTIDE P-2 CONTAINED ASP, THR, SER_3, ALA, $\frac{1}{2}CYS_2$, VAL, LEU_2, TYR AND ARG WITH AN N-TERMINAL CYSTINE. THE COMPOSITION OF P-3 WAS ASP_3, THR, SER, GLU, PRO_2, GLY_3, MET, LEU, PHE_3, HIS, ARG AND THE N-TERMINAL RESIDUE WAS ASPARTIC ACID. PEPTIDE P-4 ALSO HAD AN N-TERMINAL ASPARTIC ACID, BUT CONTAINED ONLY ASP_3, LEU, PHE, HIS AND ARG. COMPARISON OF THESE COMPOSITIONS TO THE SEQUENCE OF PORCINE CALCITONIN SHOWN IN FIGURE 29 PERMITS IDENTIFICATION OF THE PEPTIDES INVOLVED. P-1, P-2 AND P-3 ARE THE THREE PEPTIDES PRODUCED BY SPLITS AT THE C-TERMINAL

SIDE OF THE TWO ARGININE RESIDUES IN THE MOLECULE WITH P-1 AS THE C-TERMINAL PEPTIDE, P-2 AS THE N-TERMINAL PEPTIDE AND P-4 AS THE SMALLER CENTRAL PEPTIDE. THE FOURTH PEPTIDE, P-3, RESULTED FROM AN INCOMPLETE SPLIT AT THE SECOND ARGININE AND CONTAINS BOTH THE CENTRAL AND C-TERMINAL PEPTIDES.

THESE RESULTS INDICATED THAT THE ENZYME IN POOL M HAD A SPECIFICITY FOR PEPTIDE BONDS ON THE CARBOXYL SIDE OF ARGININE RESIDUES, BUT THAT OTHER FACTORS WERE INVOLVED IN THE SPECIFICITY SINCE EVEN AFTER 17 HOURS OF INCUBATION AT 37^{0} C A RELATIVELY LARGE AMOUNT OF PEPTIDE P-3 REMAINED. CLEARLY THE ARG-ASN BOND WAS SPLIT MORE READILY THAN THE ARG-PHE, AND THE SPECIFICITY WAS NOT EQUAL FOR ALL ARGININE BONDS.

THE RESULTS OF THE DIGESTIONS OF SALMON CT PROVIDED FURTHER EVIDENCE THAT NOT ALL ARGININE BONDS WERE SUSCEPTABLE, SINCE NO PEPTIDES WERE DETECTED IN THE SUPERNATES OF EITHER OF THE DIGESTS OF THIS MATERIAL. IF THE ARGININE IN THE SALMON MOLECULE HAD BEEN SUSCEPTABLE THE C-TERMINAL PEPTIDE PRODUCED WOULD HAVE BEEN READILY IDENTIFIABLE SINCE IT WOULD HAVE BEEN IDENTICAL WITH ONE OF THE TRYPTIC PEPTIDES PREVIOUSLY ISOLATED. THE PRESENCE OF A PROLINE ON THE N-TERMINAL SIDE OF THE ARGININE IN SALMON CT MAY HAVE CONTRIBUTED TO THE RESISTANCE OF THE BOND TO THE ENZYME, SINCE THIS RESIDUE CAN BLOCK THE ACTION OF PEPTIDASES WHEN IT OCCURS NEAR A NORMALLY SUSCEPTABLE BOND (68). THE FAILURE OF THE ENZYME TO SPLIT THE SALMON MOLECULE ALSO

SUGGESTED THAT THE SPECIFICITY WAS ONLY FOR ARGININE AND NOT ALL BASIC RESIDUES SINCE THE MOLECULE ALSO CONTAINS TWO LYSINES. THIS SPECIFICITY WAS CLEARLY COMPATABLE WITH THE INACTIVATING CHARACTERISTICS OF THE ENZYME SINCE HUMAN CT CONTAINS NO ARGININE AND ONLY PORCINE CT CONTAINS ARGININE RESIDUES SUSCEPTABLE TO ATTACK.

THIS SPECIFICITY ALSO SUGGESTED THE POSSIBILITY THAT THE "CALCITONINASE" MIGHT BE THROMBIN WHICH IS KNOWN TO ATTACK CERTAIN TYPES OF PEPTIDE BONDS ON THE CARBOXYL SIDE OF ARGININE (11, 77, 78). THIS POSSIBILITY WAS REINFORCED BY THE BEHAVIOR OF THE ENZYME DURING THE PURIFICATION STAGES WHERE AN ACTIVE MATERIAL WITH MOLECULAR WEIGHT AROUND 30,000 WAS RELEASED FROM A MUCH LARGER PRECURSOR MOLECULE. THE EXPERIMENTS IN THE NEXT SECTION WERE DESIGNED TO TEST THIS POSSIBILITY.

F. Comparison of "Calcitoninase" to Thrombin.

SINCE THE NATURAL SUBSTRATE FOR THROMBIN IS FIBRINOGEN AN EXPERIMENT WAS DESIGNED TO COMPARE THE ACTION OF THROMBIN AND THE "CALCITONINASE" ON THIS SUBSTRATE. TWO 1 ML SAMPLES CONTAINING 2.5 MG COHN FRACTION 1 WHICH IS 50 TO 60% FIBRINOGEN DISSOLVED IN 0.1 M AMMONIUM BICARBONATE WERE PREPARED AND 40 µL OF THE ENZYME STOCK SOLUTION DESCRIBED ON PAGE 85 ADDED TO ONE SAMPLE. AFTER INCUBATION FOR TWO HOURS AT ROOM TEMPERATURE THERE WAS NO EVIDENCE OF CLOTTING IN EITHER SAMPLE AND 10 UNITS OF AN IMPURE BOVINE THROMBIN PREPARATION FROM UPJOHN CO. KALAMAZOO, MICHIGAN WAS ADDED

TO THE CONTROL SAMPLE RESULTING IN A RAPID CLOT FORMATION IN LESS THAN 10 MIN. AFTER THREE HOURS TOTAL INCUBATION THERE WAS NO SIGN OF CLOT FORMATION IN THE "CALCITONINASE" SAMPLE AND 250 µL OF POOL M SOLUTION WAS ADDED. AFTER A FURTHER TWO HOURS OF INCUBATION THERE WAS STILL NO CLOT AND 10 UNITS OF THE BOVINE THROMBIN WAS ADDED. THIS CAUSED A RAPID CLOTTING SIMILAR TO THAT SEEN IN THE CONTROL. SUBSEQUENT TESTS OF THE POOL M SOLUTION CONFIRMED THAT IT STILL RETAINED ITS PORCINE CT INACTIVATING PROPERTIES.

THIS FAILURE OF ACTIVE "CALCITONINASE" TO CAUSE CLOTTING INDICATED THAT IT CONTAINED NO SIGNIFICANT THROMBIN ACTIVITY. THE FACT THAT THE FIBRINOGEN SOLUTION WAS STILL CLOTTED BY THROMBIN AFTER A PRIOR 5 HOUR INCUBATION WITH THE ENZYME SUGGESTED FURTHER THAT THE "CALCITONINASE" HAD NO PLASMIN ACTIVITY SINCE PRE-INCUBATION OF FIBRINOGEN WITH PLASMIN RETARDS OR PREVENTS CLOT FORMATION BY THROMBIN (11). IT IS THEREFORE UNLIKELY THAT THE"CALCITONINASE"IN POOL M CORRESPONDED TO EITHER OF THESE ENZYMES.

G. General Discussion.

IN THE INTRODUCTION TO THIS CHAPTER THE RESPONSES OF SEVERAL SPECIES OF MAMMALS TO CALCITONINS FROM A VARIETY OF OTHER SPECIES WERE DISCUSSED AND EVIDENCE WAS PRESENTED THAT THE VARIATIONS IN THE RESPONSE TO THESE STRUCTURALLY DIFFERENT CALCITONINS WERE DUE AT LEAST IN PART TO DIFFERENCES IN THE RATE AT WHICH THEY WERE INACTIVATED. FROM A PHYSIOLOGICAL STANDPOINT THESE OBSERVATIONS DO NOT APPEAR

PARTICULARLY VALUABLE SINCE THE POSSIBILITY OF A MAN SECRETING SALMON CT IS QUITE REMOTE, BUT THEY MAY BE USEFUL IN UNDERSTANDING THE ROLE OF INACTIVATION IN THE OVERALL CONTROL SYSTEM. A DETAILED KNOWLEDGE OF THE ROUTES AND MECHANISMS INVOLVED IN THE INACTIVATION OF THE HORMONE IS ALSO IMPORTANT, IF CT IS TO BE USED THERAPEUTICALLY IN MAN. IN STUDIES ON A PATIENT WITH PAGET'S DISEASE NEER ET AL (79) DESCRIBED THE USE OF OVER 200 MG OF PORCINE CT DURING AN 11 WEEK COURSE OF TREATMENT. IN STUDIES ON A PATIENT WITH A PARATHYROID CARCINOMA IN THE SAME WORK THE AUTHORS USE 80 MG OF PORCINE CT TO ACHIEVE A 1.5 MG% DROP IN THE PLASMA CALCIUM LEVEL. AN INFUSION OF 0.6 MG OF SALMON CT IN THE SAME PATIENT PRODUCED A DROP IN PLASMA CALCIUM OF NEARLY 2.5 MG%. BECAUSE, IN TERMS OF MRC U, THIS DOSE WAS ONLY 20% OF THE PORCINE CT USED, SALMON CT PROMISES TO BE A MORE EFFICIENT MEANS OF CONTROLLING BONE RESORPTION THAN PORCINE CT.

THE REASONS FOR SUCH VARIATIONS IN THE MAGNITUDE OF RESPONSE TO THE DIFFERENT CALCITONINS MUST ULTIMATELY DEPEND ON THE STRUCTURAL DIFFERENCES BETWEEN THE HORMONE MOLECULES. THE EXPERIMENTAL WORK IN THIS CHAPTER DESCRIBES AN ENZYME FROM HUMAN PLASMA WHICH RAPIDLY INACTIVATES PORCINE CT BY SPLITTING TWO PEPTIDE BONDS WHICH ARE ABSENT IN THE SALMON MOLECULE. THIS INDICATES A STRUCTURAL DIFFERENCE WHICH CONTROLS THE INACTIVATION RATE OF THE TWO HORMONES, AND PROVIDES A MECHANISM WHICH COULD EXPLAIN WHY SALMON CT HAS A MUCH GREATER POTENCY IN MAN THAN

PORCINE CT. THE CONCLUSION ONE WOULD LIKE TO DRAW FROM THIS IS THAT CALCITONINS WHICH DO NOT HAVE LABILE ARGININE BONDS ARE MORE STABLE AND THEREFORE HAVE A GREATER BIOLOBICAL ACTIVITY. UNFORTUNATELY THE PROBLEM IS MUCH MORE COMPLEX THAN THIS FOR HUMAN CT WHICH CONTAINS NO ARGININE HAS A LOWER BIOLOGICAL ACTIVITY THAN PORCINE CT IN BOTH THE STANDARD BIOASSAY AND THE AREA RESPONSE ASSAY. THE SITUATION IS SIMILAR FOR BOVINE CT WHICH HAS A LYSINE IN THE POSITION OF THE MOST LABILE ARGININE BOND IN THE PORCINE MOLECULE AND HAS THE SAME ACTIVITY IN THE STANDARD BIOASSAY. FIGURE 34 SHOWS THAT THE AREA RESPONSE TO LOW DOSES OF BOVINE CT IS ALSO SLIGHTLY LOWER THAN PORCINE, BUT THE SLOPE OF THE LINE IS GREATER. THUS THIS PLASMA ENZYME WHICH SELECTIVELY INACTIVATES PORCINE CT CAN ONLY BE CONSIDERED AS AN INDICATION OF THE TYPE OF PHENOMENA WHICH MAY BE INVOLVED IN DETERMINING THE RELATIONSHIP BETWEEN THE STRUCTURE AND FUNCTION OF CALCITONINS AND IS NOT A COMPLETE ANSWER.

THIS ENZYME DOES NOT ADEQUATELY ACCOUNT FOR INACTIVATION WITHIN THE PLASMA MUCH LESS THE OVERALL RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION. A COMPARISON OF THE INACTIVATION RATES OF SALMON CT AND HUMAN CT (FIGURE 35 (A)) NEITHER OF WHICH WERE INACTIVATED BY THIS ENZYME INDICATES THAT OTHER MODES OF INACTIVATION MUST EXIST FOR THE HUMAN HORMONE. IN THE INTRODUCTION TO THIS CHAPTER A NUMBER OF ENZYMES WERE MENTIONED WHICH SEEMED LIKELY TO DIFFERENTIATE BETWEEN THE MAMMALIAN AND SALMON CALCITONINS AND ANY ONE OF

THESE OR PERHAPS MANY OTHER ENZYMES COULD BE INVOLVED. THE ENZYME WHICH WAS STUDIED IN THIS CHAPTER DOES NOT APPEAR TO BE ANY OF THE ENZYMES SUGGESTED EARLIER. THE SPECIFICITY SUGGESTS A PLASMIN OR KALLIKREIN TYPE ENZYME, BUT ITS MOLECULAR SIZE AND CHARGE CHARACTERISTICS DO NOT COINCIDE WITH THOSE OF THE BEST STUDIED EXAMPLES OF THESE ENZYMES (108, 135), HOWEVER, THE "CALCITONINASE" MAY BELONG TO ONE OF THESE ENZYME FAMILIES. IN ANY CASE IT HAS THUS FAR PROVED IMPOSSIBLE TO RELATE THE "CALCITONINASE" TO ANY OTHER WELL CHARACGERIZED PLASMA ENZYME. THE OBSERVATION THAT AT LEAST TWO OTHER COHN FRACTIONS CONTAINED POTENT CT INACTIVATING FACTORS SUGGESTS THAT THERE MAY BE MANY OTHER "CALCITONINASES" WHICH MAY OR MAY NOT CORRESPOND TO KNOWN PLASMA ENZYMES.

EVEN A COMPLETE CHARACTERIZATION OF ALL THE PLASMA "CALCITONINASES" AND EVALUATION OF THEIR RELATIVE EFFECTS WILL NOT FULLY EXPLAIN HOW STRUCTURE INFLUENCES THE COURSE OF THE RESPONSE SINCE THERE ARE MANY OTHER FACTORS INVOLVED. IF, FOR EXAMPLE, A "SUPER" CT WERE DESIGNED FOR THERAPY WHICH WAS RESISTANT TO ATTACK BY ALL PLASMA ENZYMES, IT WOULD STILL BE CLEARED FROM THE PLASMA AND A VARIETY OF FACTORS WOULD INFLUENCE THE RATE OF CLEARANCE. LOOKING AT THE THREE COMPARTMENT MODEL DESCRIBED EARLIER IT IS EVIDENT THAT ANY DECREASE IN THE RATE OF INACTIVATION IN THE PLASMA MUST RESULT IN AN INCREASE IN THE ACTIVE CT MOVING INTO COMPARTMENT 2 AND IN THE CT ELIMINATED BY RENAL EXCRETION. THE MAGNITUDE OF THIS INCREASE WOULD BE DEPENDENT ON THE DEGREE OF BINDING OF THE CT TO PLASMA PROTEINS. IF THE

BINDING IS VERY STRONG MOST OF THE CT WILL REMAIN IN COMPARTMENT 3 WHERE IT IS UNAVAILABLE FOR EXCRETION AND CANNOT MOVE INTO COMPARTMENT 2. THUS IT MIGHT LAST A LONG TIME AND BE ESSENTIALLY USELESS SINCE THE RESPONSE OCCURS ONLY AFTER THE HORMONE REACHES THE TARGET ORGAN IN COMPARTMENT 2. IF ON THE OTHER HAND BINDING IS WEAK, THE CT WILL MOVE RAPIDLY INTO COMPARTMENT 2 AND A FAIRLY LARGE PROPORTION WILL APPEAR IN THE URINE. HOW THIS WOULD EFFECT THE RESPONSE DEPENDS ON WHAT HAPPENS IN COMPARTMENT 2. IF, AS IS SUGGESTED IN THE WORK OF DE LUISE ET AL (36), A LARGE PERCENTAGE OF THE CT IS TAKEN UP BY THE LIVER THE DURATION OF THE RESPONSE WILL DEPEND ON WHETHER IT IS INACTIVATED AND ON WHETHER THE UPTAKE IS REVERSIBLE. DE LUISE ET AL (37) ALSO SHOWED THAT CALCITONINS RESISTANT TO PLASMA INACTIVATION ARE RESISTANT TO LIVER INACTIVATION. IF UNDER THESE CONDITIONS THE LIVER UPTAKE WERE REVERSIBLE CT COULD SLOWLY RETURN TO THE PLASMA AND BE EXCRETED OR REACH THE TARGET ORGAN AND PROLONG THE RESPONSE.

THE FINAL FACTOR INFLUENCING THE EFFECTIVENESS OF THIS "SUPER" CT IS ITS ACTION ON THE TARGET ORGAN, AND SOME OF THE STRUCTURAL CHARACTERISTICS WHICH MIGHT EFFECT THIS ACTION WERE DISCUSSED IN THE PRECEEDING CHAPTER. EVALUATING THESE STRUCTURAL CHARACTERISTICS REQUIRES ELIMINATION OF INACTIVATION AS AN INFLUENCE BY USING EITHER MOLECULES WHICH ARE NOT INACTIVATED OR A SYSTEM WHICH HAS NO INACTIVATORS. EVEN IN IN VITRO STUDIES ON BONE EXPLANTS (105) INACTIVATION IS AN IMPORTANT FACTOR SINCE REPEATED SMALL

DOSES OF PORCINE CT WERE MORE EFFECTIVE IN SUPPRESSING ACTIVE RESORPTION THAN AN EQUIVALENT SINGLE DOSE. FURTHER MORE STABLE ULTIMOBRANCHIAL CALCITONINS HAD A MUCH GREATER AND MORE PROLONGED EFFECT. WHILE THIS INACTIVATION MAY HAVE RESULTED FROM THE SMALL PERCENTAGE OF SERUM USED IN THEIR INCUBATION MEDIUM, LYSOZOMAL ENZYMES RELEASED BY OSTEOCLASTIC CELLS WOULD UNDOUBTEDLY BE CAPABLE OF INACTIVATING THE MORE LABILE CALCITONINS. THIS TYPE OF INACTIVATION, WHICH WAS NOT DISCUSSED EARLIER, MAY BE CRITICAL IN DETERMINING THE EFFECTIVENESS OF A CT AFTER IT HAS REACHED THE RECEPTOR. THE PRESENCE OF SUCH INACTIVATORS IN THE TARGET ORGANS OF OTHER HORMONES HAS BEEN DESCRIBED (55). IT THUS MAY PROVE EXTREMELY DIFFICULT TO SEPARATE ACTION ON THE RECEPTOR FROM INACTIVATION AS FACTORS WHICH INFLUENCE THE POTENCY OF A GIVEN MOLECULE, AND MODIFICATIONS WHICH RESULT IN A DECRESED STABILITY CAN BE EXPECTED TO CAUSE PROFOUND CHANGES IN THE POTENCY OF THE MOLECULE WHETHER OR NOT THEY INFLUENCE ACTION AT THE RECEPTOR SITE. FURTHER STUDIES ON STRUCTURE-FUNCTION RELATIONSHIPS MUST TAKE THIS INTO ACCOUNT AND PERHAPS THE BEST APPROACH TO THE PROBLEM MAY BE THROUGH THE USE OF MOLECULES WHICH ARE AS STABLE AS POSSIBLE.

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APPENDICES

A. Statistical Methods for Calculation of Log Dose-Response Curves by Computer.

Calculation of regression line for a given bioassay required the following data:

1. The dose of CT given in mg,
2. The weight of the rat injected,
3. The plasma calcium level measured in mg % in that rat after 1 hour.

In practice the dose given was calculated on the basis of a 0.3 ml injection for an 80 g rat and the dose was corrected for rat weight by adjusting injection volume. This avoided the problem of recording rat weight, but the program was designed to compensate for variations in weight if a constant dose was used for each animal. Each set of sample daata must also include data for rats injected with vehicle only as a control. From this data calculations were carried out as shown in the printout sheets included at the end of the appendix. The mean vehicle response was calculated first (VRESAV) as the sum of the plasma calciums of vehicle injected rats divided by the number of vehicle rats (DUM/FNV) in line 18. The response (RESP) was then calculated as the difference between VRESAV and the measured plasma calcium (CA) for each sample injected rat. The log dose (NDOSE) was then calculated as the $\log_{10}$ of injected dose divided by the rat weight (RDOSE/WEIGHT). This data was then placed in the

EXECUTION ARRAY AND THE TOTALS CALCULATED (TRES AND TND). THE MEAN VALUES (MRES AND MND) WERE DETERMINED BY DIVISION BY THE NUMBER OF EXPERIMENTAL SAMPLES INCLUDED (FNE) AND WERE PLACED IN THE ARRAY. FROM THESE VALUES IT WAS POSSIBLE TO CALCULATE DEVIATIONS IN DOSES (X) AS THE DIFFERENCE BETWEEN THE LOG OF EACH DOSE (NDOSE) AND THE MEAN (MND) AND THE DEVIATIONS IN RESPONSE (Y) AS THE DIFFERENCE BETWEEN THE RESPONSE (RESP) AND THE MEAN (MRES). THESE WERE ALSO PLACED IN THE ARRAY TOGETHER WITH THE CALCULATED SQUARES OF X AND Y. THE XSQ AND YSQ TERMS WERE THEN SUMMED AND DISPLAYED BELOW THE COLUMNS IN THE ARRAY. SIMILAR PROCEDURES WERE THEN CARRIED OUT FOR THE PRODUCT XY.

THE VALUES OBTAINED TO THIS POINT ALLOWED CALCULATION OF DATA NECESSARY FOR DETERMINATION OF THE POINT ESTIMATES OF THE PARAMETERS OF THE REGRESSION POPULATIONS AND OF THEIR INTERVAL ESTIMATES BY THE METHODS DESCRIBED BY SNEDECOR (115). THE SAMPLE REGRESSION COEFFICIENT (B) OF Y ON X FOR THE EQUATION $Y = BX$ IS EQUAL TO THE SUM OF THE XY TERMS DIVIDED BY THE SUM OF THE XSQ TERMS ($B = SXY/SUM\ XSQ$) AS SHOWN IN LINE 49. THE ESTIMATED RESPONSES, YC, WERE THEN THEN CALCULATED IN LINE 57 BY THE FORMULA $YC = BX + MRES$, WHERE B IS THE SAMPLE REGRESSION COEFFICENT, X IS THE DEVIATION OF A GIVEN DOSE FROM THE MEAN DOSE, AND MRES IS MEAN RESPONSE. THE ESTIMATED RESPONSES FOR THE GIVEN DOSES WERE THEN PLACED IN THE ARRAY AND USED TO PLOT BOTH THE COMPUTER GRAPH AND THE FIGURES IN THE TEXT.

MOST OF THE REMAINING CALCULATIONS IN THE PROGRAM WERE

AIMED AT A CALCULATION OF "T" TO TEST THE HYPOTHESIS THAT THE REGRESSION COEFFICENT FOR THE POPULATION IS ZERO. THIS WAS REALLY OF LITTLE VALUE SINCE IT WOULD IMPLY THAT THERE WAS NO RESPONSE OR THAT ALL RESPONSES WERE EQUIVALENT, AND IT IS UNLIKELY THAT SUCH DATA WOULD EVER BE TESTED. IT IS ALSO POSSIBLE TO USE THE TEST TO COMPARE THE SAMPLE POPULATION REGRESSION COEFFICIENT TO ANOTHER REGRESSION COEFFICIENT (E.G. THAT OF THE STANDARD CURVE), BUT THIS WAS NOT DONE SINCE THE VARIATIONS IN THE SOURCES OF MATERIAL BEING TESTED MADE SUCH COMPARISONS OF LITTLE VALUE. THESE CALCULATIONS ALSO SUPPLIED PART OF THE DATA USED TO DETERMINE CONFIDENCE LIMITS OF ESTIMATED RESPONSES WHICH WERE THEN USED TO CALCULATE THE ACTIVITY RANGES IN TABLE 1. SINCE $(B-\beta)/SB$ FOLLOWS THE T-DISTRIBUTION WHEN β IS THE POPULATION REGRESSION COEFFICIENT AND SB IS THE STANDARD DEVIATION OF THE SAMPLE REGRESSION COEFFICIENT, IN THE NULL HYPOTHESIS WHERE β IS ZERO T IS EQUAL TO B/SB. SB IS THE SQUARE ROOT OF VARIANCE OF THE SAMPLE REGRESSION COEFFICIENT WHICH IS EQUAL TO SYXSQ/SUMXSQ. SUMXSQ IS IN THE ARRAY AND SYXSQ IS THE MEAN SQUARE DEVIATION FROM REGRESSION AND IS EQUAL TO THE SUM OF THE SQUARES OF THE DEVIATIONS FROM THE REGRESSION (RESP - YC) OR SDYXSQ DIVIDED BY THE DEGREES OF FREEDOM (SDYXSQ/(N-2)). THE DEGREES OF FREEDOM ARE EQUAL TO THE NUMBER OF SAMPLES N MINUS 2 SINCE TWO AVERAGES WERE USED IN CALCULATING THE DEVIATIONS. THE VALUE OF SDYXSQ MAY BE CALCULATED FROM THE AVAILABLE DATA BY THE FORMULA SDYXSQ = SUMYSQ - SXYSQ/SUMXSQ WHERE SXYSQ IS EQUAL TO

FIGURE 42. COMPUTER PRINT OUT AND PLOT FROM LINEAR REGRESSION PROGRAM (NEXT 4 PAGES).

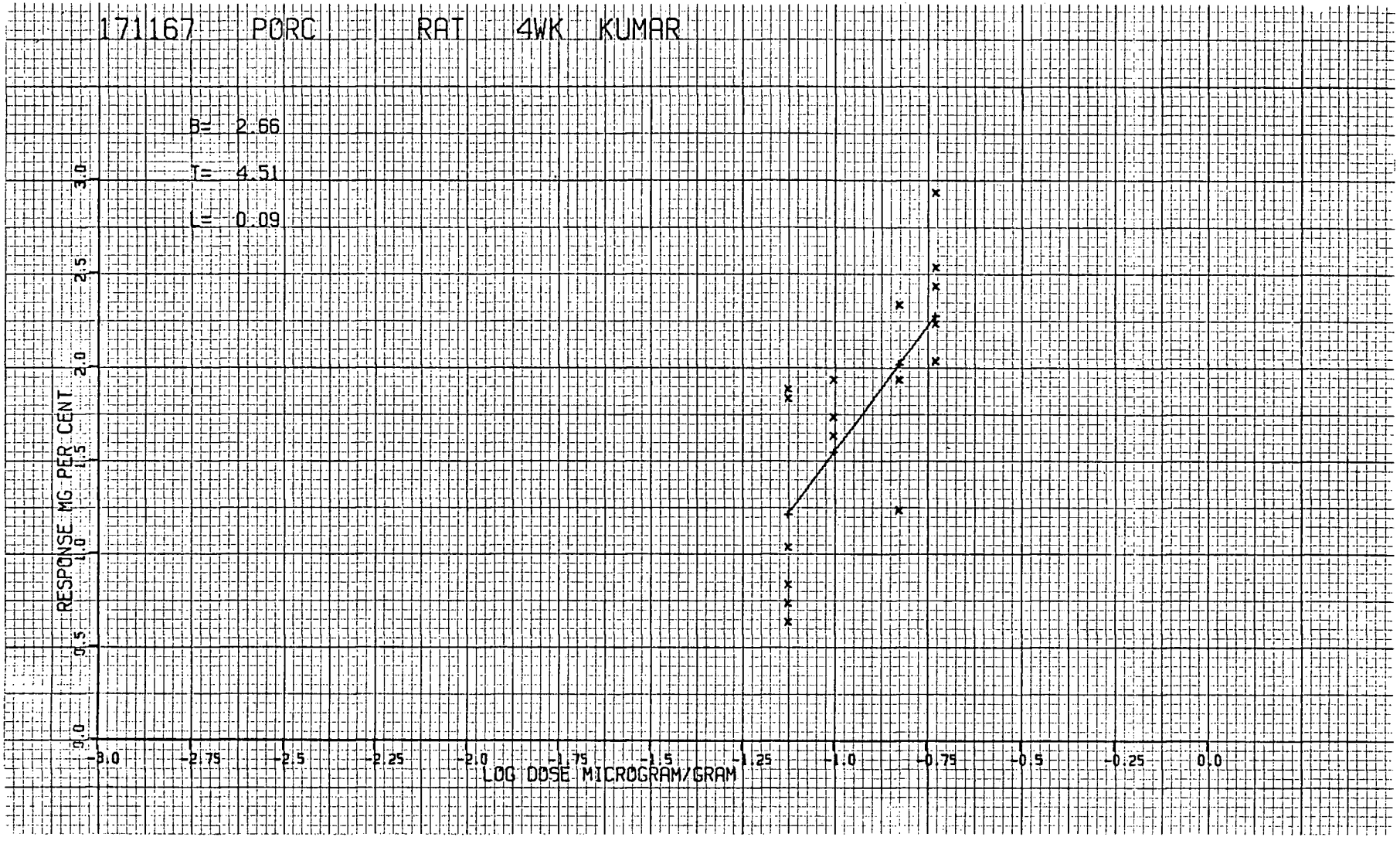
```
FORTRAN IV G COMPILER        MAIN            01-15-70        15:01:51        PAGE 0001

                C CASS1 MAIN PROGRAMME
                C PROGRAMME TO COMPUTE THE LINEAR REGRESSION OF LOGDOSE/RESPONSE
                C PROGRAMMER PETER GRAYSTONE PHYSIOLOGY DEPT. UBC  FEBRUARY 1968
                C MODIFIED BY RON COCR JANUARY 1970
                C REFERENCE  CHAPTER 6,SNEDECOR,STATISTICAL METHODS,IOWA STATE COL PRESS
                C
                C NV IS NUMBER OF VEHICLE SAMPLES, NT IS NUMBER OF TOTAL SAMPLES INC NV
                C
                C COLS 79,80 OF RUN IDENTIFIER CARD SETS THE X AXIS ORIGIN
0001                    CALL PLOTS
0002                    REAL NDOSE,MND,MRES
0003                    INTEGER DATE, STD
0004                    DIMENSION RDOSE(100),WEIGHT(100),CA(100),TITLE(15),NDOSE(100)
0005                    DIMENSION X(100),Y(100),XSQ(100),YSQ(100),XY(100),DYX(100),DYXSQ(1
                       C00),YC(100),RESP(100),DEV(100),DEVSQ(100)
0006            1       READ(5,10) DATE,NV,NT,TITLE, STD
0007            10      FORMAT(1X,I6,1X,I3,1X,I3,1X,15A4,2X,I2)
0008                    IF(DATE.LT.0)GO TO 999
0009                    WRITE(6,10)DATE,NV,NT,TITLE
0010                    READ(5,20) (RDOSE(I),WEIGHT(I),CA(I),I=1,NT)
0011            20      FORMAT(1X,F8.3,1X,F8.3,1X,F6.2)
0012                    IF(NV.EQ.0)GO TO 25
0013                    FNV=NV
0014                    DUM=0.
0015                    DO 30 I=1,NV
0016                    DUM=DUM+CA(I)
0017            30      CONTINUE
0018                    VRESAV=DUM/FNV
0019                    GO TO 35
0020            25      VRESAV=0.
0021            35      NVP=NV+1
0022                    TRES=0.
0023                    TND=0.
0024                    DO 40 I=NVP,NT
0025                    NDOSE(I)=ALOG10(RDOSE(I)/WEIGHT(I)*1000.)
0026                    RESP(I)=VRESAV-CA(I)
                C THE ARRAYS NDOSE AND RESP NOW CONTAIN THE LOG DOSE AND RESPONSE DATA
0027                    TND=TND+NDOSE(I)
0028                    TRES=TRES+RESP(I)
0029            40      CONTINUE
0030                    FNE=NT-NV
0031                    MND=TND/FNE
0032                    MRES=TRES/FNE
                C MND CONTAINS MEAN OF LOGDOSE AND MRES CONTAINS MEAN OF RESPONSE
0033                    SUMXSQ=0.
0034                    SUMYSQ=0.
0035                    SUMXY=0.
0036                    DO 50 I=NVP,NT
0037                    X(I)=NDOSE(I)-MND
0038                    XSQ(I)=X(I)*X(I)
0039                    Y(I)=RESP(I)-MRES
0040                    SUMXSQ=SUMXSQ+XSQ(I)
0041                    XY(I)=X(I)*Y(I)
0042                    YSQ(I)=Y(I)*Y(I)
0043                    SUMYSQ=SUMYSQ+YSQ(I)
```

```
0044                SUMXY=SUMXY+XY(I)
0045                SXYSQ=SUMXY*SUMXY
                  C SDYXSQ IS SUM OF RESP(I)-YC(I)
0046                SDYXSQ=SUMYSQ-(SXYSQ/SUMXSQ)
0047                RMSQ=SQRT(SUMXSQ)
0048          50    CONTINUE
                  C SAMPLE REGRESSION COEFFICIENT OF Y ON X IS B  (Y=BX)
0049                B=SUMXY/SUMXSQ
0050                DOF=FNE-2.
                  C MEAN SQUARE DEVIATION FROM REGRESSION IS SYXSQ
0051                SYXSQ=SDYXSQ/DOF
                  C SAMPLE STANDARD DEVIATION FROM REGRESSION IS SYX
0052                SYX=SQRT(SYXSQ)
                  C SAMPLE STANDARD DEVIATION OF THE REGRESSION COEFFICIENT IS SB
0053                SB=SYX/RMSQ
0054                T=B/SB
0055                SLAM=SQRT(SUMXY/FNE)/B
0056                DO 70 I=NVP,NT
0057                YC(I)=B*X(I)+MRES
0058          70    CONTINUE
                  C YC CONTAINS ESTIMATED RESPONSES
0059                WRITE(6,100)
0060          100   FORMAT(//3X,109H RAWDOSE    WEIGHT    CA ROG    LOGDOSE     RESP
                     C LD DVN     RES DVN    LD SQ      RES SQ     LD*RES     EST RESP)
0061                WRITE(6,110)(RDOSE(I),WEIGHT(I),CA(I),NDOSE(I),RESP(I),X(I),Y(I),X
                    CSQ(I),YSQ(I),XY(I),YC(I),I=NVP,NT)
0062          110   FORMAT(1X,11F10.4)
0063                WRITE(6,120)
0064          120   FORMAT(1X,3HSUM)
0065                WRITE(6,130)TND,TRES,SUMXSQ,SUMYSQ,SUMXY
0066          130   FORMAT(31X,2F10.4,20X,3F10.4/)
0067                WRITE(6,140)
0068          140   FORMAT(1X,4HMEAN)
0069                WRITE(6,150)MND,MRES
0070          150   FORMAT(31X,2F10.4//)
0071                WRITE(6,160)B
0072          160   FORMAT(1X,55HSAMPLE REGRESSION COEFFICIENT OF RESPONSE ON LOGDOSE
                    CIS,F10.4//)
0073                WRITE(6,170)SYXSQ
0074          170   FORMAT(1X,40HMEAN SQUARE DEVIATION FROM REGRESSION IS, F10.4//)
0075                WRITE(6,180)SYX
0076          180   FORMAT(1X,44HSAMPLE STANDARD DEVIATION FROM REGRESSION IS,F10.4//)
0077                WRITE(6,190)SB
0078          190   FORMAT(1X,58HSAMPLE STANDARD DEVIATION OF THE REGRESSION COEFFICIE
                    CNT IS,F10.4//)
0079                WRITE(6,200)T,DOF
0080          200   FORMAT(1X,13H,T,TEST, T IS,F10.4,13H DOF (N-2) IS,F10.4//)
0081                WRITE(6,220)SLAM
0082          220   FORMAT(1X,10H LAMBDA IS,F8.4//)
0083                CALL REGPL(NDOSE,RESP,NVP,NT,TITLE,DATE,YC,B,STD,T,SLAM)
0084                WRITE(6,55)
0085          55    FORMAT(1H1)
0086                GO TO 1
0087          999   CONTINUE
0088                CALL PLOTND
```

```
EXECUTION TERMINATED


$RUN -LOAC#**NFL+*RPLOT 9=-P
EXECUTION BEGINS
171167   6  28  PORC      RAT   4WK  KUMAR
```

RAWDOSE	WEIGHT	CA RDG	LOGDOSE	RESP	LD DVN	RES DVN	LD SQ	RES SQ	LD*RES	EST RESP
0.0060	80.0000	8.4000	-1.1249	1.8333	-0.2133	0.0523	0.0455	0.0027	-0.0112	1.2116
0.0060	80.0000	8.3500	-1.1249	1.8833	-0.2133	0.1023	0.0455	0.0105	-0.0218	1.2116
0.0060	80.0000	9.4000	-1.1249	0.8333	-0.2133	-0.9477	0.0455	0.8982	0.2022	1.2116
0.0060	80.0000	9.2000	-1.1249	1.0333	-0.2133	-0.7477	0.0455	0.5591	0.1595	1.2116
0.0060	80.0000	9.6000	-1.1249	0.6333	-0.2133	-1.1477	0.0455	1.3173	0.2449	1.2116
0.0060	80.0000	9.5000	-1.1249	0.7333	-0.2133	-1.0477	0.0455	1.0977	0.2235	1.2116
0.0080	80.0000	8.5000	-1.0000	1.7333	-0.0884	-0.0477	0.0078	0.0023	0.0042	1.5451
0.0080	80.0000	8.5000	-1.0000	1.7333	-0.0884	-0.0477	0.0078	0.0023	0.0042	1.5451
0.0080	80.0000	8.3000	-1.0000	1.9333	-0.0884	0.1523	0.0078	0.0232	-0.0135	1.5451
0.0080	80.0000	8.6000	-1.0000	1.6333	-0.0884	-0.1477	0.0078	0.0218	0.0131	1.5451
0.0120	80.0000	9.0000	-0.8239	1.2333	0.0877	-0.5477	0.0077	0.3000	-0.0480	2.0151
0.0120	80.0000	9.0000	-0.8239	1.2333	0.0877	-0.5477	0.0077	0.3000	-0.0480	2.0151
0.0120	80.0000	8.3000	-0.8239	1.9333	0.0877	0.1523	0.0077	0.0232	0.0134	2.0151
0.0120	80.0000	8.3000	-0.8239	1.9333	0.0877	0.1523	0.0077	0.0232	0.0134	2.0151
0.0120	80.0000	7.9000	-0.8239	2.3333	0.0877	0.5523	0.0077	0.3050	0.0484	2.0151
0.0120	80.0000	7.9000	-0.8239	2.3333	0.0877	0.5523	0.0077	0.3050	0.0484	2.0151
0.0150	80.0000	7.3000	-0.7270	2.9333	0.1846	1.1523	0.0341	1.3277	0.2127	2.2738
0.0150	80.0000	8.2000	-0.7270	2.0333	0.1846	0.2523	0.0341	0.0636	0.0466	2.2738
0.0150	80.0000	8.2000	-0.7270	2.0333	0.1846	0.2523	0.0341	0.0636	0.0466	2.2738
0.0150	80.0000	8.0000	-0.7270	2.2333	0.1846	0.4523	0.0341	0.2046	0.0835	2.2738
0.0150	80.0000	7.8000	-0.7270	2.4333	0.1846	0.6523	0.0341	0.4255	0.1204	2.2738
0.0150	80.0000	7.7000	-0.7270	2.5333	0.1846	0.7523	0.0341	0.5659	0.1389	2.2738
SUM			-20.0550	39.1831			0.5549	7.8424	1.4813	
MEAN			-0.9116	1.7811						

```
SAMPLE REGRESSION COEFFICIENT OF RESPONSE ON LOGDOSE IS     2.6692

MEAN SQUARE DEVIATION FROM REGRESSION IS     0.1944

SAMPLE STANDARD DEVIATION FROM REGRESSION IS     0.4409

SAMPLE STANDARD DEVIATION OF THE REGRESSION COEFFICIENT IS     0.5919

,T,TEST, T IS     4.5095 DOF (N-2) IS    20.0000

 LAMBDA IS  0.0972
```


171167 PORC RAT 4WK KUMAR
B= 2.66
T= 4.51
L= 0.09
RESPONSE MG PER CENT
0.0
0.5
1.0
1.5
2.0
2.5
3.0
-3.0
-2.75
-2.5
-2.25
-2.0
-1.75
-1.5
-1.25
-1.0
-0.75
-0.5
-0.25
0.0
LOG DOSE MICROGRAM/GRAM

SUMXY SQUARED AS SHOWN IN LINE 45. T MAY THEN BE CALCULATED AS INDICATED PREVIOUSLY.

SYXSQ AS CALCULATED ABOVE IS THE SQUARE OF SYX (LINE 52) THE SAMPLE STANDARD DEVIATION FROM REGRESSION. THE VALUES FOR SYX, N AND SUMXSQ WERE USED TO CALCULATE THE STANDARD ERROR FOR THE ESTIMATED Y OF A SELECTED X AS SHOWN IN THE TEXT IN CHAPTER 1. FURTHER CALCULATIONS AS SHOWN ALLOWED DETERMINATION OF CONFIDENCE LIMITS AND RANGES AS INDICATED IN THAT CHAPTER.

B. Simple Design for a Large, Inexpensive Sephadex Column.

SEPHADEX CHROMATOGRAPHY IS A VALUABLE TECHNIQUE FOR PROTEIN PURIFICATION, BUT IT HAS CERTAIN LIMITATIONS WHEN USED ON A PREPARATIVE SCALE, PARTICULARLY IF A HIGH DEGREE OF SEPARATION OF COMPONENTS IS REQUIRED. UNDER SUCH CONDITIONS THE ZONE OF SAMPLE APPLICATION SHOULD BE ONLY 1 TO 2% OF COLUMN LENGTH. FOR EXAMPLE, A 2.5 x 100 CM COLUMN HAS A CROSS-SECTIONAL AREA OF ABOUT 5 CM, ALLOWING A MAXIMUM SAMPLE VOLUME OF ABOUT 10 ML. THE AMOUNT OF PROTEIN WHICH CAN BE PROCESSED ON SUCH A COLUMN MAY BE LIMITED BY SOLUBILITY OR, IF SOLUBILITY IS HIGH, BY THE HIGH VISCOSITY OF THE SAMPLE WHICH LEADS TO BROAD, SKEWED PEAKS. ONE SOLUTION IS TO PROCESS LARGE SAMPLES BY REPETETIVE CHROMATOGRAPHY, BUT IN ADDITION TO MULTIPLYING THE LABOR AND TIME REQUIRED THIS MULTIPLIES THE POTENTIAL FOR ACCIDENTS AND ERRORS AND INCREASES LOSSES ON VESSEL WALLS, ETC.

THE BEST METHOD FOR HANDLING LARGER SAMPLES IS TO

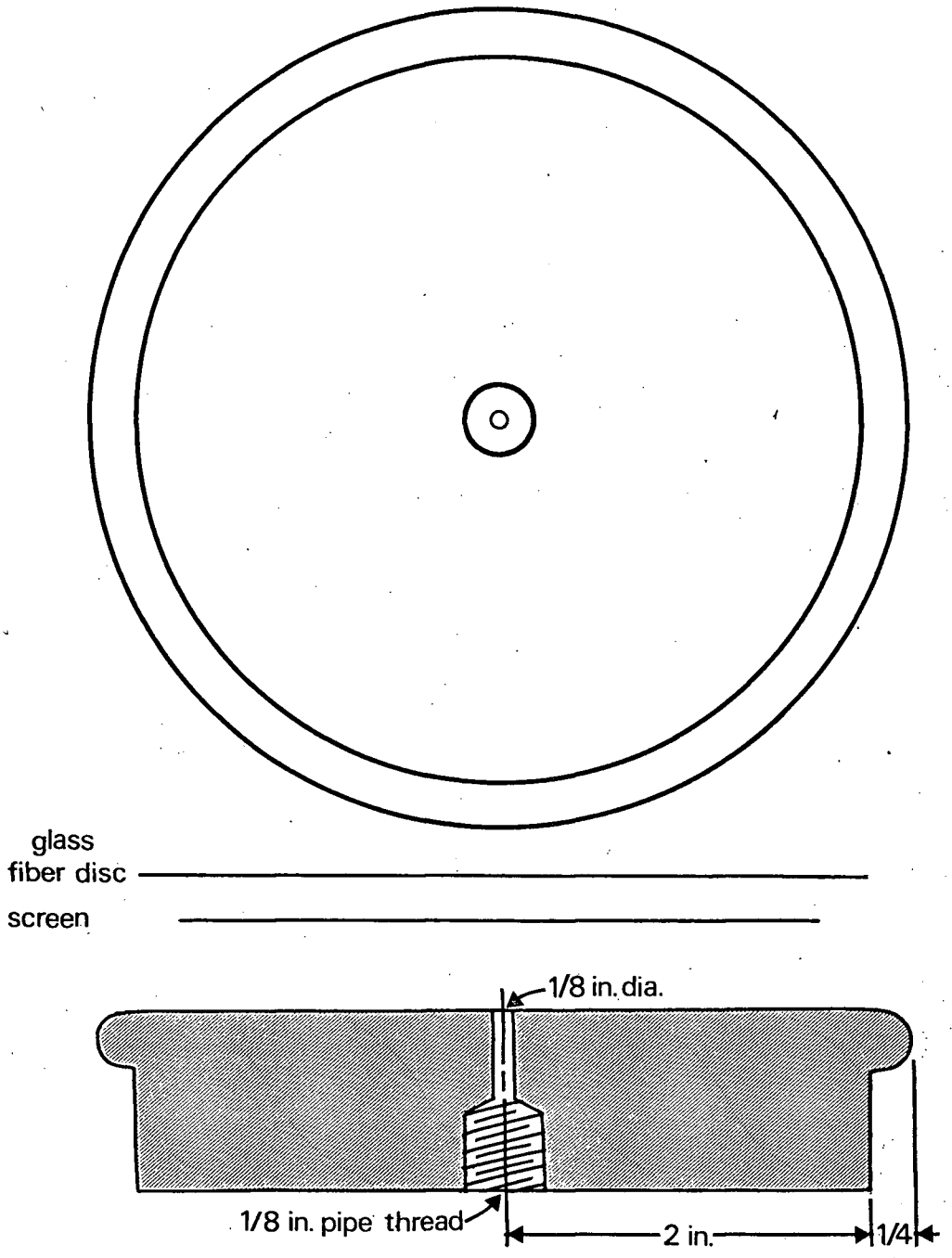


FIGURE 43. END-PIECES FOR 10 CM COLUMN--DIMENSIONS. TWO VIEWS SHOWN. DISCS MACHINED FROM 1 IN LUCITE SHEET.

INCREASE THE CROSS-SECTIONAL AREA OF THE COLUMN, BUT THIS CAN BE QUITE EXPENSIVE SINCE IT REQUIRES MUCH GREATER QUANTITIES OF GEL AND SPECIALLY DESIGNED COLUMNS. THE COST OF SUCH COLUMNS APPEARS TO INCREASE EXPONENTIALLY WITH DIAMETER, AND COMMERCIALLY AVAILABLE 10 CM COLUMNS COST WELL OVER $1000. SUCH EXPENSE CAN BE AVOIDED BY CONSTRUCTING THE REQUIRED COLUMNS FROM PARTS AVAILABLE FROM NON-SCIENTIFIC SOURCES. THE COLUMN USED FOR THE PURIFICATION OF SALMON CT IN CHAPTER III WAS 10 x 150 CM AND WAS CONSTRUCTED FOR LESS THAN $100. THE CONSTRUCTION REQUIRED THE MANUFACTURE OF ONLY TWO IDENTICAL END PIECES AND PRODUCED SEPARATIONS EQUIVALENT TO SMALLER COMMERCIAL COLUMNS ON SAMPLES AS LARGE AS 150 ML.

THE BASIC COLUMN WAS A 5 FT. SECTION OF 4 IN. DIAMETER PYREX GLASS PIPE WITH PREBEADED ENDS ORDERED FROM CORNING GLASS WORK, CORNING, N.Y. THESE PIPE SECTIONS, DESIGNED FOR CONSTRUCTING ACID PROOF DRAINS, CAN BE ORDERED IN A VARIETY OF SIZES AND ARE NORMALLY CONNECTED TOGETHER WITH TEFLON LINED RUBBER JUNCTIONS (CORNING PART #4009). TWO END PIECES DESIGNED AS SHOWN IN FIGURE 43 WERE MACHINED FROM 1 IN LUCITE SHEETS BY FIRST CUTTING A ROUGH 5 IN. CIRCLE AND THEN DRILLING A CENTER HOLE OF THE DIAMETER REQUIRED FOR ELUANT FLOW. THIS HOLE WAS FURTHER ENLARGED ON ONE SIDE AND THREADED TO TAKE A STANDARD NYLON PIPE THREAD TO HOSE CONNECTOR. A SECTION OF 1/8 IN PIPE WAS THEN TIGHTENED INTO PLACE AND USED TO CHUCK THE PLASTIC PIECE INTO THE LATHE WHERE IT WAS TURNED TO THE DIMENSIONS

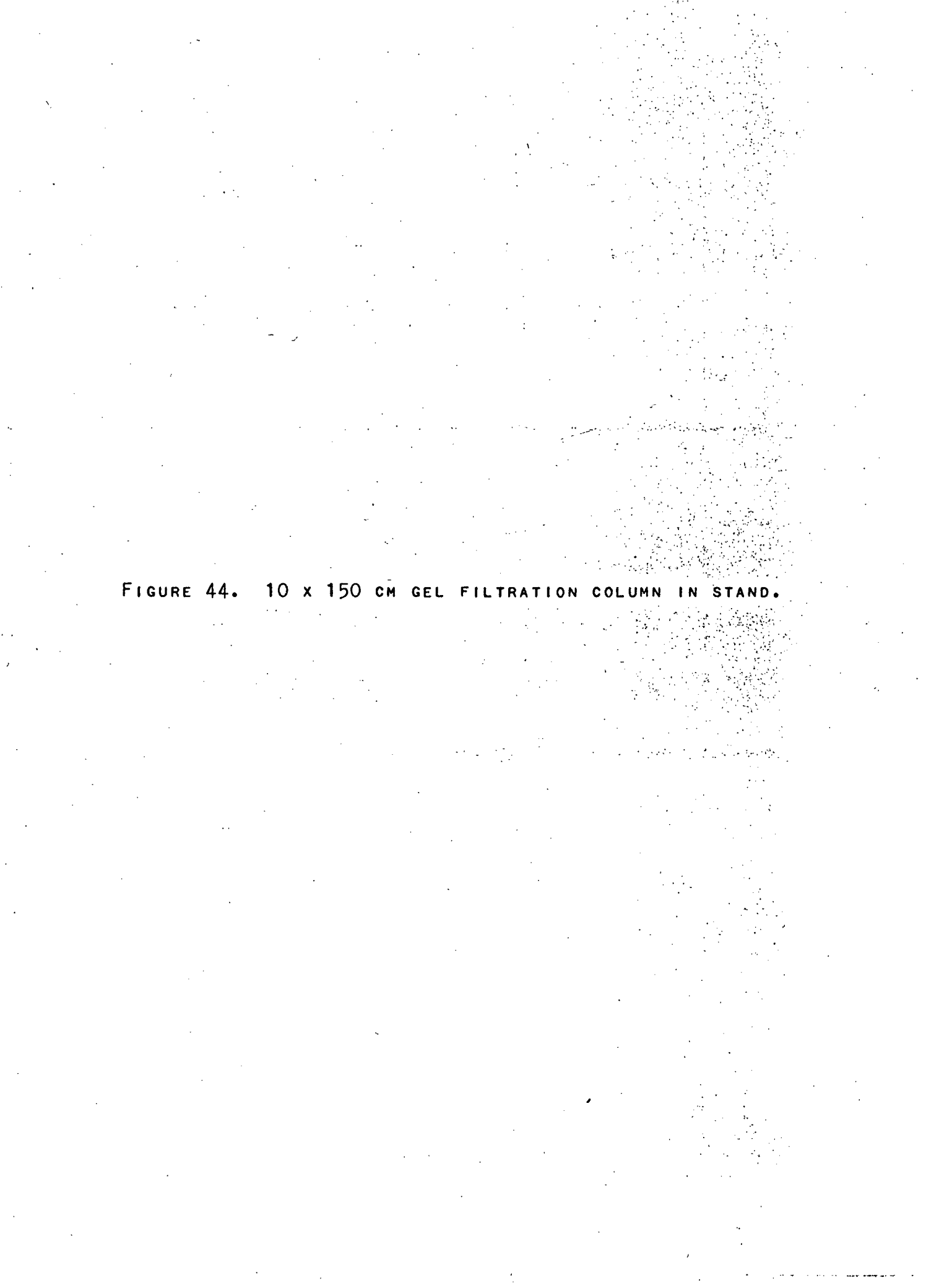
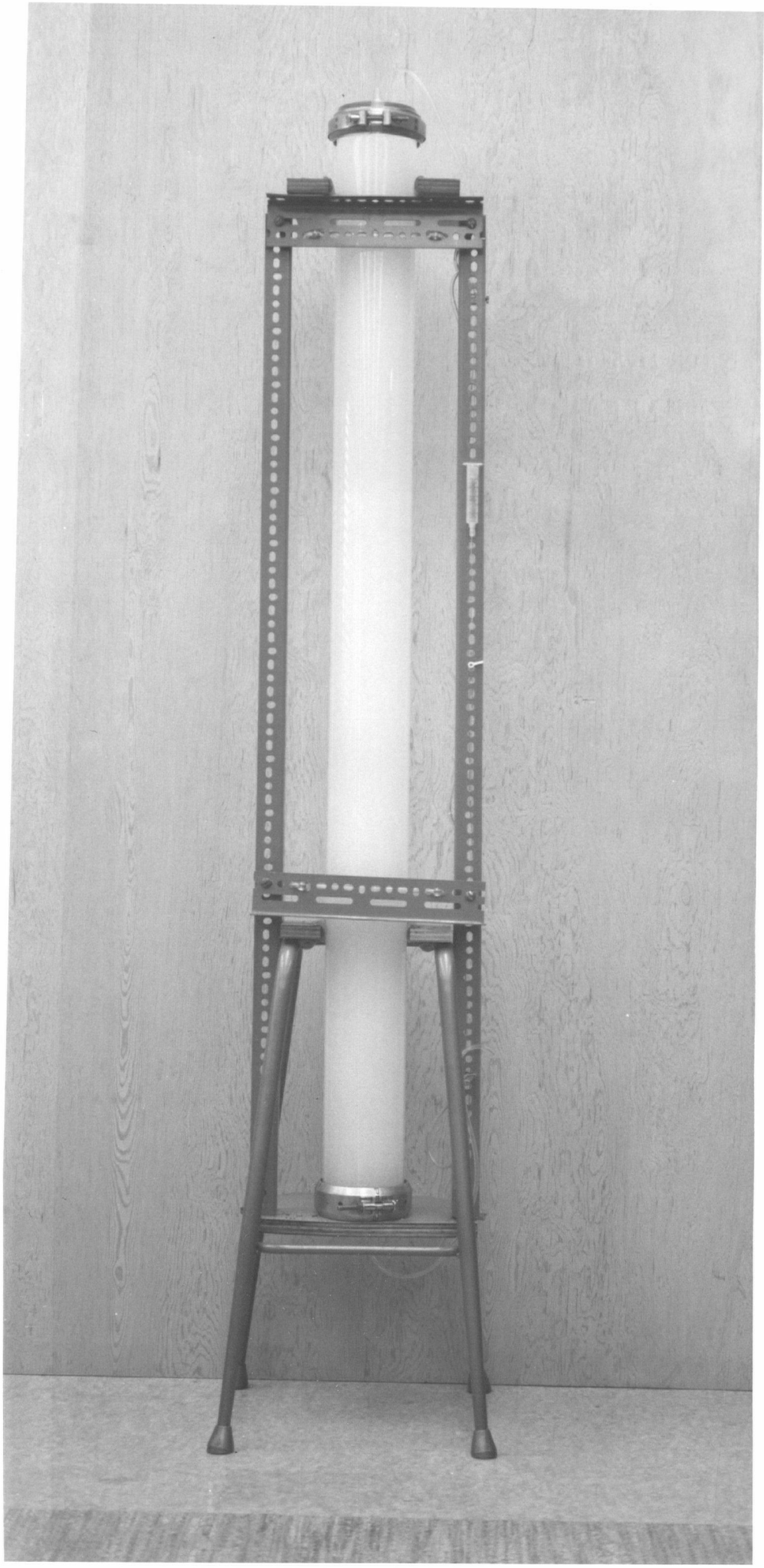


FIGURE 44. 10 x 150 CM GEL FILTRATION COLUMN IN STAND.



SHOWN. CIRCULAR PIECES OF NYLON WINDOW SCREEN AND GLASS FIBER PAPER (GFP-2, APPLIED SCIENTIFIC LABORATORIES, INC., STATE COLLEGE, PA.) WERE THEN CUT TO THE DIMENSIONS SHOWN AND PLACED ON THE END PIECES. WHEN PLACED IN THE STANDARD JUNCTION FITTINGS THE INNER LIP OF THE FITTING PROVIDED A GOOD SEAL AGAINST THE GLASS FIBER DISC PREVENTING GEL LEAKAGE AND THE NYLON SCREEN ALLOWED ELUANT FLOW FROM THE ENTIRE CROSS-SECTION TOWARD THE OUTLET HOLE.

WITH THE BOTTOM END PLATE IN PLACE THE COLUMN WAS MOUNTED ON A LABORATORY STOOL AS SHOWN IN FIGURE 44 WITH A DEXION (WESTERN FABRICATORS, BURNABY, B.C.) FRAME TO SUPPORT THE UPPER PART OF THE COLUMN. THE COLUMN WAS PLACED IN A COLD ROOM AT 4^{o} C TO MAINTAIN CONSTANT TEMPERATURE AND PARTIALLY FILLED WITH ELUANT WHICH WAS FLUSHED BACK AND FORTH THROUGH THE OUTLET HOLE TO REMOVE AIR BUBBLES FROM BENEATH THE GLASS FIBER DISK. SEPHADEX G-50 WAS ADDED TO THE COLUMN IN STAGES UNTIL THE ENTIRE COLUMN INCLUDING THE UPPER FITTING WAS PACKED WITH GEL. THE SECOND END PLATE WITH NYLON SCREEN AND GLASS FIBER DISCS WAS PRESSED INTO THE FITTING AND TIGHTENED UNTIL A GOOD SEAL WAS OBTAINED. THIS PROVIDED A 150 CM COLUMN ENTIRELY FILLED WITH GEL SO THAT EITHER NORMAL DOWNWARD OR REVERSE FLOW COULD BE USED. A THREE WAY VALVE IN THE INPUT LINE ALLOWED THE SAMPLE TO FLOW DIRECTLY ONTO THE COLUMN. AS SHOWN IN FIGURE 12 V_0 OF THE COLUMN WAS 4 L AND V_T 14 L. EXCELLENT SEPARATION WAS ACHIEVED AND THE STABILITY WAS SUCH THAT THE COLUMN SELDOM REQUIRED REPOURING.

C. Techniques for High Voltage Paper Electrophoresis.

1. Apparatus

High voltage electrophoresis was carried out in the Michl vertical strip apparatus (74, 75) modified as described by Ryle et al (110). The monograph by Leggett-Bailey contains a detailed discussion of this technique (68).

The apparatus used were constructed in all-glass Shandon Chromatanks (22.5in. x 9in. x 22.5 in. deep) from Consolidated Laboratories (Weston, Ontario). These were fitted with glass racks to support the upper glass buffer troughs, glass rods to prevent the paper contacting the walls and Lucite lids supporting the glass cooling coils.

Platinum electrodes in glass electrode holders were placed in the upper buffer troughs (cathode) and similar electrodes placed two centimeters from the bottom of the tanks which contained buffer to a depth of appoximately four centimeters. Both electrodes were attached to connectors mounted externally on the chromotanks. A Canadian Research Institute (Don Mills, Ontario) Model EPSK-200 D.C. power supply was used to maintain constant voltages up to 5 Kv at 200 mA.

The entire system was placed in a fume hood to remove toxic vapors. The hood was equiped with Lucite doors connected to an interlock safety system allowing the operator to view the paper during operation while minimizing the electrical hazard.

The two volatile buffer systems used in the present

STUDY WERE FIRST DESCRIBED BY AMBLER (134). THE BUFFERS AND COOLANTS USED WERE AS FOLLOWS:

pH 1.9

BUFFER: 2% FORMIC ACID (v/v)
8% ACETIC ACID (v/v)
IN DEIONIZED WATER

COOLANT: VARSOL

pH 6.5

BUFFER: 879 ML DEIONIZED WATER
100 ML PYRIDINE
3 ML GLACIAL ACETIC ACID

COOLANT: 92% TOLUENE, 8% PYRIDINE (BY VOLUME)

ALL REAGENTS WERE FISHER CERTIFIED GRADE. VARSOL WAS PURCHASED FROM THE IMPERIAL OIL Co.

2. STANDARDS AND MARKERS.

TWO AMINO ACID STANDARD MIXTURES WERE PREPARED CONTAINING 2 NMOLES PER µL OF EACH OF THE ACIDS LISTED.

S-1 LYSINE, ARGININE, VALINE, LEUCINE, METHIONINE, PROLINE, PHENYLALANINE, TYROSINE, CYSTEIC ACID.

S-2 HISTIDINE, GLYCINE, ALANINE, SERINE, ISOLEUCINE, THREONINE, GLUTAMIC ACID, ASPARTIC ACID, TAURINE.

5 µL OF EACH MIXTURE WAS ROUTINELY APPLIED TO THE ORIGIN LINE ON SPOTS OUTSIDE THE SAMPLE AREA. IN THE pH 6.5 BUFFER ONLY THE THREE BASIC AND THREE ACIDIC ACIDS MOVE AS INDICATED IN FIGURE 21. IN THE pH 1.9 BUFFER THE ACIDS ARE FOUND IN THE ORDER LISTED MOVING DOWN FROM THE CATHODE AS SHOWN IN FIGURE 22. THESE STANDARDS INDICATE WHETHER THE DETECTION REAGENTS

ARE WORKING AS EXPECTED AND ARE USED IN CALCULATING RELATIVE MOBILITIES.

IN ADDITION TO THE AMINO ACID MIXTURES EACH ELECTROPHORETOGRAM IS SPOTTED WITH VISIBLE MARKER DYES TO INDICATE THE CONDITIONS DURING THE RUN AND TO DETERMINE THE LENGTH OF THE RUN. FOR RUNS AT pH 6.5 THREE MARKERS ARE USED: METHYL GREEN (C.I. NO. 42590, FISHER CERTIFIED BIOLOGICAL STAIN) ORANGE G (C.I. NO. 16230, FISHER CERTIFIES BIOLOGICAL STAIN) AND -DINITROPHENYL-LYSINE (CALBIOCHEM, LOS ANGELES). CURRENT IS NORMALLY APPLIED TO THESE RUNS UNTIL THE METHYL GREEN HAS RUN TO A MARK 20 CM FROM THE ORIGIN. THE ε-DINITROPHENYL-LYSINE IS NEUTRAL AT THIS pH AND MOVES ONLY BY ELECTROENDOSMOSIS; AND ITS LOCATION ON THE ELECTROPHORETOGRAM IS USED TO INDICATE THE NEUTRAL LINE FOR CALCULATIONS OF RELATIVE MOBILITY. FOR RUNS AT pH 1.9 ONLY METHYL GREEN AND ε-DINITROPHENYL-LYSINE MARKERS ARE USED, AND THE METHYL GREEN IS ALLOWED TO TRAVEL 30 CM FROM THE ORIGIN. THE TAURINE IN THE AMINO ACID STANDARD IS NEUTRAL AT THIS pH AND IS USED TO INDICATE THE NEUTRAL LINE.

3. DETECTION REAGENTS.

THE CADMIUM-NINHYDRIN REAGENT OF HEILMAN ET AL (54) WAS USED WHEN MAXIMUM SENSITIVITY (1 TO 5 NMOLES/CM^2) WAS REQUIRED OR WHEN INFORMATION ON THE N-TERMINAL AMINO ACID WAS NEEDED. THIS REAGENT WAS PREPARED BY MIXING 15 ML OF A STOCK SOLUTION CONTAINING 5 G CADMIUM ACETATE (FISHER CERTIFIED REAGENT) IN 250 ML OF GLACIAL ACETIC ACID AND

500 ML DISTILLED WATER WITH 100 ML OF 0.5% (W/V) NINHYDRIN (PIERCE CHEMICAL CO.) IN ACETONE (FISHER REAGENT GRADE). PAPERS WERE DIPPED IN THIS SOLUTION AND ALLOWED TO AIR DRY UNTIL THE ACETONE WAS REMOVED. THE PAPER WAS THEN PLACED IN AN OVEN AT 60^{0} C FOR 20 MINUTES FOR COLOR DEVELOPMENT. IN GENERAL PEPTIDES HAVING THE N-TERMINAL AMINO ACIDS LISTED BELOW HAVE BEEN SHOWN TO GIVE THE COLORS INDICATED WITH THIS REAGENT.

YELLOW (STABLE 1 WEEK) RED	GLY, SER, THR, CYS, PRO, CYSTEIC
ORANGE	SER, HIS
SLOW RED (OVERNITE)	ILE, VAL
FAST RED	LEU, LYS, ARG, ASP, GLU, TYR, PHE, MET, $METSO_2$, TRP, ALA

THESE COLOR DIFFERENCES ARE USEFUL IN PREDICTING THE PROCEDURES NEEDED TO IDENTIFY THE DANSYL-DERIVATIVES OF THE PEPTIDES.

BECAUSE OF ITS HIGH SENSITIVITY FOR ARGININE PEPTIDES (0.1 NMOLES/CM^2) AND THE EXTRA INFORMATION IT PROVIDED, THE PAQ REAGENT WAS ROUTINELY USED IN COMBINATION WITH A SPECIAL NINHYDRIN REAGENT FOR PEPTIDE DETECTION. THE REAGENTS USED WERE DESCRIBED BY YAMADA AND ITANO (133). THE PAQ REAGENT WAS PREPARED BY MIXING EQUAL VOLUMES OF A SOLUTION CONTAINING 0.02% (W/V) PHENANTHRENQUINONE (FISHER SCIENTIFIC, CO., HIGHEST PURITY) IN ANHYDROUS ETHANOL AND 10% W/V SODIUM HYDROXIDE IN 60% ETHANOL. THE PAPERS WERE DIPPED IN THIS MIXTURE AND ALLOWED TO AIR DRY 20 MINUTES

PRIOR TO EXAMINATION UNDER A LONGWAVE UV LAMP.

AFTER AN ADDITIONAL 20 MINUTES DRYING THE PAPER COULD BE DEVELOPED WITH AN ACID NINHYDRIN REAGENT MADE BY MIXING 20 ML OF A SOLUTION CONTAINING 1 G OF CADMIUM ACETATE IN 150 ML OF GLACIAL ACETIC ACID AND 50 ML OF DISTILLED WATER WITH 80 ML OF 0.5 % (w/v) NINHYDRIN IN ACETONE. IN THIS REAGENT ALL PEPTIDES PRODUCED A VIOLET-RED COLOR ON DRYING AT 60° C FOR 20 MINUTES.

D. Amino Acid Analysis

1. Hydrolysis.

HYDROLYSES WERE CARRIED OUT ESSENTIALLY AS DESCRIBED BY MOORE AND STEIN (77). ALIQUOTS OF SOLUTIONS CONTAINING 2 TO 20 NMOLES OF PROTEIN WERE LYOPHILIZED IN 12 x 75 MM PYREX CULTURE TUBES AND REDISSOLVED IN 0.5 ML OF 5.7 M HYDROCHLORIC ACID. THE TUBES WERE THEN HEATED NEAR THE TOP WITH A FINE, HOT GAS-OXYGEN FLAME AND DRAWN OUT TO YIELD A NARROW (1MM), HEAVY WALLED NECK. THE CONTENTS WERE THEN FROZEN IN A DRY ICE-ACETONE MIXTURE TO PREVENT BUBBLING AND THE TUBES EVACUATED TO ABOUT 10^{-2} TORR BEFORE SEALING THE NECK WITH A MODERATELY HOT GAS-OXYGEN FLAME. HYDROLYSIS ON THE SEALED TUBES WAS THEN ROUTINELY CARRIED OUT FOR 17 HOURS AT 110° C IN A TEMP-BLOK FROM LAB-LINE INSTRUMENTS, MELROSE PARK, ILLINOIS. AFTER HYDROLYSIS THE TUBES WERE OPENED AND DRIED IN A VACUUM DESSICATOR OVER SODIUM HYDROXIDE. THE HYDROCHLORIC ACID USED WAS CONSTANT BOILING AZEOTROPE DISTILLED FROM BAKER AND ADAMSON REAGENT GRADE HYDROCHLORIC

ACID FROM ALLIED CHEMICALS CANADA, LTD. DILUTED WITH DISTILLED, DEIONIZED WATER. THE TUBES USED FOR HYDROLYSIS WERE WASHED IN STRONG DETERGENT AND RINSED FOUR TIMES WITH HOT TAP WATER FOLLOWED BY FOUR RINSES WITH DISTILLED WATER. THEY WERE THEN ALLOWED TO STAND OVERNITE IN 1 M HYDROCHLORIC ACID, RINSED ONCE MORE IN THE SAME ACID AND DRIED DIRECTLY FROM THE ACID RINSE IN A 60° C OVEN.

2. AIR OXIDATION OF CYSTEINE.

DRIED, HYDROLYSED SAMPLES CONTAINING CYSTEINE WERE DISSOLVED IN 200 µL OF 0.1 M PHOSPHATE BUFFER AT PH 6.5 AND ALLOWED TO STAND OPEN TO THE AIR FOR FOUR HOURS TO INSURE COMPLETE RECOVERY AS ½-CYSTINE. THE SAMPLES WERE THEN ADJUSTED TO PH 2 WITH 5 µL OF 1 M HYDROCHLORIC ACID FOR APPLICATION TO THE ANALYSER COLUMN.

3. AUTOMATED ANALYSIS.

THE FIRST DETAILED REPORT OF AN AUTOMATED SYSTEM FOR AMINO ACID ANALYSIS CAME FROM SPACKMAN ET AL IN 1958 (118) AND ALTHOUGH SEVERAL ALTERNATE SYSTEMS HAVE SINCE BEEN REPORTED THE ORIGINAL PROCEDURE IS STILL THE BASIS OF THE MAJORITY OF AMINO ACID ANALYSES PERFORMED. THERE ARE NOW A NUMBER OF COMMERCIAL INSTRUMENTS ON THE MARKET WHICH USE THIS BASIC PROCEDURE, AND THESE INSTRUMENTS VARY PRIMARILY IN THE DESIGN AND VARIETY OF ANCILLARY EQUIPMENT AVAILABLE. THEIR COMMON FEATURES INCLUDE SEPARATION OF AMINO ACIDS BY ELUTION FROM SULFONATED POLYSTYRENE RESINS, SIMILAR TO DOWEX 50 X 8, BY A SERIES OF CITRATE BUFFERS FOLLOWED BY

DETECTION AND QUANTITATION BASED ON THE REACTION OF THE ELUTED AMINO ACIDS WITH A NINHYDRIN REAGENT. THE AMOUNT OF COLORED REACTION PRODUCT IS MEASURED BY A PHOTOMETER AND RECORDED. THESE PROCEDURES ARE CARRIED OUT "ON LINE" IN A CONTINUOUSLY FLOWING SYSTEM WHICH REQUIRES A BUFFER PUMP TO MOVE THE ELUANT THROUGH THE COLUMN AND A NINHYDRIN PUMP TO MIX A CONSTANT PROPORTION OF THIS REAGENT WITH THE ELUATE. THIS MIXTURE IS THEN PASSED THROUGH A COIL OF NARROW BORE TEFLON TUBING IN A BOILING WATER BATH TO ACCELERATE THE REACTION AND THENCE TO A SERIES OF FLOW CELLS IN THE PHOTOMETER WHERE THE ABSORBANCE AT 570 NM IS RECORDED FOR THE AMINO ACIDS AND AT 440 NM FOR THE IMINO ACIDS.

THE ORDER AND DEGREE OF SEPARATION OF THE AMINO ACIDS IN THE ELUATE CAN BE VARIED BY CHANGES IN THE TEMPERATURE OF THE COLUMNS AND THE pH AND IONIC STRENGTH OF THE BUFFERS USED. MOST COMMERCIAL MACHINES HAVE PROGRAMMED SYSTEMS FOR SWITCHING BUFFERS AND TEMPERATURE AND CONTROLLING NINHYDRIN FLOW, THE RECORDER AND THE REGENERATION CYCLE FOR THE COLUMN. THE FLEXIBILITY OF THESE PROGRAMMING SYSTEMS IS ONE OF THE MAJOR VARIABLES IN THE COMMERCIAL INSTRUMENTS. THE SECOND MAJOR VARIABLE IS SENSITIVITY. MOST COMMERCIAL INSTRUMENTS ARE ROUTINELY USED FOR QUANTITATION IN THE RANGE OF 50 TO 250 NANOMOLES OF AMINO ACID, AND ALTHOUGH THEY HAVE REPORTED DETECTION LIMITS VARYING FROM 1 TO 10 NMOLES THESE LIMITS ARE OF LITTLE SIGNIFICANCE SINCE AT THESE LEVELS IT IS POSSIBLE ONLY TO DETECT THE AMINO ACIDS NOT TO QUANTITATE THEM. A THIRD VARIABLE WHICH HAS PROVED QUITE CRITICAL IS

RELIABILITY SINCE INCREASED SENSITIVITY IS OF LITTLE USE IF THE SYSTEM REQUIRES FIVE SAMPLES TO ACHIEVE ONE GOOD ANALYSIS.

THE WORK DESCRIBED IN THIS THESIS AND OTHER WORK UNDERWAY IN THIS LABORATORY REQUIRED A HIGHLY SENSITIVE PROCEDURE FOR AMINO ACID ANALYSIS SINCE THE PROTEINS UNDER STUDY WERE AVAILABLE IN ONLY QUITE LIMITED QUANTITIES. AMINO ACID ANALYSIS IS THE HEART OF ALL PROTEIN STUDIES AND IF THE ANALYSES ARE CARRIED OUT AT THE LEVELS REQUIRED BY THE COMMERCIALLY AVAILABLE EQUIPMENT IT IS THE LIMITING FACTOR IN DETERMINING THE QUANTITIES OF PROTEIN WHICH MUST BE AVAILABLE FOR STUDY. THE ADVANCES IN OTHER AREAS OF PROTEIN CHEMISTRY HAVE FAR OUTSTRIPED THOSE IN AMINO ACID ANALYSIS. AS AN EXAMPLE OF THIS THE PRESENT USE OF THE DANSYL-EDMAN METHOD (52) ALLOWS SEQUENCE DETERMINATION ON PEPTIDES USING LESS THAN HALF THE MATERIAL NECESSARY FOR A SINGLE ANALYSIS AT THE 50 NMOLE LEVEL. CLEARLY ROUTINE QUANTITATION AT OR BELOW THE 1 NMOLE LEVEL WAS ESSENTIAL TO BRING THIS PROCEDURE IN LINE WITH THE OTHER AVAILABLE TECHNIQUES.

TO ACHIEVE THIS, MODIFICATIONS WERE CARRIED OUT ON A NEWLY DEVELOPED COMMERCIAL MACHINE, THE BIOCHROM 200, WHICH HAD THE HIGHEST AVAILABLE SENSITIVITY AND THE DESIRED FLEXIBILITY IN PROGRAMMING. THE BASES OF THIS HIGH SENSITIVITY WERE LONG PATHLENGTH FLOW CELLS (14 MM VERSUS THE MORE COMMON 7 MM) AND A HIGH DEGREE OF AMPLIFICATION IN THE RECORDER CIRCUIT (5.5MV FULL SCALE DEFLECTION). EARLY EXPERIMENTS AT LOW LEVELS INDICATED THAT THIS SYSTEM WAS

INADEQUATE SINCE THE SENSITIVITY WAS STILL NOT GREAT ENOUGH AND THE SIGNAL TO NOISE RATIO OF THE SYSTEM WAS POOR. RELATIVELY SMALL PEAKS WERE NEARLY HIDDEN IN THE RANDOM FLUCTUATIONS IN THE BASE LINE. A MAJOR PART OF THIS PROBLEM WAS THE INHERENT NOISE IN THE PHOTOMULTIPLIER TUBES USED IN THE PHOTOMETER WHICH WAS GREATLY AMPLIFIED BY THE RECORDER. TO OVERCOME THIS PROBLEM A DETECTION CIRCUIT WAS DESIGNED BASED ON A NEWLY DEVELOPED PHOTOTRANSISTOR (FPT 100, FAIRCHILD SEMICONDUCTOR, MOUNTAIN VIEW, CALIFORNIA) WHICH WAS USED AS ONE SIDE OF A WHEATSTONE BRIDGE CIRCUIT. VARIATION OF THE RESISTANCE ON THE OPPOSITE SIDE OF THE BRIDGE ALLOWED ADJUSTMENT OF THE OUTPUT TO SET THE BASELINE, WHILE DRAINING PART OF THIS OUTPUT OFF TO GROUND THROUGH A SECOND VARIABLE RESISTANCE ALLOWED CONTROL OF THE GAIN IN THE CIRCUIT. D.C. CURRENT WAS SUPPLIED BY MERCURY CELLS WHICH HAVE AN EXTREMELY STABLE OUTPUT. THE NOISE LEVEL IN THESE CIRCUITS WAS UNDETECTABLE ON THE RECORDER AND THE HIGH GAIN THEY PROVIDED ALLOWED REDUCTION OF THE GAIN (55MV FULL SCALE) IN THE NOISIER RECORDER AMPLIFIER. THREE SEPARATE CIRCUITS WERE USED TO MEASURE THE ABSORBANCE AT 570 NM WITH A 14 MM FLOW CELL, AT 570 NM WITH A 7 MM FLOW CELL AND AT 440 NM WITH A 14 MM FLOW CELL.

THE ANALYSES IN TABLE IV WERE CARRIED OUT USING THIS SYSTEM AND ACCEPTABLE QUANTITATION WAS POSSIBLE AT THE 2 TO3 NMOLE LEVEL. AS INDICATED IN THE TABLE THE STANDARD TWO COLUMN SYSTEM WAS USED. THE ACIDIC AND NEUTRAL AMINO ACIDS WERE SEPARATED ON A 52 x 0.9 CM COLUMN OF BIO-RAD

AMINEX A-6 RESIN AT 52° C USING 0.2 N SODIUM CITRATE BUFFERS AT pH 3.25 AND pH 4.40 PREPARED IN DISTILLED, DEIONIZED WATER AS DESCRIBED BELOW.

	pH 3.25	4.40
SODIUM CITRATE DIHYDRATE (AMINO ACID ANALYSIS GRADE BIO-RAD LABORATORIES, RICHMOND, CALIFORNIA)	78.4 G	78.4 G
CONC. HYDROCHLORIC ACID (BAKER-ADAMSON REAGENT, ALLIED CHEMICALS CANADA, LTD.)	49.4 ML	30.0 ML
OCTANOIC ACID (A.A.A. GRADE, BIO-RAD.)	0.5 ML	0.5 ML
FINAL VOLUME	4.0 L	4.0 L

THE pH 3.25 BUFFER WAS PUMPED AT A RATE OF 70 ML/HR FOR 60 MIN. AND pH 4.40 BUFFER FOR AN ADDITIONAL 100 MIN., FOR A TOTAL RUN TIME OF 2 HOURS AND 40 MINUTES. REGENERATION WITH 0.2 M SODIUM HYDROXIDE AND RE-EQUILIBRATION REQUIRED AN ADDITIONAL 70 MINUTES. BASIC AMINO ACIDS WERE SEPARATED ON A 14 x 0.9 CM COLUMN OF BIO-RAD AMINEX A-5 RESIN AT 52° C USING A 0.35 N SODIUM CITRATE BUFFER AT pH 5.28 PREPARED AS DESCRIBED BELOW.

pH 5.28

SODIUM CITRATE DIHYDRATE (A.A.A. GRADE, BIO-RAD)	137.2 G
CONC. HYDROCHLORIC ACID (B & A REAGENT)	26 ML
OCTANOIC ACID (A.A.A. GRADE, BIO-RAD)	0.5 ML
FINAL VOLUME	4.0 L

ELUTION OF THESE ACIDS AT 70 ML/HR REQUIRED 70 MIN AND A TOTAL CYCLE TIME OF 110 MIN.

NINHYDRIN REAGENT PREPARED AS INDICATED BELOW WAS PUMPED AT A RATE OF 35 ML/HR AND MIXED WITH THE ELUATES FOR

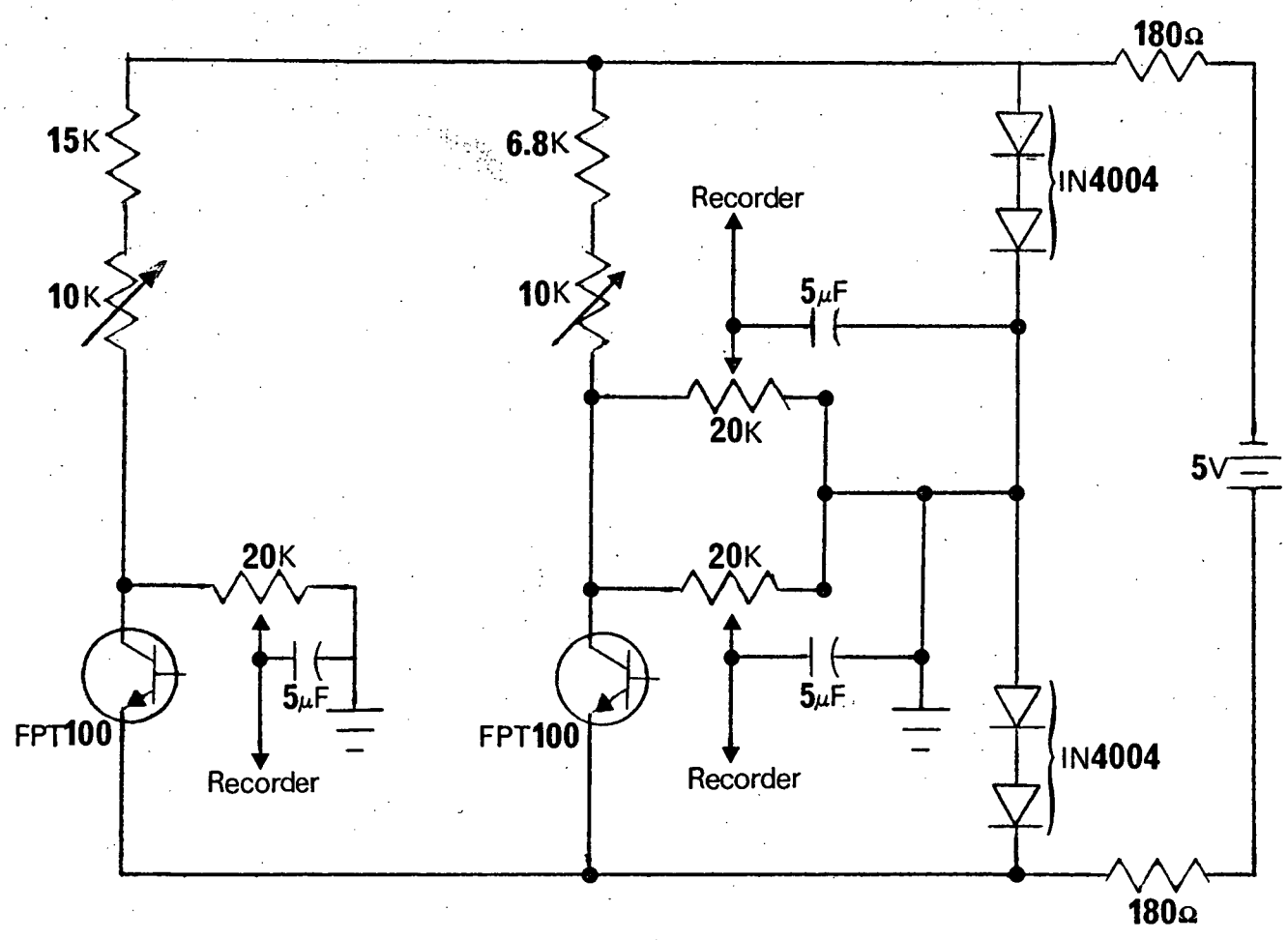


FIGURE 45. IMPROVED PHOTOTRANSISTOR DETECTOR FOR AMINO ACID ANALYSER--CIRCUIT DIAGRAM.

DETECTION.

NINHYDRIN REAGENT:

METHYL CELLOSOLVE (SEQUANAL GRADE, PIERCE CHEMICAL CO., ROCKFORD, ILLINOIS)	3 L
4 M SODIUM ACETATE (FISHER CERTIFIED)	1 L
NINHYDRIN (REAGENT GRADE, PIERCE CHEM.)	80 G
STANNOUS CHLORIDE DIHYDRATE (A.A.A. GRADE, BIO-RAD)	1.60 G

OXYGEN WAS REMOVED BY BUBBLING THE MIXTURE WITH NITROGEN (L GRADE, CANADIAN LIQUID AIR LTD.) FOR 30 MIN AND THE REAGENT WAS STORED UNDER NITROGEN. UNDER THESE CONDITIONS A COMPLETE ANALYSIS REQUIRED A MINIMUM OF 5 NMOLES OF PROTEIN AND A TOTAL OF ALMOST 6 HOUR. THUS WITH CAREFUL PLANNING AND FULL USE OF AUTOMATED PROCEDURES TWO ANALYSES COULD BE CARRIED OUT IN A NORMAL WORKING DAY.

ALTHOUGH THIS SYSTEM WAS A MARKED IMPROVEMENT OVER THE ORIGINAL THE MATERIAL REQUIREMENTS WERE STILL TOO HIGH, AND THE NOISE LEVEL IN THE BASELINE WAS UNSATISFACTORY WHEN MAXIMUM GAIN WAS USED, PARTICULARLY ON THE 440 CHANNEL SINCE ABSORBANCE OF THE IMINO ACID PRODUCT IS LESS THAN THAT OF THE AMINO ACIDS. THE PHOTOTRANSISTOR CIRCUIT HAD PROVED TO BE MUCH QUIETER THAN THE REST OF THE SYSTEM AND IT WAS THEREFORE DECIDED TO INCREASE THE GAIN IN THIS CIRCUIT BY REDESIGN AS SHOWN IN FIGURE 45 AND TO CUT DOWN THE NOISE WHICH WAS NOW KNOWN TO COME PRIMARILY FROM THE HYDRAULIC SYSTEM. HAMILTON(51) HAD PRESENTED A DETAILED DISCUSSION OF THE DIFFICULTIES ENDOUNTERED IN HIGH SENSITIVITY ANALYSIS

AND EFFORTS WERE MADE TO CORRECT MANY OF THE FAULTS HE HAD POINTED OUT. THE TWO BASIC CAUSES OF NOISE IN THE BASELINE ARE RHYTHMICAL FLUCTUATIONS DUE TO MINUTE VARIATIONS IN PUMPING RATES AND RANDOM FLUCTUATIONS DUE TO PARTICULATE MATTER AND BUBBLES IN THE FLUIDS. RHYTHMICAL FLUCTUATIONS WERE ELIMINATED BY CAREFULLY MATCHING THE SPEED OF THE BUFFER AND NINHYDRIN PUMPS, BUT THE PROBLEM OF PARTICULATE MATTER WAS MORE COMPLEX. IMPROVED PROCEDURES FOR FILTRATION OF BUFFERS AND NINHYDRIN REAGENT HELPED AND IT WAS POSSIBLE TO REDUCE THE TIME IN THE REACTION BATH FROM 15 TO 5 MIN WITHOUT EFFECTING COLOR DEVELOPMENT (117) BY SHORTENING THE COIL. THIS HAD THE EFFECT OF REDUCING BUBBLE FORMATION AND PRECIPITATION. THESE CHANGES REDUCED BUT DID NOT ELIMINATE THE PROBLEM SO THAT MORE BASIC CHANGES WERE REQUIRED. THE PHOTOMETER WAS REDESIGNED TO MINIMIZE THE IMPORTANCE OF THE HYDRAULIC FLUCTUATIONS BY DECREASING THE LENGTH OF THE FLOW CELLS TO 7 MM AND COMPENSATING THIS REDUCTION BY INCREASED GAIN IN THE QUIET ELECTRONIC SYSTEMS. FURTHER IMPROVEMENTS WERE OBTAINED BY REDUCING THE NUMBER OF FLOW CELLS TO TWO AND DIVIDING THE OUTPUT OF THE 570 CHANNEL ELECTRICALLY TO PROVIDE A SUPPRESSED CHANNEL. THIS REDUCED MIXING VOLUME AND INCREASED PEAK HEIGHT RELATIVE TO BASE LINE VARIATIONS. A CHANGE IN THE CONFIGURATION OF THE PHOTOMETER SET UP ALLOWED BOTH FLOW CELLS TO BE ILLUMINATED FROM A SINGLE LAMP RATHER THAN THE THREE LAMPS USED ORIGINALLY. THIS REDUCED THE POWER REQUIREMENTS FOR THE VOLTAGE STABILIZER SUPPLYING THE LAMPS TO ABOUT

ONE FOURTH OF ITS RATED CAPACITY AND ELIMINATED LAMP NOISE WHICH HAD OCCURED PREVIOUSLY DURING SWITCHING OPERATIONS. IT ALSO ELIMINATED RANDOM VARIATIONS VETWEEN CHANNELS DUE TO INSTABILITIES IN THE LAMPS.

AN ADDITIONAL CHANGE WHICH HALVED THE MATERIAL REQUIREMENTS WAS THE ADOPTION OF AN ACCELERATED SINGLE COLUMN SYSTEM SIMILAR TO THAT OF DEVENYI (38) WHICH ALLOWED DETERMINATION OF ALL AMINO ACIDS IN A SINGLE RUN. THIS PROCEDURE REQUIRED THE USE OF A THIRD BUFFER WITH INCREASED IONIC STRENGTH (0.8 N IN SODIUM IONS) PREPARED AS SHOWN BELOW AND ELEVATION OF OPERATING TEMPERATURE TO 58° C. THE

pH 6.35

SODIUM HYDROXIDE (FISHER CERTIFIED REAGENT)	160 G
CITRIC ACID (A.A.A. GRADE, BIO-RAD)	259 G
OCTANOIC ACID (A.A.A. GRADE, BIO-RAD)	0.5 ML
FINAL VOLUME	4 L

REDUCED VISCOSITY AT THIS ELEVATED TEMPERATURE ALLOWED THE PUMPING RATES TO BE INCREASED TO 80 ML/HR FOR BUFFER AND 40 ML/HR FOR NINHYDRIN. THIS SYSTEM WAS USED FOR THE ANALYSES OF THE OXIDIZED SAMPLE OF SALMON CT AND ALLOWED COMPLETE QUANTITATIVE AMINO ACID ANALYSIS ON AS LITTLE AS 2 NMOLES OF PROTEIN. THE TIME REQUIRED FOR A COMPLETE ANALYSIS WAS REDUCED TO LESS THAN 5 HOURS (pH 3.25, 50 MIN; pH 4.40, 60 MIN; pH 6.45, 95 MIN; RECYCLE, 70 MIN).

THESE MODIFICATIONS ALLOWED ROUTINE OPERATION AT THE 5 NMOLE LEVEL, A 50 FOLD INCREASE IN SENSITIVITY, BUT IT WAS STILL NOT POSSIBLE TO WORK AT THE 1 NMOLE LEVEL.

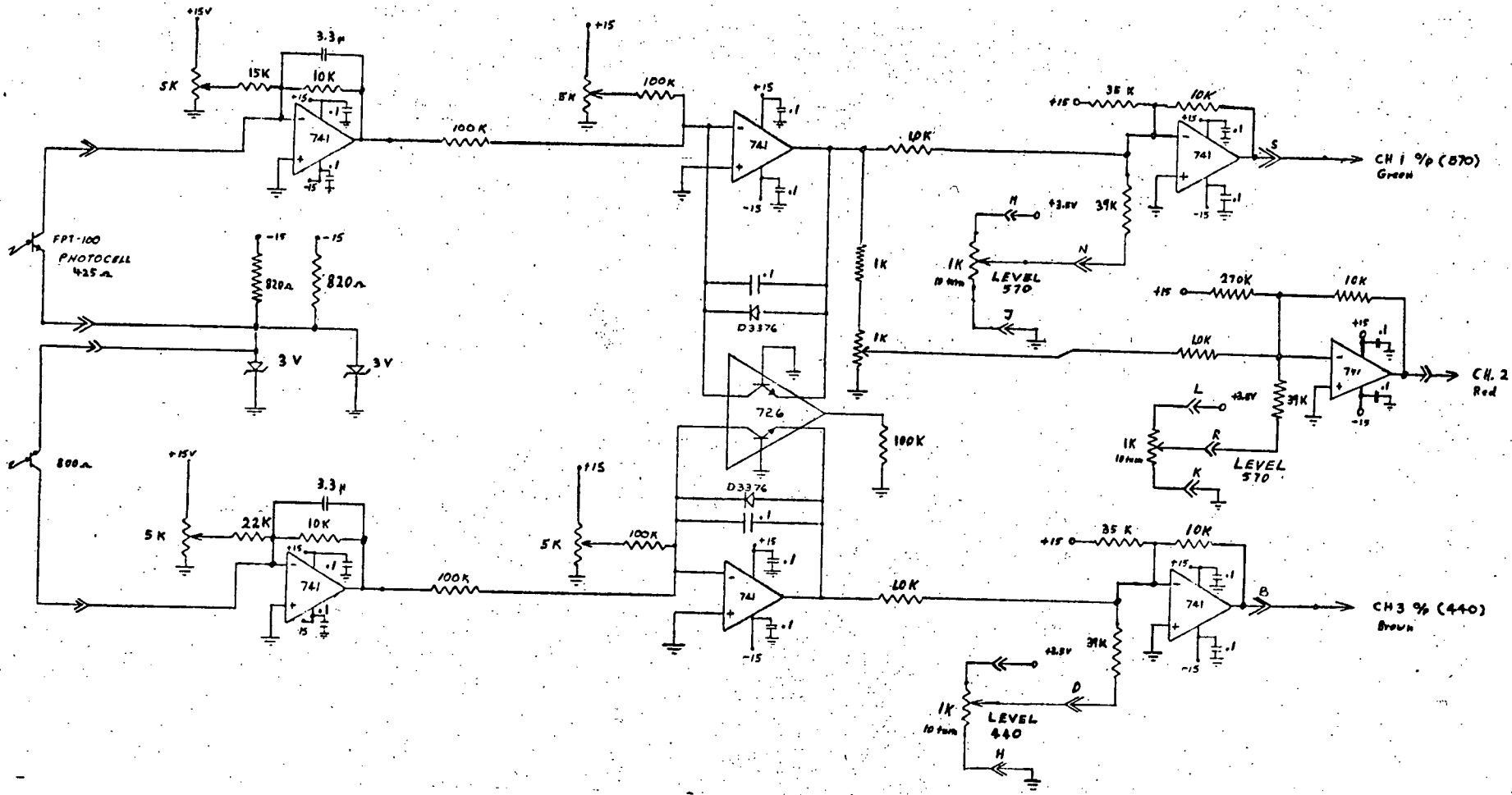


FIGURE 46. PHOTOTRANSISTOR--LOG AMPLIFIER DETECTOR FOR AMINO ACID ANALYSER--CIRCUIT DIAGRAM.

BASELINE NOISE FROM THE HYDRAULIC SYSTEM WAS THE MAJOR PROBLEM SINCE THE SOLID STATE ELECTRONICS WERE ESSENTIALLY NOISE FREE AND COULD PROVIDE ALMOST UNLIMITED GAIN. LINEAR AMPLIFICATION OF THE OUTPUT SIGNAL HOWEVER, MAGNIFIED THE NOISE AS WELL AS THE SIGNAL DUE TO THE NINHYDRIN PRODUCTS. CALCULATIONS OF AMINO ACID CONCENTRATIONS IN THE ELUATE OF THIS SYSTEM ARE BASED ON THE BEER-LAMBERT LAW ($I/I_0 = e^{-UCX}$) WHICH STATES THAT THE RATIO OF THE INTENSITY OF TRANSMITTED LIGHT (I) TO THAT OF INCIDENT LIGHT (I_0) IS A LOGARITHMIC FUNCTION OF THE ABSORPTION COEFFICENT (μ) OF THE DISSOLVED COMPOUND, THE CONCENTRATION OF THAT COMPOUND (c) AND THE PATHLENGTH (x). WHEN μ, I_0 AND x ARE CONSTANT, I, THE QUANTITY MEASURED, IS A LOG FUNCTION OF THE CONCENTRATION. IN THIS SYSTEM A CONCENTRATION CHANGE FROM 0 TO1 NMOLE AND FROM 15 TO 20 NMOLES BOTH RESULT IN A RECORDER DEFLECTION WHICH IS 10% OF FULL SCALE. THUS BUBBLES AND PARTICLES CAUSE LARGE FLUCTUATIONS IN THE BASELINE, BUT LARGE CHANGES IN CONCENTRATION SOMETIMES HAVE ONLY A RELATIVELY SMALL EFFECT. IT IS THEREFORE POSSIBLE TO IMPROVE THE SIGNAL TO NOISE RATIO OF THE SYSTEM BY USING AN AMPLIFIER WITH AN OUTPUT WHICH IS A LOGARITHMIC FUNCTION OF ITS INPUT. THE USE OF SUCH A LOG-AMPLIFIER MEANS THAT PEAK HEIGHT BECOMES DIRECTLY PROPORTIONAL TO CONCENTRATION AND THAT SMALL VARIATIONS NEAR THE BASELINE ARE NO LONGER EXAGGERATED. FIGURE 46 SHOWS THE CIRCUIT DIAGRAM OF A LOG-AMPLIFIER DESIGNED BY TECHCAL ELECTRONIC SERVICES, VANCOUVER, B.C. FOR USE WITH THE PHOTO-TRANSISTOR DETECTION SYSTEM.

FURTHER IMPROVEMENTS MADE TO THE SYSTEM AT THIS TIME INCLUDED REDUCTION OF COLUMN DIAMETER TO 0.6 CM WHICH REDUCED ELUTION VOLUME BY HALF, EFFECTIVELY DOUBLING SAMPLE CONCENTRATION IN THE ELUATE; AND THE USE OF A TWO BUFFER-SINGLE COLUMN ELUTION SYSTEM. THE FIRST BUFFER WAS THE SAME AS THAT PREVIOUSLY USED BUT THE pH 4.40 BUFFER WAS MADE UP TO 0,8 N IN SODIUM IONS BY THE ADDITION OF 35.1 G/L OF SODIUM CHLORIDE (FISHER REAGENT GRADE). IT HAD BEEN OBSERVED EARLIER THAT BOTH SODIUM CITRATE AND CITRIC ACID APPARENTLY CONTAINED MINOR CONTAMINANTS WHICH WERE NINHYDRIN POSITIVE. THIS MEANT THAT INCREASING IONIC STRENGTH WITH CITRATE BUFFERS INCREASED THE BACKGROUND COLOR IN THE PHOTOMETER AND IN THE HIGH SENSITIVITY SYSTEM THIS RESULTED IN A MARKED SHIFT IN BASELINE DURING THE THIRD BUFFER WHICH WAS AN INCONVENIENCE. THIS WAS ELIMINATED BY MAINTAINING THE CONCENTRATION OF CITRATE IN BOTH BUFFERS CONSTANT AND INCREASING IONIC STRENGTH WITH SODIUM CHLORIDE WHICH CAUSED NO CHANGE IN BACKGROUND COLOR.

THIS SYSTEM WAS USED FOR THE ANALYSES ON THE SALMON CT TRYPTIC PEPTIDES (TABLE VI) AND FOR PORCINE CT PEPTIDES (CHAPTER IV), AND IS RUN ROUTINELY AT THE 5 NMOLE LEVEL. PUMPING RATES OF 50 ML/HR FOR BUFFER AND 25 ML/HR FOR NINHYDRIN ALLOW COMPLETE AMINO ACID ANALYSES IN SLIGHTLY OVER 4 HR ON SAMPLES OF LESS THAN 1 NMOLE WHEN THE FULL GAIN OF THE LOG-AMPLIFIER IS USED. THE DECREASED-ANALYSIS TIME ALSO MEANS THAT THE AUTOMATED SYSTEM ALLOWS COMPLETION OF THREE ANALYSES DURING A NORMAL DAY. A FURTHER ADVANTAGE OF THE

SYSTEM IS THAT THE LINEAR RELATIONSHIP BETWEEN PEAK HEIGHT AND CONCENTRATION ALLOWS DIRECT DETERMINATION OF CONCENTRATION FROM MEASUREMENTS OF PEAK HEIGHT. VARIATION IN PEAK HEIGHT BETWEEN AMINO ACIDS CAN BE COMPENSATED FOR BY THE USE OF CORRECTION FACTORS AND THE TEDIOUS PROCESS OF AREA CALCULATIONS AND THE INACCURACIES OF INTEGRATING UNITS ARE ELIMINATED. COMPARISONS OF THE ACCURACY OF AREA AND HEIGHT MEASUREMENTS IN THIS SYSTEM HAVE SHOWN NO SIGNIFICANT VARIATION BETWEEN THE TWO METHODS.

E. Preparation and Identification of Dansyl Derivatives.

N-TERMINAL AMINO ACIDS WERE DETERMINED BY A MODIFICATION OF THE DANSYL PROCEDURE OF GREY AND HARTLEY (47). AN ALIQUOT OF THE SAMPLE TO BE ANALYSED CONTAINING APPROXIMATELY 1 NMOLE OF PEPTIDE WAS PLACED IN A 4 x 50 MM PYREX CULTURE TUBE WHICH HAD BEEN CLEANED BY THE SAME METHODS DESCRIBED FOR THE HYDROLYSIS TUBES. THE SAMPLE WAS DRIED IN VACUUO AND REDISSOLVED IN 10 µL OF 0.2 M SODIUM BICARBONATE (BAKER CHEMICAL, REAGENT GRADE) IN DEIONIZED; DISTILLED WATER. 10 µL OF DANSYL CHLORIDE (1-DIMETHYLAMINO-NAPHTHALENE-5-SULFONYL CHLORIDE, CAL BIOCHEM, LOS ANGELES) IN FISHER REAGENT GRADE ACETONE (1 MG/ML) WAS THEN ADDED AND THE TUBE SEALED WITH PARAFILM. THE SAMPLE WAS THEN INCUBATED AT 37°C FOR 2 HOURS AND DRIED IN A VACUUM DESICCATOR OVER SULFURIC ACID. AFTER DRYING 50 µL OF CONSTANT BOILING HYDROCHLORIC ACID WAS ADDED AND THE TUBE DRAWN OUT AND FLAME SEALED. AFTER FOUR HOURS OF HYDROLYSIS AT 105° C THE TUBE WAS OPENED

AND DRIED IN A VACUUM DESSICATOR OVER SODIUM HYDROXIDE. THE RESIDUE WAS THEN DISSOLVED IN 10 µL OF AN ACETONE-GLACIAL ACETIC ACID MIXTURE (3:2) FOR APPLICATION TO THE POLYAMIDE PLATES USED FOR SEPARATION.

THE SYSTEM USED FOR IDENTIFYING THE DANSYL DERIVATIVES PRODUCED WAS SIMILAR TO THAT OF WOODS AND WANG (132) AS MODIFIED BY HARTLEY(52). THE BASIC PROCEDURE IS TWO-DIMENSIONAL, THIN-LAYER CHROMATOGRAPHY ON POLYAMIDE PLATES (CHENG-CHIN TRADING CO. LTD. TAIPEI, TAIWAN) USING FOUR SOLVENT SYSTEMS WHICH ALLOW POSITIVE IDENTIFICATION OF ALL NATURAL AMINO ACID DERIVATIVES FROM A SINGLE SAMPLE. THE ORIGINAL 15 CM SQUARE PLATES ARE CUT INTO NINE 5 CM SQUARE PLATES WHICH ARE USED TO RUN THE SAMPLES. THIS REDUCES BOTH RUNNING TIME AND COST, THOUGH SINCE PLATES CAN BE WASHED AND REUSED THE COST FACTOR IS NOT REALLY SIGNIFICANT. 0.5 µL OF A STANDARD SOLUTION CONTAINING 60 PICOMOLE/µL OF EACH DANSYL AMINO ACID IS APPLIED TO ONE SIDE OF THE PLATE 0.5 CM IN FROM THE SIDES IN ONE CORNER, AND A SIMILAR SAMPLE OF THE UNKNOWN CONTAINING 10 TO 50 PMOLES IS APPLIED TO THE OPPOSITE SIDE AT THE SAME POINT. THE FOUR SOLVENT SYSTEMS USED ARE LISTED BELOW.

1. 1.5% AQUEOUS FORMIC ACID
2. BENZENE, ACETIC ACID, 9:1
3. N-HEXANE, N-BUTANOL, ACETIC ACID, 3:3:1
4. 0.1 M AMMONIUM HYDROXIDE, ETHANOL, 9:1

THE BASIC TWO-DIMENSIONAL RUN CONSISTS OF SOLVENT (1) IN DIMENSION (1) FOR 3 TO 5 MINUTES, FOLLOWED BY SOLVENT (2) IN DIMENSION (2) FOR 5 TO 7 MINUTES. EXTREME CARE MUST BE TAKEN TO DRY THE PLATE THROUGHLY AFTER SOLVENT (1).

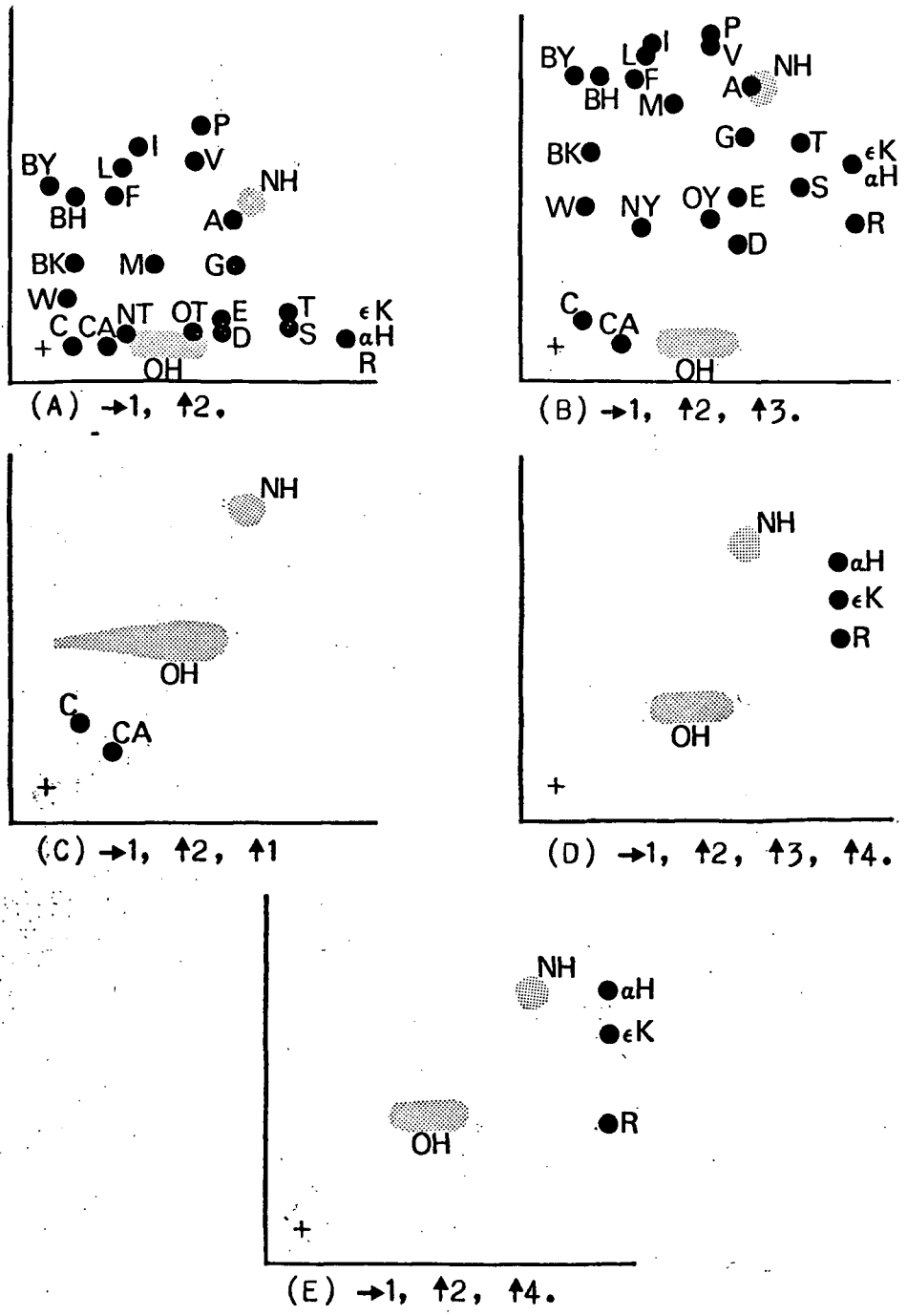


FIGURE 47. MIAGRATION OF DANSYL DERIVATIVES OF AMINO ACIDS ON POLYAMIDE LAYERS IN VARIOUS SOLVENTS. SOLVENTS ARE DESCRIBED IN THE TEXT AND WERE RUN IN THE DIMENSIONS INDICATED. SINGLE LETTER NOTATION FOR THE PARENT AMINO ACIDS ARE USED FOR MOST DERIVATIVES. ADDITIONAL ABBREVIATIONS INCLUDE; OH, DNASYL HYDROXIDE; NH, DANSYL AMINE; BY, BIS-DNS-TYR; BH, BIS-DNS-HIS; BK, BIS-DNS-LYS; CA, DNS-CYSTEIC ACID; NY, N-DNS-TYR; OY, O-DNS-TYR; αH, α-DNS-HIS; ϵK, ϵ-DNS-LYS.

FIGURE 47 (A) SHOWS THE POSITIONS OF THE FLOURESCENT SPOTS VISIBLE AFTER THESE TWO STAGES. THE DANSYL DERIVATIVES OF PROLINE, ISOLEUCINE, LEUCINE, VALINE, PHENYLALANINE, GLYCINE, METHIONINE, TRYPTOPHAN AND ALANINE CAN BE POSITIVELY IDENTIFIED AT THIS POINT. THE DI-DANSYL DERIATIVES OF TYROSINE, LYSINE AND HISTIDINE CAN ALSO BE IDENTIFIED, AS CAN DANSYL AMINE AND DANSYL HYDROXIDE. IF IDENTIFICATION CANNOT BE MADE AT THIS STAGE SOLVENT (3) MAY BE RUN IN DIMENSION (2) FOR ABOUT 8 MINUTES TO SEPARATE THE DERIVATIVES OF GLUTAMIC ACID FROM ASPARTIC ACID; THREONINE FROM SERINE; ARGININE FROM ε-LYSINE AND α-HISTIDINE; AND CYSTINE, α-TYROSINE AND O-TYROSINE FROM DANSYL HYDROXIDE. THE POSITIONS OF THE SPOTS AT THIS STAGE ARE SHOWN IN FIGURE 47 (B). AT THIS STAGE THE CYSTEIC ACID DERIVATIVE MAY STILL BE HIDDEN BENEATH THE DANSYL HYDROXIDE STREAK IF THE STREAK IS LARGE AND THE ε-LYSINE AND α-HISTIDINE DERIVATIVES ARE UNRESOLVED. DANSYL CYSTEIC ACID MAY BE SEPARATED FROM DANSYL HYDROXIDE BY RUNNING SOLVENT (1) IN DIMENSION (2) FOR 3 TO 5 MINUTES (FIGURE 47C). THE DERIVATIVES OF ε-LYSINE AND α-HISTIDINE MAY BE SEPARATED BY RUNNING SOLVENT (4) IN DIMENSION (2) FOR 3 TO 5 MINUTES (FIGURE 47D). α-HISTIDINE, ε-LYSINE AND ARGININE DERIVATIVES MAY ALSO BE SEPARATED BY RUNNING SOLVENT (4) IMMEDIATELY AFTER SOLVENT (2), AND IF THESE ARE THE ONLY EXPECTED DERIVATIVES THIS PROCEDURE IS PREFERABLE SINCE IT GIVES A BETTER SEPARATION AS SHOWN IN FIGURE 47 (E). THE PLATES MAY BE WASHED FOR REUSE BY STANDING OVERNITE IN SOLVENT (1) AND WASHING TWICE IN A MIXTURE OF 1 M AMMONIUM

HYDROXIDE AND ACETONE, 1:1.

THE STANDARD DANSYL AMINO ACIDS USED WERE PREPARED FROM THE AMINO ACIDS(CALBIOCHEM, LOS ANGELES) BY THE FOLLOWING PROCEDURE. 6.5 μMOLES OF AN AMINO ACID WERE DISSOLVED IN 1 ML OF 0.1 M SODIUM BICARBONATE SOLUTION AND MIXED WITH 1 ML OF DANSYL CHLORIDE IN ACETONE (6 MG/ML). THE MIXTURE WAS ALLOWED TO STAND OVERNITE AND THE SODIUM BICARBONATE PRECIPITATED WITH 8 ML OF ACETONE. THE STOCK SOLUTIONS THUS CONTAINED 650 PMOLES OF DERIVATIVE PER μL. AN ALIQUOT OF THE HISTIDINE SAMPLE WHICH CONTAINED PRIMARILY THE BIS-HISTIDINE DERIVATIVE WAS HYDROLYSED IN 5.7 M HYDROCHLORIC ACID FOR 17 HOURS TO PRODUCE THE α-HISTIDINE DERIVATIVE (47). ε-DANSYL-LYSINE WAS PURCHASED FROM CALBIOCHEM, LOS ANGELES AND MADE UP TO A SIMILAR CONCENTRATION IN ACETONE. THE ABOVE SOLUTIONS WERE MIXED IN EQUAL VOLUME TO PRODUCE THE STANDARD SOLUTION USED IN THIN-LAYER CHROMATOGRAPHY.

F. Polyacrilamide Gel Electtrophoresis.

POLYACRILAMIDE GEL ELECTROPHORESIS WAS CARRIED OUT USING THE METHOD OF JOHNS (61). THE GELS WERE PREPARED IN 5 X 75 MM GLASS TUBES USING 1 ML OF A SOLUTION CONTAINING 10 ML OF MONOMER STOCK (40% W/V ACRILAMIDE, 0.6% W/V N,N'-METHYLENEBISACRILAMIDE IN DISTILLED WATER), 10 ML OF CATALYST 1 (0.5% V/V N,N,N'N'-TETRAMETHYLENEDIAMINE IN 4.6 N ACETIC ACID) AND 6 ML OF CATALYST 2 (0.6% W/V AMMONIUM PERSULFATE) FOR EACH TUBE. ORGANIC REAGENTS WERE PURCHASED FROM EASTMAN ORGANIC CHEMICALS, ROCHESTER, N.Y.,

AND AMMONIUM PERSULFATE WAS FISHER REAGENT GRADE. THE GEL SOLUTION WAS DEGASSED FOR 30 MINUTES IN A VACUUM DESSICATOR BEFORE BEING PLACED IN THE TUBES. DISTILLED WATER WAS LAYERED CAREFULLY ON THE TOP OF THE GEL WITH AN INFUSION PUMP TO ELIMINATE THE MINISCUS, AND THE GELS ALLOWED TO POLYMERIZE. ELECTROPHORESIS WAS CARRIED OUT IN A SHANDON MODEL 12734 APPARATUS (COLAB CANADA LTD., WESTON, ONTARIO). GELS WERE PLACED IN THE APPARATUS SO THAT SAMPLE MIGRATION WAS TOWARD THE CATHODE AND THE GELS WERE EQUILIBRATED WITH 0.01 M ACETIC ACID FOR 3 HOURS AT 32 VOLTS. AFTER THE EQUILIBRATION BUFFER WAS REPLACED WITH FRESH BUFFER 20 µG OF PROTEIN DISSOLVED IN 10 µL OF 1 M SUCROSE IN 0.002 M ACETIC ACID WAS APPLIED TO THE TOP OF THE GEL AND THE APPLICATION ZONE SHARPENED IN A 32 VOLT FIELD FOR 25 MIN. ELECTROPHORESIS WAS THEN CARRIED OUT AT 320 V FOR 70 MIN AND THE TUBES REMOVED. THE TUBES WERE THEN CRACKED IN A VISE AND THE GELS PLACED IN 12.5% TCA FOR 30 MIN TO FIX THE PROTEIN FOR STAINING. THE STAIN USED WAS 0.05% (W/V) COOMASSIE BRILLIANT BLUE R250 (COLAB LABORATORIES, INC., CHICAGO HEIGHTS, ILLINOIS) IN 12.5% TCA (22). AFTER 1 HOUR IN THE STAINING SOLUTION THE GELS WERE DESTAINED OVERNITE IN 12.5% TCA.

G. Single Letter Notation for Amino Acids. (59)

A – Alanine

B – Aspartic acid or asparagine

C – Cysteine

D – Aspartic acid

E – Glutamic acid

F – Phenylalanine

G – Glycine

H – Histidine

I – Isoleucine

K – Lysine

L – Leucine

M – Methionine

N – Asparagine

P – Proline

Q – Glutamine

R – Arginine

S – Serine

T – Threonine

V – Valine

W – Tryptophan

X – Unknown or "other"

Y – Tyrosine

Z – Glutamic acid or glutamine