PROTEIN BOUND BROMINE

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IN

BLOOD SERUM

By

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By a tracer study, using ^{82}Br, it is demonstrated that bromine is bound to serum proteins in vivo. $^{82}Br^-$ of high specific activity was injected into rabbits and serum removed one day later. Approximately ½% of the total ^{82}Br in the serum was found to be protein-bound at this stage. The application of various separation methods (electrophoresis, bromide exchange, denaturation followed by desalting) showed that one-third of the protein-bound bromine is loosely attached whereas two-thirds are firmly bound. After partial and complete enzymatic hydrolysis the bromine was found in the amino acid fraction. On the basis of the elution pattern of the amino acids on calibrated cation exchange resin columns it is concluded that the main portion of the radioactivity appeared to be associated with 3-bromo-L-tyrosine. Little, if any, bromine was observed in the serum lipids and in the thyroxine fraction isolated from serum proteins.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS USED

C_1	first order proteolytic coefficient
E	enzyme concentration
EDTA	ethylenediamine tetraacetic acid
Na_2EDTA	disodium ethylenediamine tetraacetate
GuHCl	guanidine hydrochloride
K_1	first order rate constant
PBBr	protein bound bromine
RT_4	^{131}I-labelled L-thyroxine
TRIS	tris(hydroxymethyl)-aminomethane

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

In the mineral world as well as in the biosphere bromine ranks intermediate in abundance between chlorine and iodine. The relative amounts of chlorine, bromine and iodine are the same in plant and animal tissue and are 1000:1:0.01 respectively. Tissue from terrestrial animals contains approximately 1 to 9 ppm bromine[1] whereas aquatic organisms contain higher concentrations. Bromine is found in all species of the animal kingdom investigated. It occurs mainly as bromide with the exception of two organic bromine compounds in marine organisms. Dibromotyrosine was discovered in skeletons of corals and sponges[2,3,4,5,6]. Dibromoindigo was found in certain molluscs[7] and is the famous dye of ancient times, Tyrian Purple. No other organisms are known to contain organically bound bromine. Its precise physiological role is still unknown.

In general, the bromine content of the organs of mammals does not vary greatly, being 4-8 µg/g. However, some investigators found a strikingly high concentration in the pituitary gland (hypophysis),[8,11] whereas others could not confirm it[15,30,31]. But all agree on the bromine accumulation in the thyroid gland[1].

The analysis for bromine in animal tissue presented a serious problem for a long time. It was not until 1920 that DAMIENS convincingly demonstrated the presence of bromine in animal tissue[16,17]. The methods used in the early analyses were all subject to criticism. Bromine analyses in blood usually produced values which are now regarded as too high. The average concentration range which most investigators

agree upon today is 2-5 µg Br/ml blood serum. No doubt variation in the blood bromine concentration may depend on the dietary intake. For instance, table salt is the richest source of bromine for man.

The greatest stimulus for developing methods for bromine analysis in blood was created by ZONDEK and BIER around 1932. They pointed out that in certain cases of mental disorder (particularly of the manic depressive type) blood bromine content was 40-60% lower than the then-accepted range[9-14]. Unfortunately, they used the method of PINCUSSEN and ROMAN[18,19] which was later shown to be unreliable[20,21,27,28,29]. ZONDEK and BIER's claim was supported by SACRISTAN and PERAITA[22] and KLIMKE and HOLTHAUSEN[23] who used the same method of analysis, and by WIKOFF et al.[24], HANNELLEY and YATES[80], MEIER and SCHLIENTZ[81], and CHATAGNON and CHATAGNON[82] who used different methods. Other investigators employing different methods found the same range of blood bromine in healthy persons as well as in psychotics[15,30,87]. The question of the lowering of the blood bromine concentration in mental disorders remains unanswered because investigators appear to have lost interest in this problem since 1945.

Another issue of bromine metabolism was the question whether or not an organic bromine compound exists in blood or in any organ. Bromine was only found as bromide. It is, however, difficult to generalize since some of the bromine determined as bromide in ashes of blood or organs may well have been present originally in the living material in an organic compound. The majority of the micro-analytical techniques were not designed to differentiate between bromide and organically bound bromine.

It was again ZONDEK and BIER[9-14] who advanced the hypothesis that there was a bromine-containing organic compound in blood which functions, like thyroxine, as an important catalyst in the organism. The pituitary gland was thought to be the site of the formation of this substance. Furthermore, they claimed to have isolated from the pituitary gland a protein-free substance containing bromine, which, when injected into dogs, produced signs of fatigue and asthenia. Unfortunately, the isolation procedure and the experimental details were never published. They suggested that this "bromine hormone" was tetrabromothyronine, by analogy to the iodine situation in blood, since tetrabromothyronine had the same physiological effects on the dogs as the isolated substance. This theory was supported by GUILLAUMIN and MEREJKOWSKY[25], and by EWER[26] who claimed to have demonstrated the existence of organically bound bromine in blood.

Detailed studies by LEIPERT and WATZLAWEK[30,31], authorities in the field of bromine analysis at that time, using a more reliable micro-technique, showed no indication of any bromine-containing protein in blood.

DOERING in 1938[32] presented the first conclusive analytical evidence for organically-bound blood bromine. His method involved wet ashing of the blood with nitric acid/silver nitrate. This was followed by conversion of the resulting silver bromide into soluble bromide with zinc, oxidation of bromide to bromate by means of hypochlorite, and iodometric estimation of bromate. He carried out this method under two different ashing conditions. First, nitric acid ashing at 100°C in an open beaker and, second, nitric acid ashing

in a bomb tube at 250°C. The ashings at 100°C in open beakers produced bromine values which were always lower than those produced by ashings at 250°C in bomb tubes. He found the remaining bromine in the filtrate after the silver halide precipitation. When this filtrate was treated in a bomb tube the remaining bromine could be precipitated. Bromide was readily converted to silver bromide by both methods. He concluded that the bromine in the filtrate (ca. 0.5 µg/ml blood) was organically bound and 100°C open ashing was not sufficient to destroy this compound. To ensure that the organically bound bromine was not the result of the oxidation action of the nitric acid during the initial wet ashing, he carried out control analyses in blood which was hemolyzed and hydrolyzed prior to analysis. With the open ashing method he found the same low values. Thus, organically bound bromine was originally present in the blood. He suggested speculatively that an aromatic bromine-containing compound existed.

This important work was never quoted by later investigators. The prevailing opinion at the present time is reflected in a statement by GROSS made in a review article on bromide metabolism in 1962: "In effect, then, bromine metabolism in most living organisms is that of the bromide ion"[33].

It was not until 1967 that SKLAVENITIS and COMAR[34], using neutron activation analysis of precipitated serum proteins, re-discovered this phenomenon and found 0.07-0.15 µg bromine/ml to be bound to protein. FRITZE and ROBERTSON also observed considerable amounts of bromine in carefully desalted serum proteins in their qualitative neutron activation studies[35].

When radioactive bromine isotopes (^{80}Br and ^{82}Br) became available investigations of a more physiological character were carried out. In vivo animal experiments were conducted in which the partition of injected $^{82}Br^-$ in various organs of the body was studied. PERLMAN[36], VERKHOVSKAYA[37,38] and YAGI[39] found that $^{82}Br^-$ was concentrated in the thyroid gland. A more intensive study by YAGI also showed ^{82}Br in liver, stomach, kidney, heart, spleen and brain[40]. No protein bound bromine in serum was detected. Their results were confirmed by SÖREMARK[41].

LA ROCHE and BROWN, 1963,[42] studied the ability of the thyroid gland of rats to concentrate bromine using ^{82}Br. After injection of $^{82}Br^-$ into rats, proteins were isolated from the thyroid gland and separated by paper electrophoresis. The proteins did not contain any ^{82}Br. It was concluded that no incorporation of bromine into proteins takes place in the thyroid gland.

No in vivo-^{82}Br tracer experiment reported so far has shown the presence of bromine in serum proteins. But, in view of the new evidence favouring the binding of bromine to proteins[34,35] it was considered of interest to investigate the nature of this phenomenon. The final objective was to isolate from the serum proteins one or more constituents containing bromine. The first approach to the problem was a strictly analytical one. The combination of gel chromatography and neutron activation analysis was used to identify "bromine-containing proteins" in the way metal proteins have been identified as reported by EVANS and FRITZE[43]. This approach failed. It was not possible to obtain a sufficiently low "bromine background" in the effluent

emerging from gel chromatographic columns. The cleanest effluent contained 0.1-0.3 ppm bromine which, of course, does not allow accurate analysis for 0.1 ppm protein bound bromine in the same solution. However, the modest results obtained for protein bound bromine, 0.12-0.20 µg bromine/ml serum, are consistent with those of SKLAVENITIS and COMAR.

This failure led to tracer studies in vivo with the radioactive isotope ^{82}Br. These were confined to the blood serum proteins only.

To aid the experimental work the following four working hypotheses regarding site and manner of bromine binding may be proposed:

1. bromine is bound as bromide only;

2. bromine has replaced iodine in thyroxine;

3. bromine is bound to unsaturated compounds, e.g., squalene, cholesterol; and

4. bromine occurs in some unusual amino acid, be it within the peptide chain of the proteins or only attached to it. Possibilities are brominated tyrosine or tryptophan.

The plan adopted was to inject $K^{82}Br$ of high specific activity into a rabbit and study the occurrence and distribution of ^{82}Br within the serum proteins. These were subjected to various biochemical separations (bromide exchange, gel filtration, electrophoresis) and degradation procedures (denaturation, partial and complete enzymatic hydrolysis). Experimental evidence is given to support or reject each hypothesis.

2. MATERIALS AND INSTRUMENTATION

2.1. Materials

Chemicals and Buffers. Potassium bromide purchased from FISHER Scientific Co. was used in the preparation of ^{82}Br. It has been found advantageous over ammonium bromide, since potassium bromide did not show obvious chemical decomposition during the ten days of neutron irradiation.

L-Tyrosine, Lot No. 52567, from MERCK & Co., Rahway, N.J., was used to prepare 3-bromo-L-tyrosine, 3,5-dibromo-L-tyrosine as well as the ^{82}Br-labelled tyrosines. Their syntheses are described in Appendices 7.9 and 7.10.

Glycyl-L-proline was used as substrate in the estimation of the prolidase activity. It was obtained from Lot No. 128B-1890, SIGMA, St. Louis, Mo.

Ninhydrin (Triketohydrindene Hydrate), Lot No. 765504, was obtained from FISHER Scientific Co.

Hydrindantin was prepared in the following manner[44,45]: to 5 g ninhydrin dissolved in 125 ml water at 90°C a solution of 5 g ascorbic acid in 25 ml water was added with stirring. Crystallization of hydrindantin started immediately as the solution cooled down to room temperature. The product was filtered off, washed with water and dried over P_4O_{10} protected from light.

"Analar" Urea, Lot No. 810256, was obtained from B.D.H. Picric acid was obtained from Eastern Chemical Co., Pequmock, N.J.

Cellosolve (monomethylether of ethylene glycol), Lot. No. 786015, was supplied by FISHER Scientific Company.

Guanidine hydrochloride was obtained from BAKER and was fully soluble in water.

Amido Black 10-B, Lot No. 25583, B.D.H., was used as the stain for proteins on the electrophoresis paper strips. The staining solution was prepared in the following manner: 2 g Amido Black were dissolved in a solvent which was composed of 454 ml methanol, 454 ml water and 92 ml glacial acetic acid.

TRIS-barbital-sodium-barbital buffer pH 8.8, supplied by GELMAN Instruments, Ann Arbor, Mich., was used as electrophoresis chamber buffer.

The 0.1 M sodium citrate buffer pH 5 was prepared from a 0.2 M stock solution (500 ml) and 500 ml water to which 100 mg Na_2EDTA were added. The stock solution was prepared from 21 g citric acid monohydrate and 200 ml of N sodium hydroxide diluted to 500 ml and stored cold[46].

The 0.1 M sodium phosphate buffer pH 6.8 was prepared from 500 ml 0.1 M Na_2HPO_4, 450 ml 0.1 M $NaH_2PO_4 \cdot H_2O$ and 100 mg Na_2EDTA.

The 4 N sodium acetate buffer pH 5.5 was prepared in the following manner[47]: 340 g sodium acetate$\cdot 3\ H_2O$ were dissolved in 250 ml water under heating and stirring. After cooling to room temperature, 63 ml glacial acetic acid was added and the solution made up to 625 ml. The buffer was stored at 4°C.

FOLIN-CIOCALTEU reagent was prepared according to the method by LOWRY et al.[48]:

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

B: ½% $CuSO_4 \cdot 5H_2O$ in 1% sodium-tartrate

C: 50 ml of solution A plus 1 ml of solution B. Discarded after one day.

D: "Phenol Reagent", obtained from Harleco, Philadelphia, Pa., was titrated with 1 N sodium hydroxide solution (phenolphthalein as indicator) and diluted to 1 N in acid.

Radiochemicals. The ^{131}I-labelled L-thyroxine was obtained from Radiochemical Centre Amersham, England. The material was dissolved in a 50% aqueous solution of propylene glycol and contained 20 µg of L-thyroxine in 5 ml. At the time of use the compound contained 0.5 µCi of ^{131}I per mg.

The preparation and identification of ^{82}Br labelled [^{82}Br]3-bromo-L-tyrosine and [^{82}Br]3,5-dibromo-L-tyrosine are described in Appendices 7.12 and 7.13.

Gel chromatographic material was supplied by BIO-RAD Laboratories, Richmond, Cal. The following types were used:

Resin	Lot. No.	Hydrated bed vol.
Bio-Gel P-2 (100-200 mesh)	5409	3.8 ml/dry gram
Bio-Gel P-6 (100-200 mesh)	43133	7.4 ml/dry gram
Bio-Gel P-200 (100-200 mesh)	47493	34.0 ml/dry gram

It had been found that the performance of the P-200 type varies with the production batch.

Ion exchange chromatographic resins (sulfonated or quarternary ammoniated polystyrene) was supplied by BIO-RAD Laboratories. The following types were used:

1. Strongly basic anion exchange resin:
   AG 2-X8, 100-200 mesh, Cl^- form, Lot No. 5618-48, B-1623, capacity 3.2 meq/dry gram.
2. Acidic cation exchange resins:
   AG 50W-X8, 200-400 mesh, H^+ form, Lot No. 7253, capacity 5.1 meq/dry gram; and
   AG 50W-X12, 200-400 mesh, H^+ form, Lot No. 4101, B-324, capacity 5.0 meq/dry gram.

Before use in amino acid separation columns the AG 50W-X8 resin was converted into the sodium form. According to MOORE and STEIN[49], this is easily accomplished by stirring the resin with 2 N NaOH for several hours followed by washing with water until neutral reaction of the wash water.

Enzymes. Papain (E.C.3.4.4.10), Lot No. 97B-1870, was purchased from SIGMA, St. Louis, Mo. The material was obtained as twice recrystallized and suspended in 0.05 M sodium acetate, adjusted to pH 4.5. Specific activity: 1 mg liberates approximately 3.7 µmoles NH_3 per min. from benzoyl-L-arginine amide at pH 6 at 37°C.

Pronase, Lot No. 801929, was supplied by CALBIOCHEM, Los Angeles, Cal.

Subtilisin (E.C.3.4.4.16), Lot No. 59B-0020, was obtained from SIGMA, St. Louis, Mo. Specific activity: 1 mg hydrolyzes casein to produce colour approximately equivalent to 12 µM of tyrosine per min at pH 7.5

at 37°C. Colour per FOLIN-CIOCALTEU reagent.

Leucine aminopeptidase (E.C.3.4.1.1), Lot No. 109B-8140, was obtained from SIGMA, St. Louis, Mo. The enzyme was chromatographically purified and delivered as suspension in 0.75 saturated ammonium sulfate, 0.1 M TRIS and 0.005 M magnesium chloride at pH 8. Specific activity: 1 mg hydrolyzes 86 µmoles L-leucine amide per min. at pH 8.5 at 25°C. Before use the enzyme was dialyzed against 0.005 M TRIS buffer and 0.005 M magnesium acetate at pH 8.5 for 4 hours.

Prolidase (E.C.3.4.3.7) was prepared from pork kidney according to the isolation procedure of DAVIS and SMITH[51], described in Appendix 7.8. The enzymatic activity was estimated with glycyl-L-proline[52] supplied by SIGMA, as substrate. It is expressed as the first order proteolytic coefficient, $C_1 = K_1/E$, where K_1 is the first order rate constant calculated in minutes and decimal logarithms. E is the enzyme concentration in mg of protein N per ml of the test solution. Protein N was determined by the WARBURG and CHRISTIAN method (see Appendix 7.18) assuming that the proteins contain 16% protein N.

The enzyme was prepared twice. Proteolytic coefficients of $C_1 = 23$ and $C_1 = 15$ were obtained.

2.2. Instrumentation

The electrophoresis equipment was the GELMAN Rapid electrophoresis chamber No. 51101 in conjunction with GELMAN DC Power supply No. 38201.

Effluents from separation columns were collected by means of a L.K.B. Ultro-Rac fraction collector. The ultraviolet absorption of

the effluent at 254 nm was continuously monitored with a L.K.B. Uvicord ultraviolet spectrometer coupled to a BAUSCH & LOMB recorder.

Individual fractions were also checked for ultraviolet absorption at 280 nm using a BAUSCH & LOMB Spectronic 600 with 1 cm cells. When ninhydrin colour at 570 nm was measured a BAUSCH & LOMB Spectronic 20 with a 1 cm cell was used.

Protein estimation by UV absorption at 280 nm and 260 nm was carried out using a Unicam S.P. 800 ultraviolet spectrometer.

The radioactivity was measured in four ways:

1. A 3×3 inch NaI(Tl) scintillation detector in conjunction with a Victoreen linear amplifier (DD2, Model 851 A) and Nuclear Data 256 channel analyzer was used. The counts reported are based on the intensity of the 540 keV gamma energy peak of ^{82}Br. The counts in the channels, in which this peak occurs, are summed for evaluation.
2. Also a 3×3 inch NaI(Tl) well type scintillation detector in conjunction with a Victoreen linear amplifier (DD2, Model 851 A) and Nuclear Chicago scaler-timer was used. The single channel analyzer accepts pulses which correspond to gamma energies between 500 and 800 keV. In this arrangement mainly test tubes with radioactive effluents were counted.
3. A beta proportional counter with end window (Nuclear Chicago) in conjunction with a Nuclear Chicago scaler-timer was used.
4. In one case, a coaxial Ge(Li) solid state detector with an Ortec 118 A preamplifier, Hewlett-Packard (5583 A) linear amplifier and Nuclear Data (series 2200) 1024 channel analyzer was used.

The counting results obtained in either one of the four units were

corrected for background radioactivity. Whenever the measurements of a number of fractions extended over a period more than one hour an appropriate half-life correction was applied. Although longer counting times were used in most cases, the radioactivity data are reported as cpm throughout the experimental section.

Nuclear reactor. The potassium bromide irradiations were carried out in the McMaster swimming pool reactor (flux 2×10^{13} neutrons sec^{-1} cm^{-2}, with a cadmium ratio of 20).

2.3. Animals

All animal experiments were performed on three male New Zealand white rabbits (2-3 kg body weight).

3. EXPERIMENTAL PROCEDURES AND RESULTS

3.1. Preparation of the ^{82}Br-labelled serum proteins *in vivo*

In a typical experiment, a crystal of potassium bromide (7.5 mg) was neutron-irradiated for 10 days building up a specific activity of about 5 Ci ^{82}Br per gram potassium bromide. After about 4 hours the potassium bromide crystal was dissolved in 1-2 ml of saline solution and injected into the rabbit via the dorsal ear vein. During preliminary experiments, which are described in Appendix 7.1, it was found to be advantageous to draw the blood 1 day after injection. By this time, the uptake of ^{82}Br has not yet reached its maximum[34] but the amount incorporated can be conveniently measured. The 36 hours half-life of ^{82}Br presents an obvious limitation for the experiments. To standardize the procedure, all blood samples were taken from the ear artery 1 day after injection. The blood was kept in quartz centrifuge tubes at 37°C for 3 hours and the serum then separated by centrifugation. The separation of the serum proteins from ionic and low molecular weight components including radioactive bromide was carried out by gel chromatography on Bio-Gel P-2, 1.8 × 19.5 cm, columns (see Appendix 7.2). The columns were pre-equilibrated with a 0.15 M ammonium acetate buffer (pH 6.9) which also served as elution buffer. Sodium acetate buffer (0.15 M, pH 7) was used in cases when enzymatic reactions followed. The effluent was continuously monitored at 254 nm, collected in test tubes in fractions of 2 ml and their respective radioactivity measured.

In a typical desalting run, serum (1-4 ml) was applied to the

column and the effluent from the 12th to 18th ml was combined. This fraction contains the *in vivo*-^{82}Br-labelled serum proteins and will be referred to as "^{82}Br-labelled serum protein solution" throughout the text (see Fig. 1). It served as starting material for the experiments that followed. This protein fraction contained 0.2 - 0.5% of the total ^{82}Br present or, expressed as specific activity, approximately 40 cpm per mg protein; counted on top of a 3×3 inch NaI(Tl) scintillation detector coupled to a single channel analyzer.

To ensure that bromine binding by the serum proteins *in vivo* is not an artifact, a pick-up experiment *in vitro* described in Appendix 7.3 was carried out. Only 0.01% of the total ^{82}Br present was recovered in the protein fraction.

3.2. The distribution of ^{82}Br in serum proteins

This experiment was undertaken in order to see if any protein takes up bromine preferentially.

A solution of ^{82}Br-labelled serum proteins (0.8 ml) was applied to a Bio-Gel P-200 column, 1.7 × 37.5 cm in size. The column was pre-equilibrated with 0.15 M sodium acetate buffer which also served as elution buffer. The column was operated at a flowrate of 2-3 ml/hr. The effluent was monitored at 254 nm and collected in 2 ml fractions, which were checked for ^{82}Br activity. The ^{82}Br activity pattern is given in the histogram in Fig. 2. The total activity put on the column was 1.6×10^6 cpm, of which ¼% were found in the fractions of the protein region. The first ^{82}Br containing fraction (16-18 ml) contained 100 cpm ^{82}Br over a background of 300 cpm. The isotope was unambiguously

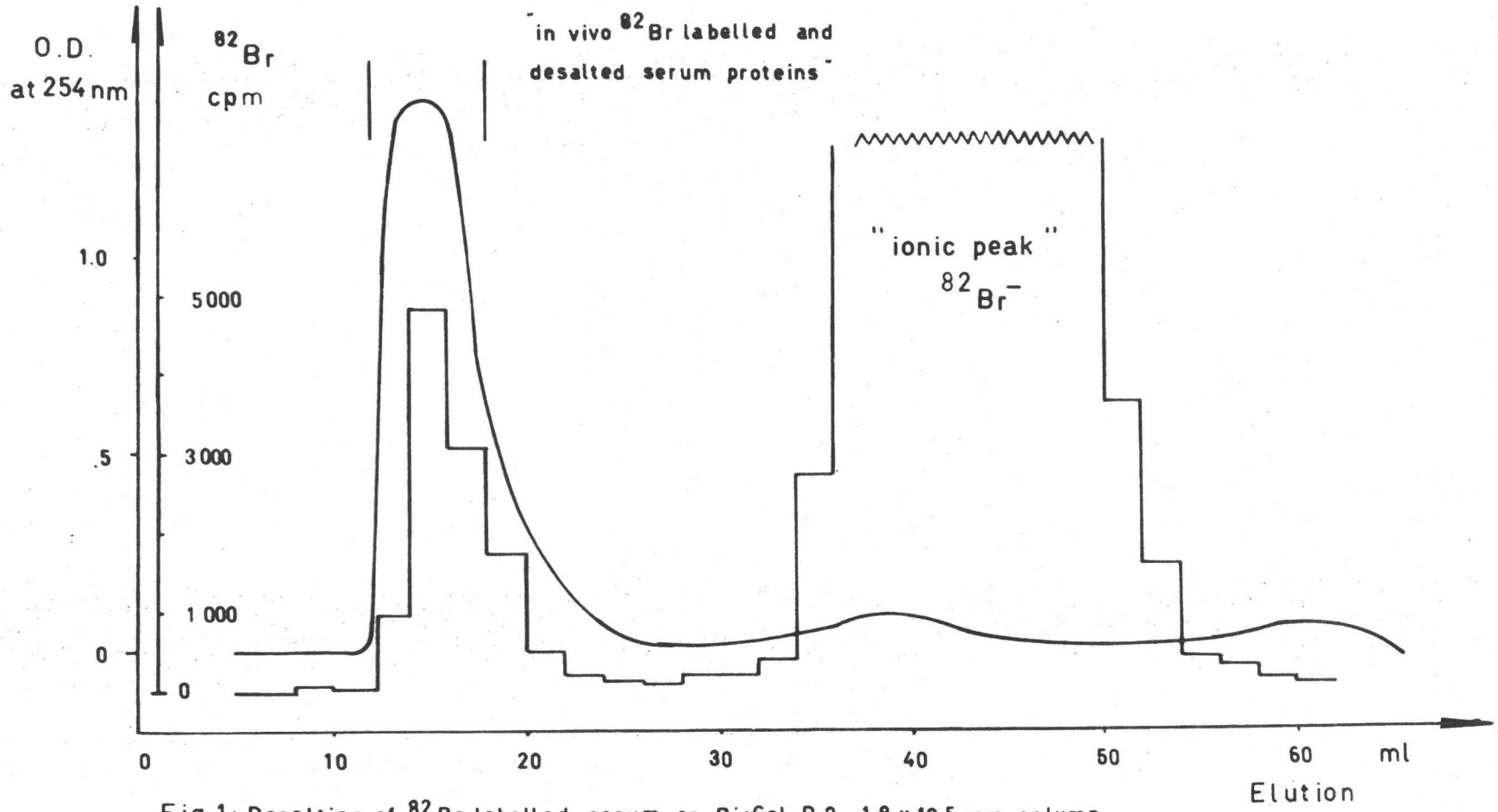


Fig.1: Desalting of ^{82}Br labelled serum on BioGel P-2, 1.8 x 19.5 cm, column

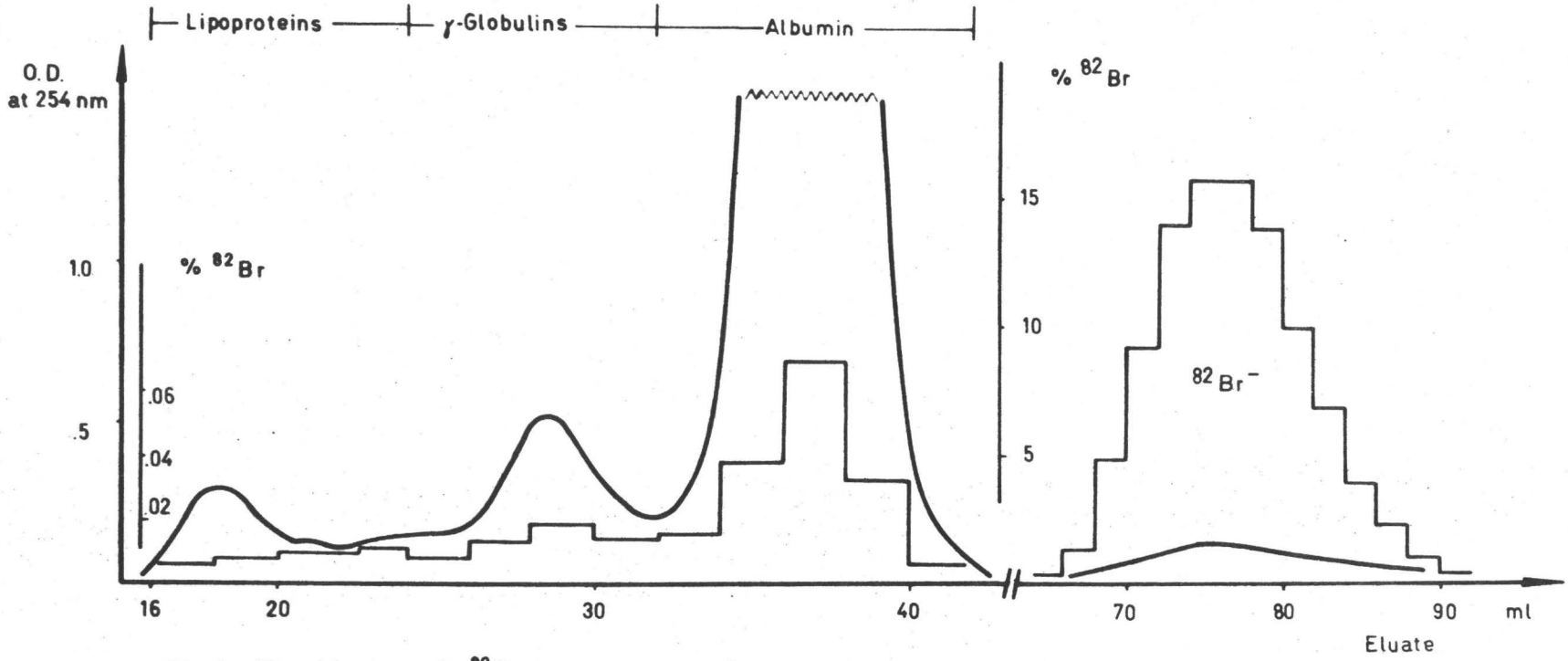


Fig 2 Distribution of ^{82}Br in serum proteins

identified in all fractions by gamma-ray spectroscopy. For further quantitative evaluation, the fractions comprising each of the three peaks were combined. The protein contents were estimated, using UV absorption at 280 nm and 260 nm[53] and the FOLIN-CIOCALTEU reaction[48], and the ^{82}Br concentration determined by counting. Protein estimation procedures are described in Appendices 7.17 and 7.18. The results are shown in Table 1.

3.3. Exchange of protein-bound ^{82}Br with non-radioactive bromide

Any ^{82}Br which is present as bromide should exchange with added non-radioactive bromide. In a desalting run following the exchange, the exchanged radioactive bromide should show up in the "ionic peak".

To two samples of 4.5 ml, each of a ^{82}Br-labelled serum protein solution, containing 16 mg protein per ml, approximately 0.5 mg potassium bromide were added. One sample was desalted 2 hours later in the way described earlier. The radioactivity of the fractions was measured and the counts in the protein and ionic peak expressed as percentage of the total (protein + ionic). The "protein peak" was found to contain 67% of the total radioactivity and the "ionic peak" 33%. The second sample was allowed to react for 24 hours and then desalted in the way described in Chapter 3.1. The same result was obtained.

To ensure that the radioactivity in the ionic fraction was not due to degradation of the proteins during handling, the following control experiment was carried out.

A sample of ^{82}Br-labelled serum protein solution was kept for 24 hours at room temperature. A second desalting was performed with the

Table 1. Distribution of ^{82}Br in serum proteins after separation on Bio-Gel P-200 (Fig. 2)

	Elution volume (ml)	Proteins[83]	MW	Protein estimate in peak (mg)		
				UV-method	FOLIN	Mean
First peak	18-21	Lipoproteins	>200 000	3.6	2.7	3.1 ± 0.5
Second peak	26-32	γ-Globulins	∿140 000	13.5	13.7	13.6 ± 1.3
Third peak	33-42	Albumin	∿ 70 000	29.5	28.8	29.0 ± 3

	^{82}Br total activity (cpm)	"specific activity" cpm/mg protein
First peak	383 ± 32	121 ± 24
Second peak	826 ± 40	61 ± 7
Third peak	2551 ± 49	89 ± 9

result that only 8% of the total ^{82}Br showed up in the ionic peak; the rest remained bound to the proteins.

3.4. Electrophoresis

Electrophoresis is a more powerful tool than exchange to liberate ionically bound ^{82}Br from the proteins.

A solution of ^{82}Br-labelled serum proteins (7 ml), containing approximately 35 mg protein per ml, was concentrated by addition of 2.2 g of dry Bio-Gel P-2 beads. From the concentrated solution, aliquots of 20 µl and 30 µl were taken and put on Whatman 3 MM electrophoresis paper strips (4 × 19 cm). Electrophoresis was carried out in barbital buffer pH 8.8 at 180 V and approximately 2 mA/strip for 1 hour. In a calibration experiment, described in Appendix 7.4, it was determined that bromide migrates 7-9 cm from the origin whereas the proteins only progress 3 cm (Fig. 8). The paper strip was dried in air and cut into 1 cm pieces perpendicular to the direction of migration. The paper pieces were counted using an end window beta proportional counter. In four runs similar results were obtained. A typical radioactivity pattern in shown in Fig. 3.

3.5. Protein bound ^{82}Br under denaturing conditions

The ratio of "weakly" bound to "strongly" bound ^{82}Br was investigated by destroying the native structure of the proteins. Desalting under denaturing conditions served as the separation method between bromide and protein-bound bromine. Again, the effluent was monitored at 254 nm to indicate the proteins and collected in 2 ml fractions. They were taken

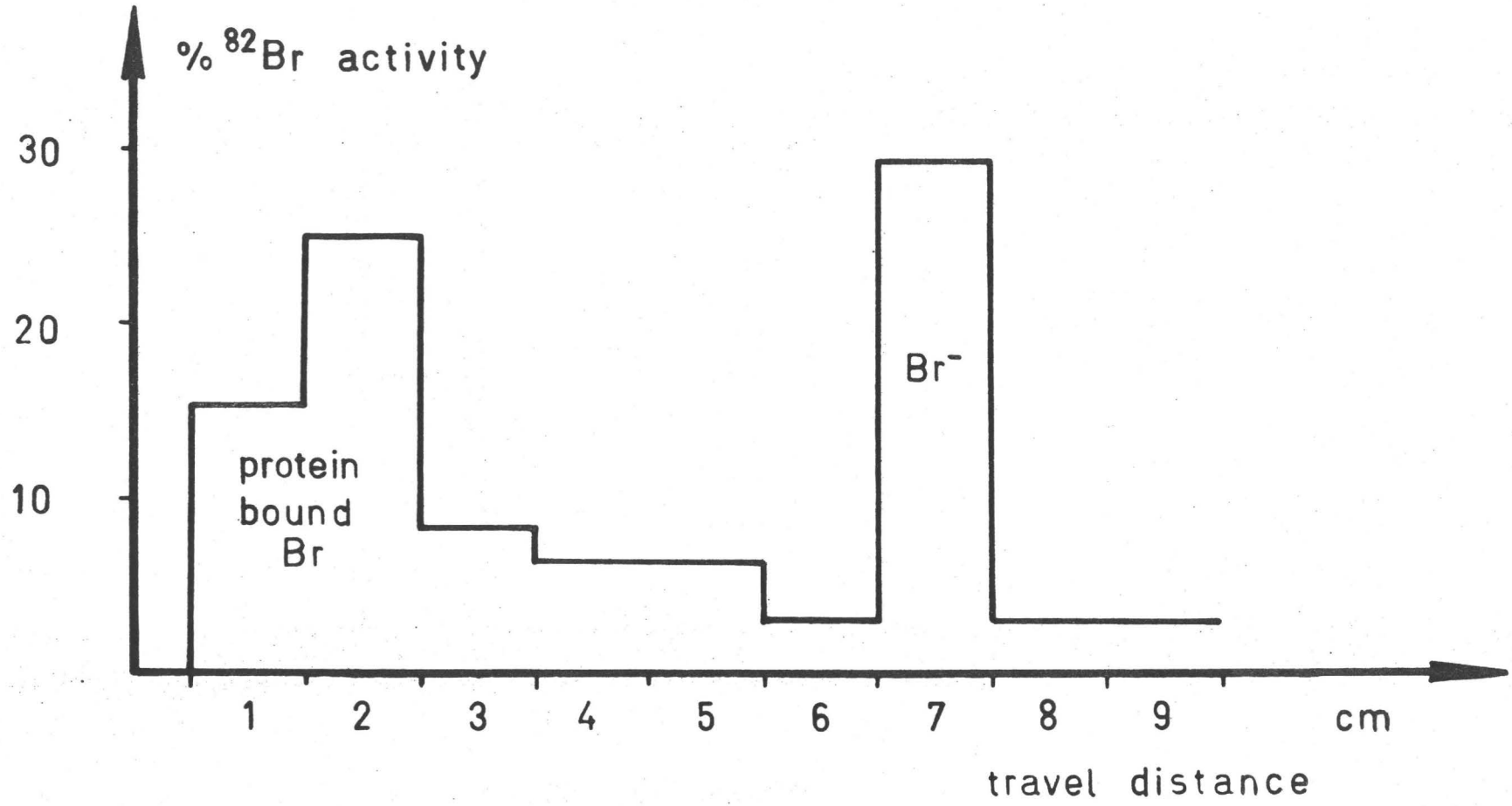


Fig.3: Electrophoresis of ^{82}Br labelled serum proteins

for radioactivity measurement (3×3 inch NaI(Tl) scintillation detector coupled to single channel analyzer). The counts in the ionic fraction are compared with the total ^{82}Br activity emerging from the column (protein plus ionic peak) and expressed as percent of that total.

A solution of ^{82}Br-labelled serum proteins (2 ml) containing 28 mg protein per ml was adjusted to an urea concentration of 7 M and a few mg cysteine were added. The mixture was allowed to stand for 2 hours at room temperature. For desalting, the mixture was passed over a Bio-Gel P-2, 1.8 × 19.5 cm, column previously equilibrated with 0.15 M ammonium acetate -7 M urea solution (pH 7). About 14% of the radioactivity occurred in the ionic peak and 86% remained protein bound.

A ^{82}Br-labelled serum protein solution (3 ml) was brought to 7 M in urea by the addition of 1.2 g of crystallized urea plus 5 mg cysteine. The mixture stood for 16 hours before it was desalted, yielding 15% of the activity in the ionic peak and 85% in the proteins.

In further investigation, denaturation and exchange with non-radioactive bromide was combined.

A 2 ml sample of desalted and denatured ^{82}Br-labelled serum protein solution (21 mg protein/ml) received 10 mg of potassium bromide. After 4 hours the sample was desalted again. As a result, 38% of the protein-bound ^{82}Br was liberated as bromide; 62% remained firmly bound to the proteins.

Guanidine hydrochloride (GuHCl) is known as a more powerful denaturing agent than urea[79]. Its application was analogous to the urea denaturation.

To a 3 ml ^{82}Br-labelled serum protein solution (24 mg protein/ml)

1.8 g GuHCl were added to bring the GuHCl-concentration up to 6 M along with a few mg of cysteine. An increase in viscosity and volume was observed. After 4 hours at room temperature, the mixture was desalted on Bio-Gel P-2 as described in Chapter 3.1. with 0.15 M ammonium acetate/6 M GuHCl as buffer. The ionic peak contained 22% and the protein peak 78% of the radioactivity.

After treatment with urea or GuHCl the denatured proteins remained soluble. In the following experiment, the denaturation in an acidic medium leads to precipitation of the proteins. The proteins are precipitated with 10% sulfosalicylic acid according to COMAR's procedure[34].

A solution of ^{82}Br-labelled serum proteins (1 ml) containing 27 mg protein/ml was introduced dropwise into 3 ml of 10% sulfosalicylic acid. A white precipitate was formed immediately and filtered off. The filtrate was checked for radioactivity. It contained 36% of the total. The precipitate was washed twice with 4 ml water. No activity was found in the wash water. The precipitate contained 64% of the total radioactivity. Table 2 summarizes the results obtained in the preceeding desaltings under denaturing conditions.

3.6. Papain hydrolysis followed by gel-chromatography in search of ^{82}Br incorporation into thyroxine

The method by LISSITZKY and BISMUTH[54,55] was used to liberate thyroxine from serum proteins. The method consists of papain hydrolysis and gel chromatography in aqueous and organic solutions. Thyroxine is eluted from the gel column with an organic solvent. The method was tested using ^{131}I-labelled thyroxine (RT_4) and the results are described

Table 2. Denaturation

Denaturing reagent	Desalting after denaturation. Percentage of ^{82}Br released as bromide	Percentage of ^{82}Br remaining in the proteins
7 M urea 2 hours	14	86
16 hours	15	85
7 M urea + KBr	38	62
6 M GuHCl	22	78
sulfosalicylic acid	36	64*

*The precipitated proteins were centrifuged off and counted in comparable geometry.

in Appendix 7.5.

Eight ml of a ^{82}Br-labelled serum protein solution (35 mg/ml) were adjusted to pH 4.5 and 0.25 ml of a suspension of papain, 0.25 ml 0.1 M solution of cysteine and 0.25 ml 0.1 M solution of EDTA were added. At this time 10,000 ± 100 cpm were observed for ^{82}Br activity. The counting was done by means of a 3×3 inch NaI(Tl) scintillation detector in conjunction with the 256 channel analyzer. The mixture was incubated at 40°C for 20 hours. The hydrolysis was terminated by adjusting the pH to about 7. During the digestion the mixture became cloudy but cleared on neutralization. Six ml of the digest, equivalent to 180 mg original protein were chromatographed on a Bio-Gel P-6 column, 1.8 × 25 cm, with 0.15 M sodium acetate pH 7 as buffer and tert.-amyl alcohol saturated with ammonia. About 4100 ± 100 cpm ^{82}Br activity were placed on the column. The change in elution solvent is indicated in Fig. 4. The UV-absorption of the effluent at 254 nm was recorded and 3 ml fractions were collected which were checked for radioactivity. Thyroxine should occur with the tert.-amyl alcohol front (see Appendix 7.5). In this experiment, no radioactivity could be detected in the amyl alcohol effluent. The radioactivity placed on the column was recovered in the effluent up to the 75th ml: after correction for half life and geometry changes 4000 ± 200 cpm were counted.

3.7. Attempt to detect ^{82}Br incorporation into the serum lipids

Delipidation was performed according to SCANU *et al.*[56] with ethanol/ether at -18°C and chloroform/methanol at room temperature[57].

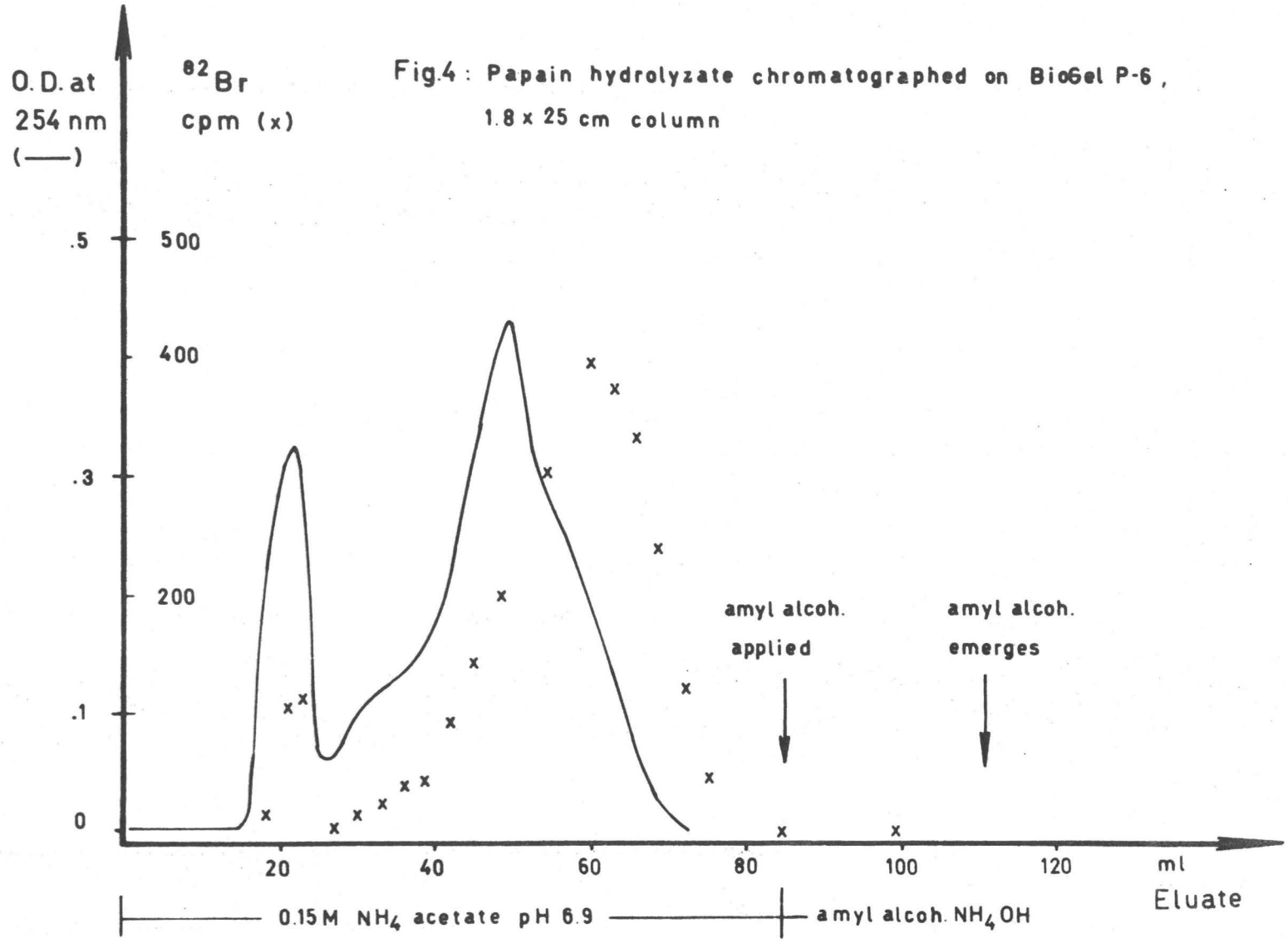


Fig.4 : Papain hydrolyzate chromatographed on BioGel P-6, 1.8 x 25 cm column

3.7.1. Extraction of desalted serum proteins with alcohol/ether

Two ml of ^{82}Br-labelled serum protein solution (35 mg/ml) previously stored at 0°C were extracted with 10 ml of a mixture of ethanol/ether (3:1 by volume). During the 2 hours processing the mixture was shaken frequently in the cold chamber at -18°C. The precipitate was collected on filter paper and washed with ether (-18°C) twice. Each filtrate was checked for ^{82}Br activity in comparable geometry. A 3×3 inch NaI(Tl) scintillation detector with 256 channel analyzer was used. All filtrates were combined and the solvent evaporated at 60°C. The remaining white crystallized residue amounted to 18.4 mg. The results are given in Table 3.

Table 3. Counting results

^{82}Br of sample before extraction	1272 ± 24 cpm
^{82}Br of filtrate	28 ± 12 cpm
^{82}Br in the first ether wash	20 ± 12 cpm
^{82}Br in the second ether wash	-
Sum	48 ± 20 cpm
	= 4%

3.7.2. Extraction of a serum protein hydrolyzate with chloroform/methanol

To 1 ml ^{82}Br-labelled serum protein solution (20 mg/ml) approximately 1 mg pronase was added and hydrolysis allowed to progress at 40°C for 24 hours. At the initial pH of 7 the digest was extracted twice with chloroform/methanol (2:1 volume). After separating, the organic extract was checked for radioactivity.

^{82}Br activity of the hydrolyzate	745 ± 20 cpm
Aqueous layer	620 ± 20 cpm
Chloroform/methanol layer	36 ± 5 cpm

Background 97 ± 9 cpm

Only 5% of the total activity could be extracted.

3.8. Protein-bound ^{82}Br under the condition of enzymatic hydrolysis

Since the methods under 3.3., 3.4. and 3.5. on the average remove only one-third of the bromine from the serum proteins, the question of whether the remaining two-thirds are organically bound or simply trapped as ions in a blocked conformational site should be answered. The approach used is to break down the peptide chains to various extents by enzymatic hydrolysis and follow the fate of ^{82}Br by ion exchange separation procedures. Enzymatic hydrolysis was chosen because of its advantage over acid and alkaline hydrolysis. Acid hydrolysis would not only turn the reaction mixture black on account of the carbohydrates in the glycoproteins but also destroy labile amino acids. Alkaline hydrolysis could possibly liberate organically bound bromine by a substitution reaction. Ion exchange columns are employed to separate the hydrolysis products from the released radioactive bromide.

3.8.1. Pronase hydrolysis followed by cation exchange chromatography

To three 2.5 ml samples of a ^{82}Br-labelled serum protein solution (40 mg protein/ml) 3 mg pronase were added. The almost clear solution was left at 40°C for 23 hours. The enzyme was inactivated by adjusting the pH to 2.2 with 3 M acetic acid. The mixture was counted for radio-

activity and brought on top of an AG 50W-X12, 0.9 × 23 cm, column and washed in with a few drops of 3 M acetic acid. Distilled water was used to elute $^{82}Br^-$ from the column quantitatively. Proteins, peptides and amino acids are adsorbed on the resin under these conditions. The radiobromide emerges with the void volume. An experiment testing this method of separation is outlined in Appendix 7.6. The effluent was collected (10 ml) and its ^{82}Br content measured (Table 4). The effluent containing ^{82}Br was checked for ninhydrin active compounds and found to be free of them. One of the columns (III) was washed with 0.3 M hydrochloric acid, containing a few mg of sodium sulfite as a reducing agent to ensure the conversion of all "inorganic bromine" to bromide.

Table 4. Pronase hydrolyzate on AG 50W-X12, 0.9 × 23 cm, columns

	Column		
	I	II	III
^{82}Br applied on the column, cpm	1690 ± 20	1484 ± 20	1368 ± 20
^{82}Br eluted from the column as Br^-, cpm	710 ± 15	540 ± 15	540 ± 15
$^{82}Br^-$ as per cent of the amount applied	41	36	40

The eluate was found to contain 41, 36 and 40% of the total ^{82}Br; the remaining approximately 60% were found to be adsorbed in the top third of the resin bed. A control experiment showed that pronase does not pick up radioactive bromide (see Appendix 7.7). The time dependence of the ^{82}Br release during the pronase attack had been roughly determined using the same procedure (Table 5). In addition, three aliquots of a

Table 5. $^{82}Br^-$ released as function of time during pronase attack

Time in hours	^{82}Br applied to the column	^{82}Br released as bromide	
			%
12	952 cpm	330 cpm	35
20	2030 cpm	620 cpm	31
24	2970 cpm	2690 cpm	36

^{82}Br-labelled serum protein solution were incubated with pronase at 40°C for 12, 20 and 24 hours. Radiobromide was separated from "strongly bound" bromine as described above. About 35, 31 and 36% respectively of the ^{82}Br activity was liberated from the proteins and appeared in the ionic fraction.

3.8.2. Pronase-, subtilisin- and papain-hydrolysis followed by anion exchange chromatography

In order to isolate any ^{82}Br containing organic material from hydrolyzates a separation method by STEIN and MOORE[58] was adopted. The method consists of deproteinization of the hydrolyzate with picric acid and separating bromide, picrate and the rest of protein from amino acids and small peptides on an AG 2-X8 column. The anions are bound to the resin whereas the amino acids appear in the effluent.

The experimental details are given in Table 6. All three hydrolyses had the following procedure in common: the solution of ^{82}Br-labelled serum proteins was adjusted to the working pH and the amount of enzyme added. The enzyme attack was terminated by adjusting the pH to about 2. Ten ml of a 1% aqueous picric acid solution were added. A precipitate

formed almost immediately. At this point, the radioactivity was measured and taken as 100%. A 3x3 inch NaI(Tl) scintillation detector in conjunction with a single channel analyzer was used for counting. The precipitate was centrifuged off and the supernatant poured over a AG 2-X8, 1.8 x 2.5 cm resin bed. The amino acids are eluted with approximately 5 ml of 0.03 M hydrochloric acid. The effluent was checked for radioactivity.

Table 6. Hydrolysis with pronase, subtilisin and papain

	Pronase	Subtilisin	Papain
Amount serum proteins	100 mg	50 mg	100 mg
Dissolved in 0.15 N sodium acetate buffer	3 ml	3 ml	10 ml
Adjusted to pH	8	7.3	5.2
Enzyme added	5 mg	2 mg	10 mg
Hydrolysis time	65 hrs	65 hrs	24 hrs
^{82}Br activity, total	1800±42 cpm	920±30 cpm	575±10 cpm
^{82}Br activity in ppt.	5%	-	1%
^{82}Br in effluent	335±20 cpm	250±20 cpm	75±10 cpm
Per cent of total	18%	27%	13%
ninhydrin reaction	positive	positive	positive

3.8.3 Complete enzymatic hydrolysis followed by anion exchange chromatography

The method by HILL and SCHMIDT[59] of complete enzymatic hydrolysis of proteins was applied to the ^{82}Br-labelled serum proteins. It uses sequential application of papain, leucine aminopeptidase and prolidase

to achieve cleavage of every peptide bond. As HILL and SCHMIDT have shown, the method yields hydrolyzates which are identical to those obtained by acid hydrolysis.

Six ml of a ^{82}Br-labelled serum protein solution (18 mg/ml) were diluted with 20 ml water and the proteins heat denatured by incubating in a 95°C water bath for 5 min. After adjusting the pH to 5.2, 9.8 mg potassium cyanide and 0.3 ml of a suspension of 30 mg papain in 1 ml 0.05 M sodium acetate were added and the mixture incubated at 40°C for 23 hours. The enzyme attack was stopped by adjusting the pH to 2 with a few drops of 3 M hydrochloric acid. Hydrocyanic acid, hydrochloric acid and most of the water were removed from the mixture by rotary evaporation at 40°C and approximately 15 torr vacuum, resulting in a 2 ml turbid solution. The pH of the digest was adjusted to 8.5 with 2 N NaOH. TRIS buffer (0.5 M) at pH 8.5 and manganese chloride were added in amounts to give each a final concentration of about 0.005 M. Leucine aminopeptidase and 2-5 mg of the prolidase preparation with an enzymatic activity of C_1 = 23 were added and the mixture was incubated at 40°C for 28 hours. The enzymes were inactivated by adjusting the pH to 2. At this point, the radioactivity was measured. The proteins were removed by picric acid precipitation. To the mixture 15 ml picric acid (1% aqueous solution) were added. The immediately-formed precipitate was centrifuged off, washed once with water and checked for radioactivity. Only 4% of the total activity appeared in the precipitate. The supernatant was passed over an AG 2-X8, 1.8 × 2.5 cm, column and washed with a few ml of 0.03 M hydrochloric acid. The emerging colourless solution was checked for radioactivity. This hydrolysis was done in two batches.

About 33 and 31% of the total radioactivity emerged from the AG 2-X8 column. The column effluent was also tested for ninhydrin reaction in the way described in Appendix 7.16 using 1 ml aliquots from each batch. The details are given in Table 7.

Table 7. Complete enzymatic hydrolysis with papain, leucine aminopeptidase and prolidase. Experimental details.

	Batch 1	Batch 2
Amount of serum protein	108 mg	108 mg
Total hydrolysis time	85 hrs	85 hrs
^{82}Br before deproteinization	2422±50 cpm	2364±50 cpm
^{82}Br in precipitate	102±10 cpm	95±10 cpm
^{82}Br in effluent	749±26 cpm	781±26 cpm
Per cent of the total	31%	33%
Ninhydrin reaction	positive	positive

3.8.4. Complete enzymatic hydrolysis followed by amino acid separation

The results reported in the Chapters 3.6., 3.7., 3.8.1. and 3.8.2. suggest the possibility of brominated amino acids in the serum proteins. Only a few amino acids can be thought to be brominated: phenylalanine, tyrosine and possibly tryptophan.

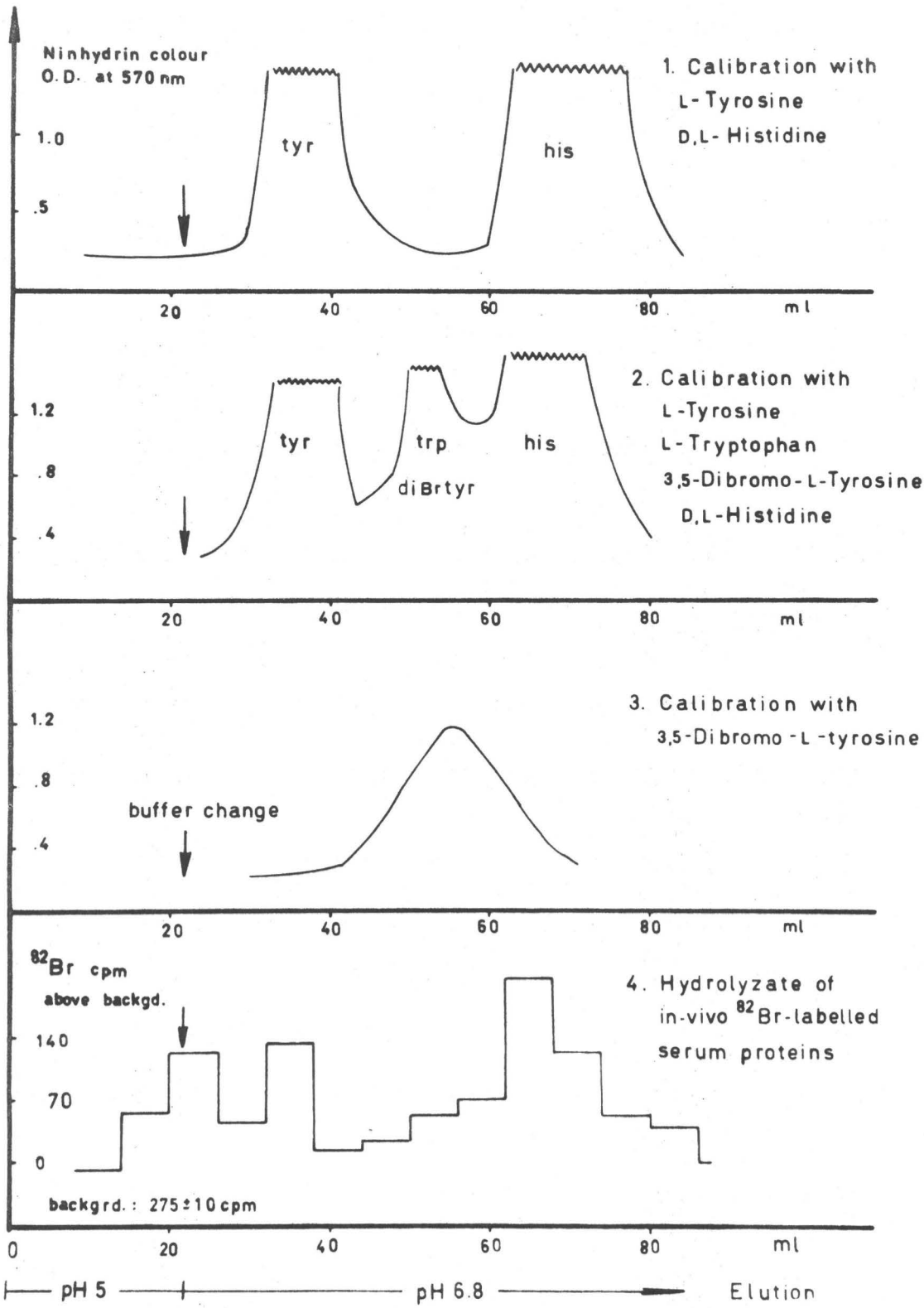
The main objective in the set of experiments reported in this chapter is to isolate a "radioactive amino acid". For separation a chromatographic method by MOORE and STEIN[49] was adopted. These authors have separated the basic amino acids by means of a cation exchange resin column, eluting with pH 5 and pH 6.8 buffers.

Since 3,5-dibromo-L-tyrosine had been found in nature previously[2-6], an experiment was carried out in search for ^{82}Br containing 3,5-dibromo-L-tyrosine in the ^{82}Br-labelled serum protein hydrolyzate. For this purpose an AG 50W-X8 (200 to 400 mesh, Na^+ form), 0.9 × 15 cm, column was calibrated with 3,5-dibromo-L-tyrosine. The preparation and identification of 3,5-dibromo-L-tyrosine are described in Appendix 7.10. The elution positions of L-tyrosine, 3,5-dibromo-L-tyrosine, histidine, and tryptophan were marked in separate runs (Figs. 5.1, 5.2 and 5.3).

The radioactive amino acid mixture obtained after deproteinization (Chapter 3.8.2.) was chromatographed on the same AG 50W-X8, 0.9 × 15 cm column with the buffers indicated in Fig. 5.4. The effluent after buffer change was collected in 6 ml fractions and taken for ^{82}Br measurement on top of a 3×3 inch NaI(Tl) scintillation detector coupled with a single channel analyzer. The histogram in Fig. 5.4 represents the ^{82}Br distribution in the effluent. In fact, there is a basic amino acid which is radioactive and emerges from the column in the vicinity of 3,5-dibromo-L-tyrosine, tryptophan and histidine. The result is not clear enough to associate the radioactivity with any of these amino acids. The slight shift of the radioactivity peak could mean both an accidental inconsistency due to varying experiment conditions or a different "radioactive amino acid".

To make this distinction, a chromatographic column capable of separation of 3,5-dibromo-L-tyrosine from tryptophan and histidine is required. As it is outlined in Appendix 7.11, an AG 50W-X8 column, 1.4 × 24 cm, was found to be satisfactory in resolution. In calibration chromatograms under conditions which simulate a real protein hydrolyzate

Fig. 5 : Amino Acids on an AG50W-X8, 0.9×15 cm, column



the elution position of [^{82}Br]3,5-dibromo-L-tyrosine and [^{82}Br]3-bromo-L-tyrosine as well as phenylalanine, tyrosine and tryptophan was established.

A complete enzymatic hydrolysis of ^{82}Br-labelled serum proteins was carried out according to the procedure described in Chapter 3.8.3. A prolidase preparation with C_l = 15 was used. Two batches with approximately 230 mg ≅ 2 μmoles serum protein each were hydrolyzed. The hydrolyzate was acidified to pH 2 and the precipitate formed was centrifuged off. It contained only 5 and 9% respectively of the protein bound radioactivity. The two batches were combined in order to increase the radioactivity for the amino acid separation which amounted to 5850±100 cpm. The approximately 50 ml almost clear hydrolyzate were evaporated down to 10 ml by rotary evaporation and 30 mg 3,5-dibromo-L-tyrosine were added to mark clearly the 3,5-dibromo-L-tyrosine elution position. The mixture was adjusted to pH 2 and brought on top of the AG 50W-X8, 1.4 × 24 cm column. The acidic and neutral amino acids were eluted with 142 ml of 0.1 M sodium citrate pH 5 buffer. The very first 40 ml contained a radioactivity of 2000±50 cpm. No radioactivity was detected in the 102 ml that followed. Then, the 0.1 M sodium phosphate pH 6.8 buffer was employed to elute the tyrosine related amino acids and tryptophan. The effluent after the 142nd ml was collected in 2 ml fractions which were checked for radioactivity and UV-absorption at 280 nm. The radioactivity measurement was made with a 3×3 inch well type NaI(Tl) scintillation detector coupled to a single channel analyzer. The result is shown in Fig. 6 and also in Table 8.

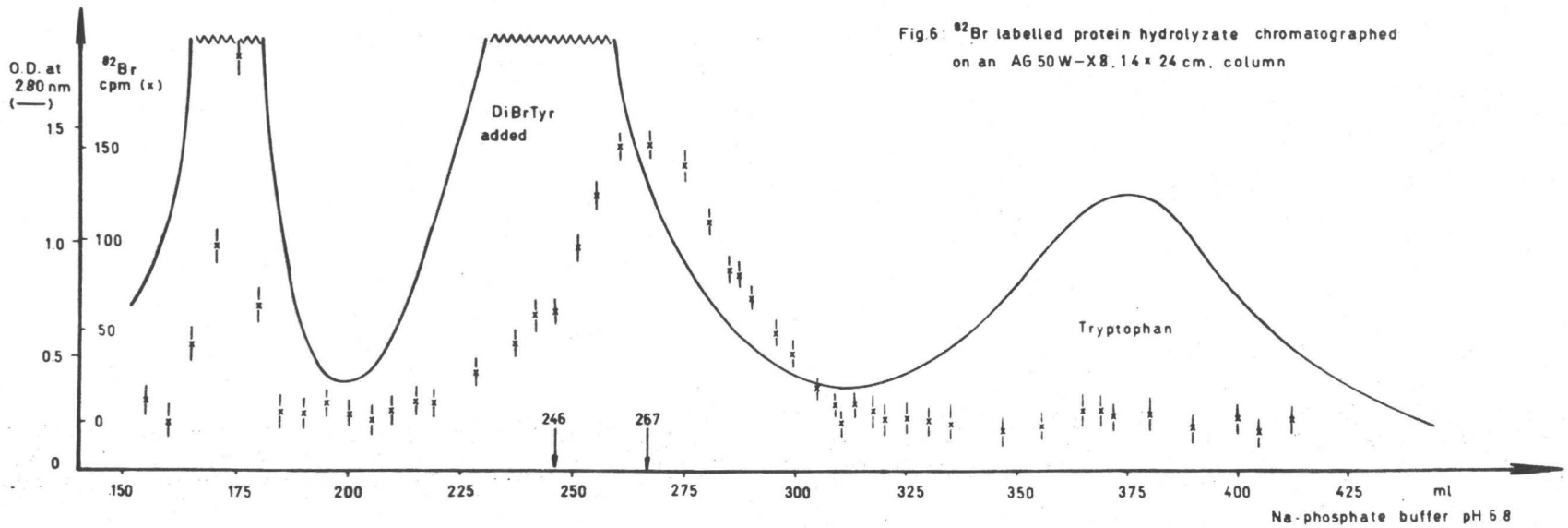


Fig.6: ^{82}Br labelled protein hydrolyzate chromatographed on an AG 50 W–X8, 1.4 × 24 cm. column

Table 8. Amino acid elution positions in ml

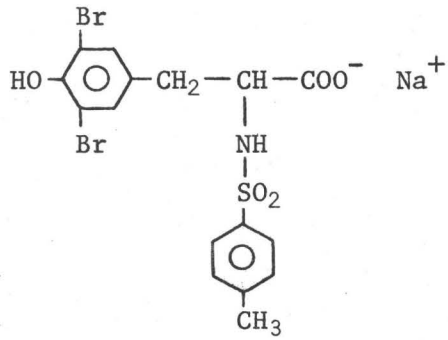
Amino Acid	Elution positions obtained in Calibration I	Calibration II	Hydrolyzate analysis
3,5-dibromo-L-tyrosine	233	235	246
3-bromo-L-tyrosine	-	265	267 (^{82}Br)
d,l-tryptophan	360	365	375
unidentified	-	-	175

As in the experiment described on page 34, the dibromotyrosine peak does not line up with any radioactivity peak. In fact, these are well separated. The radioactivity emerges from the column in the elution position of 3-bromo-L-tyrosine.

An account of the radioactivity shows that the amount applied to the column could be recovered after the peak at the 267th millilitre.

^{82}Br applied to the column	:	5850 ± 100 cpm
^{82}Br eluted with the first 40 ml	:	2000 ± 50 cpm
^{82}Br eluted with the two peaks at 175th and 267th millilitre	:	3410 ± 60 cpm
Sum	:	5410 ± 110 cpm

To ensure that the radioactive compound in the peak at the 267th millilitre is really a tyrosine-like amino acid, a derivative should be prepared which is specific for tyrosines. The derivative chosen was the sparingly soluble sodium salt of p-toluenesulfonyl-3,5-dibromo-L-tyrosine.



Under the preparative conditions applied only tyrosine, phenylalanine, dibromotyrosine and monobromotyrosine form the sparingly soluble sodium salt. The sodium salts of all other amino acid p-toluenesulfonates do not precipitate[60].

The "radioactive fractions" collected during the amino acid separation from 242 to 292 ml were combined and evaporated down to 12 ml using a rotary evaporator at 40°C and 15 torr vacuum. 3,5-Dibromo-L-tyrosine (0.5 g) was added and the solution made 1 N in sodium hydroxide. A solution of 2 g p-toluenesulfonyl chloride in 22 ml ether was also added and the reaction mixture stirred mechanically. After 4 hours the white precipitate (sodium salt of N(p-toluenesulfonyl)-3,5-dibromo-L-tyrosine) was separated from the aqueous solution and the ether layer by centrifugation. The precipitate was washed twice with 1 N sodium hydroxide and counted for radioactivity which was found to be 974 ± 30 cpm. The aqueous layer only contained 28 ± 10 cpm. The counting was done in comparable geometry. Thus, most of the activity was found in the derivative. As shown in Appendix 7.16, $^{82}Br^-$ remains in the aqueous layer under these preparative conditions.

4. DISCUSSION

It is clearly shown that ^{82}Br is incorporated into serum proteins. The extent is small after 24 hours (approximately 0.5% of the total bromine in serum) but sufficient to study the system further. Previous workers did not observe any bromine incorporation because they injected considerably smaller doses. As far as figures were given, 1-60 µCi ^{82}Br per ml animal blood were injected (VERKHOVSHAYA[37,38]; SÖREMARK[41]; LA ROCHE[42]; YAGI[39,40]). In the experiments reported here 150 µCi ^{82}Br per ml animal blood were injected. The amount of specific radioactivity (5 Ci ^{82}Br per gram potassium bromide) represents the maximum attainable in a typical research reactor. The rabbit's blood bromide concentration was doubled by the injection. The rabbit suffered no obvious ill effects. In fact, one of the animals was used seven times over a period of 15 months.

It may be argued that the entire effect of ^{82}Br binding to the proteins is artificially created by the injection of radioactive bromide. During the production of the isotope some elementary ^{82}Br may have been formed[41] which rapidly binds to the proteins after injection. There is even a protein labelling method which uses bromine Br_2[61]. Furthermore, an experiment relating to the incorporation of ^{82}Br into organic compounds resulting from chemical activation of ^{82}Br by the isomeric transition of ^{82m}Br to ^{82}Br has been reported[62].

The *in vitro* experiments recorded in Appendix 7.2 indicate that indeed 0.01% of the radioactivity enters the proteins, whereas the incorporation *in vivo* is greater by more than an order of magnitude, being approximately 0.5%. It should be noted that the incorporation of 0.01%

of the total radioactivity cannot be entirely attributed to ^{82}Br. Since proteins are known to bind a variety of cations and anions, the *in vitro* binding of radioactive impurities from potassium bromide could also contribute to the 0.01% incorporation. The 0.01%, therefore, represents a maximum value for *in vitro* ^{82}Br incorporation. How the major amount of ^{82}Br is incorporated *in vivo*, whether by exchange between originally bound bromine and $^{82}Br^-$ or by any enzymatic oxidation of $^{82}Br^-$ to ^{82}Br following incorporation, cannot be deduced from the experiments. However, YAGI[39,40], after failing to observe bromine binding in thyroid proteins, has suggested that this might be due to the rather high reduction-oxidation potential of $2Br^- - 2e^- = Br_2$ of -1.06 volts, whereas no oxidizing enzymatic system is known which matches this potential.

On the other hand, there are a few marine forms which apparently oxidize bromide to bromine and incorporate it into 3-monobromo-L-tyrosine and 3,5-dibromo-L-tyrosine[2,3,4,5,6]. Recently, two experiments have been reported in which Br^- was oxidized enzymatically using the H_2O_2/chloroperoxidase system leading to bromine incorporation into the thiazole ring[63] and into 16-dehydroestradiol 3-methyl ether 17-acetate[85]. An enzyme which catabolizes bromine containing organic compounds is known. The debrominase was first isolated from rat liver but has been described also in the thyroid[64]. This enzyme debrominates hydrocarbons as well as mono- and dibromo-L-tyrosine[65,71,72,73].

The data in Fig. 2 indicate an even distribution of ^{82}Br within the serum proteins. But the specific activity figures (Table 1) show a higher activity in the lipoproteins. The lipoproteins are known to contain considerable amounts of unsaturated compounds, e.g., cholesterol, which on

account of its double bond may be considered as a bromine binding species. This led to the extraction experiments (Chapter 3.7.) designed to separate the lipid moiety from the serum proteins or serum protein hydrolyzate. Only insignificant amounts of ^{82}Br (4% and 5%) could be extracted. The hypothesis of bromine binding to the unsaturated compounds, then, does not appear very significant.

The results of the exchange, electrophoresis, and denaturing experiments allow a distinction between "weakly and strongly" bound ^{82}Br (see Table 9).

About one-third of the protein-bound bromine can be easily removed from the proteins and, therefore, should be termed "weakly bound bromine". This most likely represents $^{82}Br^-$ ions attached to the polar groups on the surface of the native protein molecules. The rapid exchange points in this direction also. To explain the remaining strongly bound part simply as another kind of bromide binding, the assumption of a blocked conformational site arising from the tertiary structure would be required. This site would trap bromide preferentially. This type of bromide should be freed when the native structure is destroyed, since the protein is "unfolded". The results of the denaturing experiments show that again only 14-38% can be recovered as bromide. Furthermore, even after destruction of the protein chain by pronase attack, only 41% ^{82}Br can be liberated. Therefore, the concept of a blocked conformational site holding bromide can be excluded.

It is important to note that SKLAVENITIS' and COMAR's analytical values for protein bound bromine represent the strongly bound bromine only (3-5% of the total plasma bromine). It appears probable that under

Table 9. Fractionation of protein-bound bromine

Method	Fraction #1	Fraction #2
Gel chromatography (Fig. 1)	no specific bromine binding protein, i.e., PBBr essentially evenly distributed over protein fractions	
Exchange	33% Br^-	67% in proteins
Electrophoresis (Fig. 2)	30% Br^-	50% in proteins
Denaturation		
(a) urea	14% Br^-	86% in proteins
(b) urea plus exchange	38% Br^-	62% in proteins
(c) GuHCl	22% Br^-	78% in proteins
(d) sulfosalicylic acid	36% Br^-	64% in proteins
Partial hydrolysis	41% Br^-	60% in "peptides"
(a) with pronase, 24 hrs	36% Br^-	not measured
	40% Br^-	not measured
(b) with pronase, 12 hrs	35% Br^-	not measured
(c) with pronase, 20 hrs	31% Br^-	not measured
(d) with pronase, 24 hrs	36% Br^-	not measured
Partial hydrolysis with		
(a) pronase	not measured	18% in amino acids
(b) subtilisin	not measured	27% in amino acids
(c) papain	not measured	13% in amino acids
Complete enzymatic hydrolysis	not measured	33% in amino acids
		31% in amino acids

actual physiological conditions the strongly bound bromine, relative to the weakly bound bromide, accounts for much more than the two-thirds found in the tracer experiments. The weak binding should occur faster, possibly by way of exchange with natural protein bound bromide. On the other hand, the strongly bound bromine forms more slowly. This is indicated by two observations. First, after 24 hours only approximately ½% of the total serum bromine is in this form. Second, SKLAVENITIS and COMAR[34] showed that after oral administration of 1 g potassium bromide the protein-bound bromine reached a maximum concentration after 12 days in humans.

For an investigation of protein bound bromine the obvious working hypothesis is the assumption of bromine substituting iodine in the thyroid hormones. YAGI's observation that injected radiobromide is concentrated in the thyroid gland of rats indicates this possibility. The experiment described in Chapter 3.6. failed to show any radioactivity in thyroxine. The method is able to easily detect ½% of the ^{82}Br applied to the column in the thyroxine fraction. This result is in accordance with LA ROCHE and BROWN[42] who could not find any bromine bound to organic compounds in the tissue proteins of the thyroid gland and in rat serum. EVANS[66] conducted a $^{131}I^-$ *in vivo*-tracer experiment. After injection of $^{131}I^-$ into a rabbit, the serum proteins were obtained 1 day later and separated by gel chromatography. He showed that only the albumin fraction contained ^{131}I radioactivity. The ^{82}Br distribution in the serum proteins obtained in this study does not follow the ^{131}I pattern. It is, therefore, concluded that bromine does not replace iodine in thyroxine.

The nature of the "strongly bound" bromine has been investigated by

various enzymatic hydrolyses and ion exchange separations. The pronase hydrolysis followed by cation exchange (Chapter 3.8.1.) showed that there was ^{82}Br which remained bound to the hydrolysis products. In order to isolate and analyze defined hydrolysis products, it was desirable to achieve complete enzymatic hydrolysis. The action of papain, pronase or subtilisin only yields a partial hydrolyzate which is not suitable for analysis. Therefore, the complete enzymatic hydrolysis method of HILL and SCHMIDT was used. This method still is regarded as the most efficient one to date[50]. Its advantage is reflected in the yield of "radioactive amino acids" after deproteinization, 33% of the protein bound bromine could be obtained in the amino acid fraction compared with only 13-27% when pronase, subtilisin or papain were used alone. The important result is that there are ninhydrin active hydrolysis products, mainly free amino acids, which are radioactive. The radioactivity cannot be due to $^{82}Br^-$ which is adsorbed on the anion exchange resin under these conditions.

It should be noted, however, that the AG 2-X8 resin also binds at least some of the amino acids. MOORE and STEIN pointed out that tryptophan can only be recovered in yields of to 85% in the effluent[49]. The situation concerning 3,5-dibromo-L-tyrosine binding to this resin was investigated in an experiment described in Appendix 7.20. Only 50% of the 3,5-dibromo-L-tyrosine applied to the column could be recovered in the effluent. This explains the low yield obtained in the experiment described in Chapter 3.8.2.: 13 of up to 27% of the total protein-bound bromine. Theoretically about 60% of the protein-bound bromine, corresponding to the "strongly bound" bromine, should have been recovered in the effluent. This low yield was the reason that the picric acid-AG 2-X8 deproteinization was not used

when amino acid analysis was intended after hydrolysis.

The amino acid separation designed to isolate a "radioactive amino acid" was successful. Three ^{82}Br containing substances were eluted from the separation column. Firstly, the ^{82}Br in the first 40 ml of the effluent is undoubtedly due to $^{82}Br^-$. The cation exchange resin does not bind Br^-. In fact, this property was utilized to separate Br^- from hydrolysis products (see Appendix 7.6). The presence of $^{82}Br^-$ in the hydrolyzate represents the "weakly bound" ^{82}Br. It again is roughly one-third of the total protein bound ^{82}Br. Secondly, a ^{82}Br radioactivity peak appears around 175 ml (Fig. 6). This is consistent with the result of the amino acid separation on the AG 50W-X8, 0.9 × 15 cm, column where also a radioactivity peak prior to the 3,5-dibromo-L-tyrosine peak was observed (Fig. 5.4). Its nature cannot yet be explained. It may be another brominated amino acid.

Thirdly, the most important result is that ^{82}Br radioactivity emerges in the elution position of authentic 3-bromo-L-tyrosine. The elution positions obtained in the calibration chromatograms and hydrolyzate analysis show variations from run to run. The pH of the amino acid mixture applied to the column greatly influences the elution position. Small deviations from pH 2 cause sizeable shifts in elution position. Although careful attention was given to accurate pH adjustment, the elution positions have an estimated error of ± 6 ml. In the actual analysis, a ^{82}Br peak at millilitre 267 was obtained, which can neither be associated with 3,5-dibromo-L-tyrosine nor with an amino acid other than brominated tyrosine. The peak is well separated from 3,5-dibromo-L-tyrosine. All other amino acids have different positions. The only

possibility is to associate this peak with 3-bromo-L-tyrosine for which the elution position (265 ml) fits.

The preparation of the specific derivative (Chapter 3.8.3.) offers an additional proof for a tyrosine-like brominated amino acid. Under the preparative conditions other amino acids do not form the sparingly soluble sodium salt. Although the derivative was prepared with 3,5-dibromo-L-tyrosine as carrier, the result is still valid since all tyrosines form this sodium salt[60]. Most of the radioactivity was found in the sodium salt precipitate. $^{82}Br^-$ does not co-precipitate under these conditions (Appendix 7.16). The ^{82}Br found in the precipitate is not necessarily in the form of the sodium salt of p-toluenesulfonyl derivative of [^{82}Br]3-bromo-L-tyrosine; it also could be a co-precipitate of this minor amount of [^{82}Br]3-bromo-L-tyrosine on the large amount of sodium salt of p-toluenesulfonyl 3,5-dibromo-L-tyrosine.

It is concluded, then, that the major portion of the protein-bound ^{82}Br occurs in a brominated tyrosine, in all likelihood 3-bromo-L-tyrosine. A future experiment which would offer more conclusive evidence in favour of [^{82}Br]3-bromo-L-tyrosine in serum proteins involves the addition of 3-bromo-L-tyrosine instead of 3,5-dibromo-L-tyrosine before the amino acid separation. Conclusive evidence will be established when the ^{82}Br radioactivity emerges from the amino acid separation column in the same elution position as the added 3-bromo-L-tyrosine.

It is, however, too early to claim the discovery of 3-bromo-L-tyrosine as a natural constituent of the serum proteins. Although the series of experiments described here presents strong evidence in favour of 3-bromo-L-tyrosine, its presence in the serum proteins still has to

be shown without using ^{82}Br tracers. This can be accomplished by using the hydrolytic procedure with natural serum proteins and analyzing the effluent from the amino acid separation column in fractions for bromine. If bromine can be found in the elution position of 3-bromo-L-tyrosine this, then, can only be attributed to a natural occurring 3-bromo-L-tyrosine. In 1931, VICKERY and SCHMIDT[67] have set standard criteria for the acceptance of a newly discovered amino acid as a protein constituent. The main criteria are: 1. the substance be liberated from a protein by hydrolysis; 2. the substance must be isolated from the hydrolyzate and the identity between isolated natural product and the established synthetic amino acid must be demonstrated; 3. the new amino acid must be isolated by some worker other than its discoverer. The first and second requirement are partly met in this study. In order to verify the result obtained in this study, a different chromatographic technique can be applied.

The method of choice would be gas-liquid chromatography. Amino acids have been successfully chromatographed by this method. Furthermore, the isolation of related compounds such as thyroxine, mono- and diiodotyrosine from serum and their gaschromatographic separation and identification has also been accomplished[68,69]. The application of this method seems to be very promising for further work on protein bound bromine.

The question immediately arising is whether 3-bromo-L-tyrosine is a constituent of the protein chain or only attached to it like thyroxine can not clearly be answered on the basis of the results obtained in this study. A definitive proof that 3-bromo-L-tyrosine is present in peptide

linkage in proteins rests on the demonstration of 3-bromo-L-tyrosine in a peptide isolated from a partial hydrolyzate. Although the experiment described in Chapter 3.6. was not designed to separate hydrolysis products of the serum proteins, it throws some light upon the preceding question. A partial enzymatic hydrolysis was carried out yielding peptides of various sizes as well as free amino acids. The papain hydrolyzate of ^{82}Br-labelled serum proteins was chromatographed on a Bio-Gel P-6 column (Fig. 4). ^{82}Br radioactivity was found in the "first peak" (proteins and peptides with a molecular weight >6000) as well as all over the region where peptides of various sizes emerge from the column. This may be taken as a strong indication that 3-bromo-L-tyrosine is present in peptide linkage in proteins. In addition, it can only be released by complete enzymatic hydrolysis of the serum proteins.

5. CONCLUSIONS

1. The phenomenon of bromine binding to blood serum proteins is demonstrated with a ^{82}Br-tracer study *in vivo*.
2. ^{82}Br does not bind preferentially to one protein.
3. ^{82}Br does not bind to thyroxine as a substitute for iodine.
4. After *in vivo* labelling there is a ^{82}Br containing amino acid in serum proteins.
5. Approximately one-third of the protein bound ^{82}Br is in the form of a tyrosine-like amino acid one day after injection. This amino acid shows the same ion-exchange chromatographic behaviour as 3-bromo-L-tyrosine.

6. THOUGHTS ON THE BIOLOCIGAL SIGNIFICANCE OF THE RESULTS

The results of this study - incorporation of injected $^{82}Br^-$ into monobromotyrosine - is in a peculiar way consistent with those of DOERING[32,33]. Based on his analysis he had suspected that some of the blood bromine is bound to amino acids. Furthermore, ZONDEK's idea of a "bromine hormone" in blood[9-14] seems to go through a revival. The "bromine hormone" circulating in blood did not turn out to be tetrabromothyronine as ZONDEK and BIER had claimed. Instead, strong evidence has been obtained in favour of monobromotyrosine along with an other still unidentified bromine containing compound.

The question where monobromotyrosine is produced in the body can only be speculated on. In view of ZONDEK's claims, i.e. the pituitary gland is the site of the bromine metabolism, it may be thought that the pituitary produces monobromotyrosine which is carried through the blood stream to the liver and there is built into the peptide chain of the serum proteins, be it via a natural biochemical process or be it by lack of specificity in the protein synthesis. This explanation would require the co-existence of both "free" monobromotyrosine and protein incorporated monobromotyrosine. The experiments carried out in this study could not distinguish between those two forms.

It is well established that the tyrosine metabolism is linked with the function of the nervous system. Tyrosine is hydroxylated and decarboxylated to dopamine, a brain active amine. Whether monobromotyrosine has a connection with this metabolism can only be speculated on. But it seems significant that the body produces monobromotyrosine instead of dibromotyrosine. 3-Monobromotyrosine, having an open 5 position, can still be hydroxylated and decarboxylated to a bromine containing dopamine.

It is interesting to note that $^{82}Br^-$ injected into rats enters the brain noticeably later than other organs[41]. The possible link between bromine, tyrosine metabolism and neurological function deserves investigation. The fact that manic depressive psychotics were treated with bromide in the past and with dopamine today can only be mentioned.

ZONDEK and BIER created the bromine controversy of the early '30s as they had demonstrated a decrease in blood bromine content in cases of manic depressive psychosis by means of an analytical technique that produced too high bromine values and was proven to be unreliable. One explanation of their results may be that the method has different sensitivities towards bromide and organically bound bromine in blood. A change in the Br^-/PBBr ratio from healthy persons to psychotics would be reflected in different bromine values produced by this method. Other analytical techniques for bromine, e.g., LEIPERT's, produced the right value but were not sensitive to changes in the chemical state of bromine present in blood. They, therefore, could not confirm ZONDEK and BIER's findings.

In view of the high likelihood that monobromotyrosine is present in blood, it is considered to be of medical value to investigate the ratio between monobromotyrosine and bromide in healthy persons and in psychotics. Gas-liquid chromatography would be very helpful in analyzing for monobromotyrosine.

It may very well be that the discovery of monobromotyrosine in blood serum proteins contributes to elucidate the material base of the manic depressive psychosis.

7. APPENDICES

7.1. ^{82}Br *in vivo* uptake by serum proteins as function of time after injection

Two mg neutron-irradiated potassium bromide (6 days) were dissolved in 1 ml saline solution and injected into a rabbit (2.4 kg body weight). Blood samples of 2-3 ml were drawn 1, 2, 3 and 4 days after injection. One ml of the gained serum was chromatographed on Bio-Gel P-200, 1.8 × 49 cm, column which was operated at a flowrate of 6 ml/hr. The effluent was continuously monitored at 254 nm to indicate the proteins and collected in 3 ml fractions which were checked for radioactivity. A 3×3 inch NaI(Tl) scintillation detector in conjunction with a 256 channel analyzer was used for counting. The counts in the channels corresponding to a gamma energy of 200-1000 keV were summed up representing the ^{82}Br activity of the fraction. The protein fraction emerges from the column between 21 and 66 ml and the ionic fraction from 108 to 144 ml. The counts in the protein and ionic fractions are combined and reported below.

Table 10. ^{82}Br uptake as function of time

	Days after injection			
	1 cpm	2 cpm	3 cpm	4 cpm
total counts, containing all activity emerging from the column	710,000	403,000	189,000	86,000
counts in the protein fraction	2,250	1,310	850	830
per cent of the total	0.32	0.33	0.45	0.95

7.2. The separation of the serum proteins from bromide

It is shown in a tracer experiment that Bio-Gel P-2 columns, 1.8 × 19.5 cm in size, are suitable to separate bromide from serum proteins. To 1 ml of blood serum 10 μg $K^{82}Br$ containing 35 μCi ^{82}Br were added. The mixture was put on top of a Bio-Gel P-2, 1.8 × 19.5 cm, column and eluted with 0.15 M ammonium acetate pH 6.9 buffer. The effluent was continuously monitored at 254 nm. The collected 2 ml fractions were checked for radioactivity. As indicated in Fig. 7, the protein fraction and the bromide fraction are clearly separated.

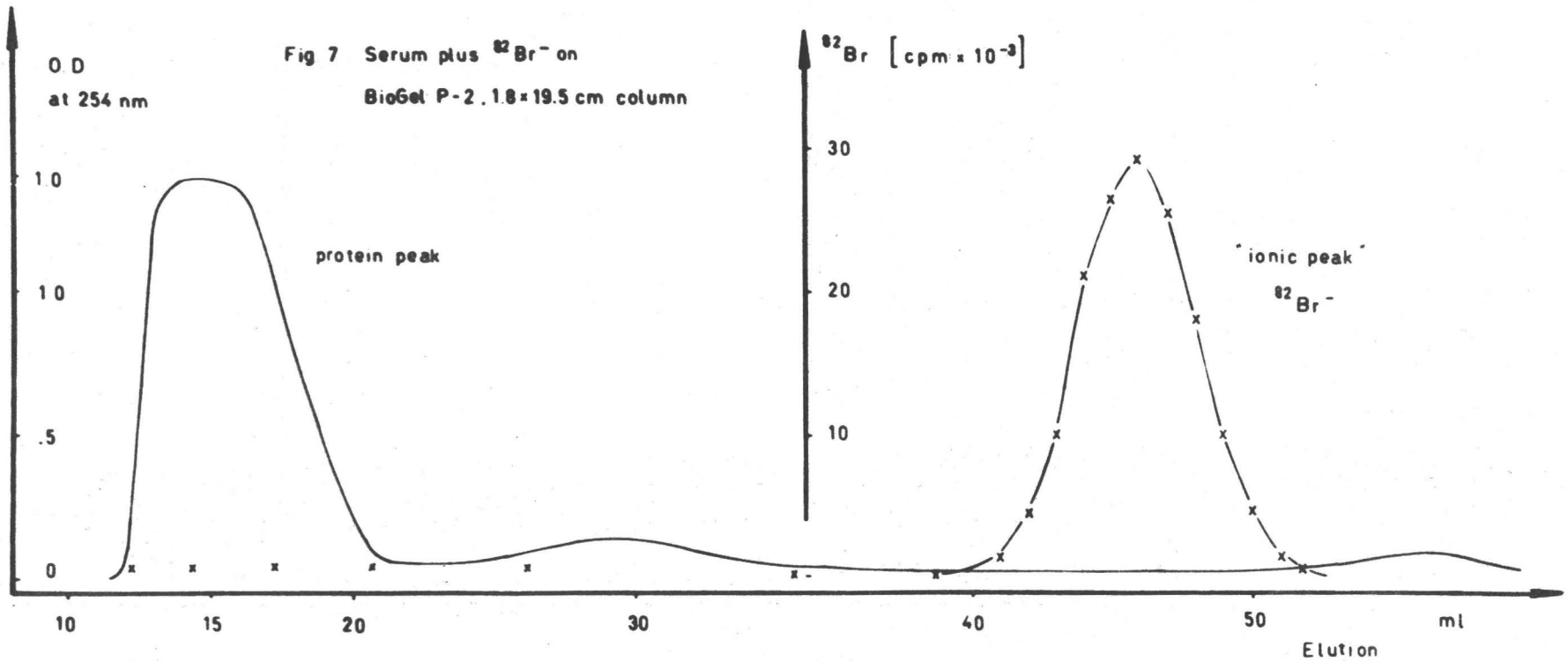


Fig 7 Serum plus $^{82}Br^-$ on BioGel P-2, 1.8 x 19.5 cm column

7.3. *In vitro* ^{82}Br uptake by serum proteins

To 1 ml fresh serum a solution containing 30 µg of $^{82}Br^-$ of the same specific activity as used in the *in vivo* experiment were added. This amount simulates the *in vivo* condition. In one case the $^{82}Br^-$ was treated with 20 mg sodium sulfite in 0.04 ml aqueous solution at pH 1 in order to reduce any bromine that has probably formed, then neutralized and added to the serum. In another case untreated $^{82}Br^-$ was used. The mixture was allowed to stand for 24 hours at 37°C followed by desalting on Bio-Gel P-2, 1.8 × 19.5 cm, column. The effluent was continuously monitored at 254 nm and collected in 2 ml fractions which were checked for radioactivity. Instead of a schematic elution pattern the actual counting results are given in Table 11.

Table 11. Desalting of *in vitro* serum-$^{82}Br^-$ mixture

Fr. #	ml		$^{82}Br^-$ pretreated with Na_2SO_3 cpm		$^{82}Br^-$ untreated cpm	
5	10		-		4	
6	12		3		15	
7	14	protein	37	116 cpm	82	145 cpm
8	16	peak	54	= 0.01%	30	= 0.01%
9	18		22		18	
10	20		5		11	
16	32		106		165	
17			6,228		7,015	
18	36		50,214		66,801	
19			488,430		512,683	
20	40	ionic	682,228	1.408×10^6 cpm	751,232	1.806×10^6 cpm
21		peak	103,995	= >99%	335,120	= 99%
22	44		62,708		87,324	
23	46		11,520		23,870	
24			6,003		10,427	
25	50		2,123		5,832	
26			718		1,120	
27	54		120		683	

In both cases the protein fraction contains only 0.01% of the total radioactivity present.

7.4. Electrophoretic separation of serum proteins from ^{82}Br

To 1 ml desalted serum protein solution some $^{82}Br^-$ was added. Twenty µl of this mixture were put on Whatman 3 MM electrophoresis paper strip (4 × 19 cm) and electrophoresed in barbital buffer pH 8.8 at 180 V and 2 mA per strip for one hour. The paper strip was dried and cut into 1 cm pieces perpendicular to the direction of migration. The paper pieces were counted using an end window beta proportional counter. Fig. 8 shows the radioactivity pattern along the paper strip. Below, the strip itself is shown schematically as stained with Amido Black to make the proteins visible.

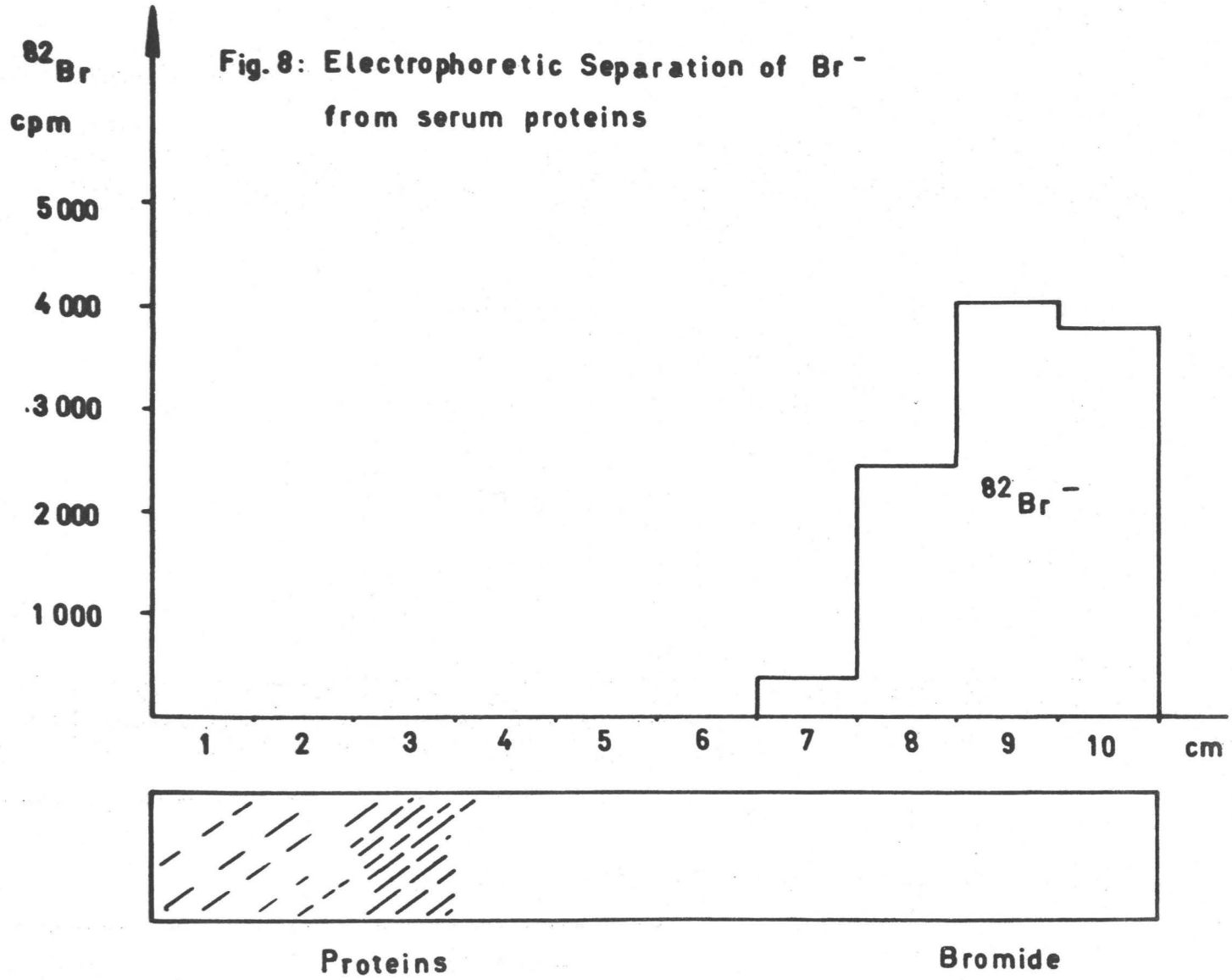


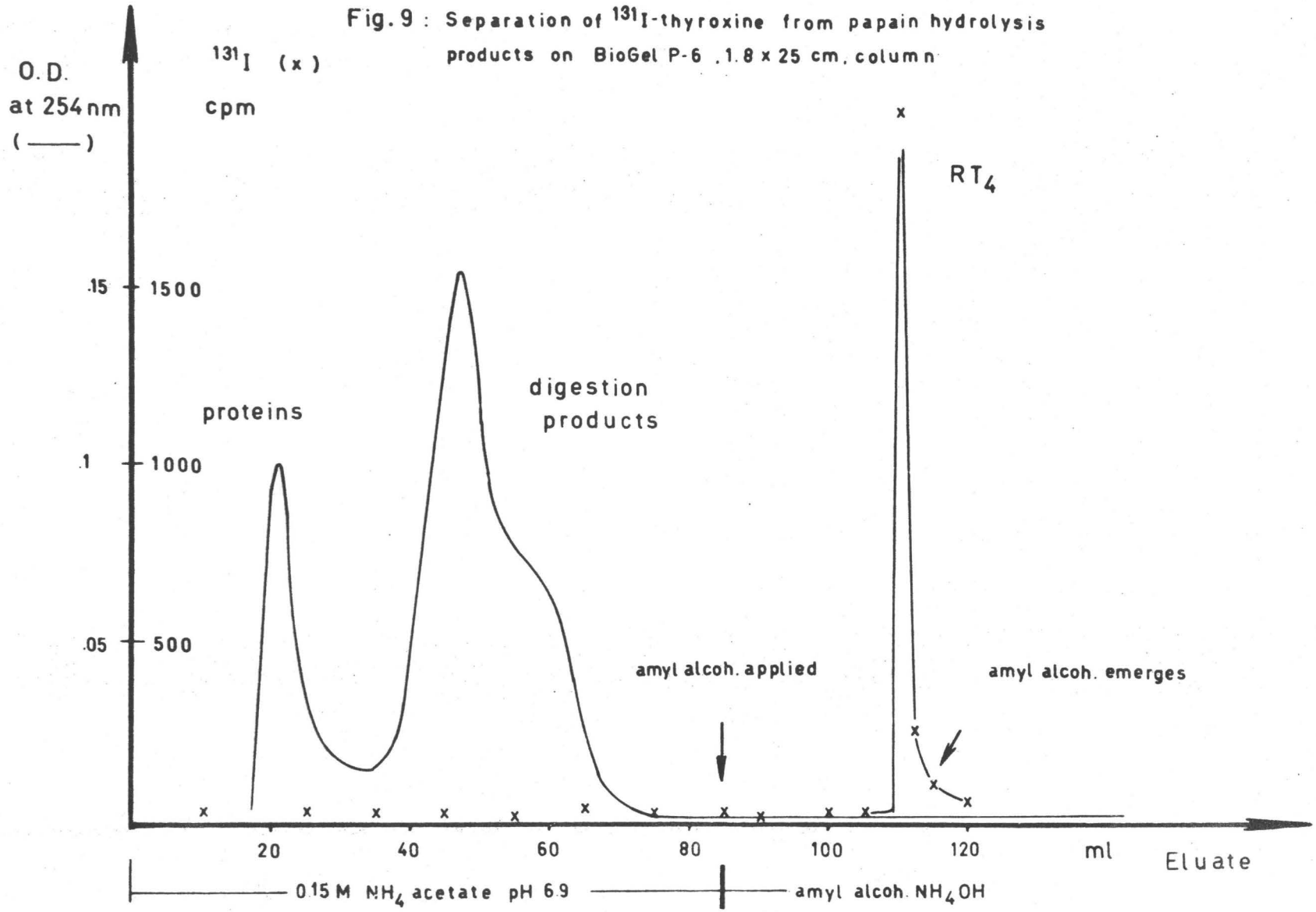
Fig. 8: Electrophoretic Separation of Br^- from serum proteins

7.5. Separation of ^{131}I-labelled thyroxine(RT_4) from blood serum by papain digestion and gel chromatography

To 1 ml blood serum 3.2 μg RT_4 dissolved in 0.8 ml 50% aqueous propyleneglycol solution is added. The mixture was desalted on Bio-Gel P-2, 1.8 × 19.5 cm, column in the way described in the experimental section 3.1., to separate the proteins which bind RT_4 from I^-. The protein fraction was collected (6 ml), adjusted to pH 4.5 with 2 N HCl and 0.25 ml of the papain suspension, 0.25 ml 0.1 M solution of cysteine, and 0.25 ml 0.1 M solution of EDTA were added. The mixture was incubated at 37°C for 23 hours. The hydrolysis was terminated by adjusting the pH to about 7 with KOH. From the total solution of 10 ml a 3 ml sample was chromatographed on a Bio-Gel P-6, 1.8 × 25 cm, column with 0.15 M sodium acetate pH 7 as the elution buffer. The effluent was continuously monitored at 254 nm and collected in 5 ml fractions which were counted for radioactivity (3×3 inch NaI(Tl) scintillation detector with single channel analyzer). At 85 ml the elution buffer was changed to tert.-amyl alcohol saturated with 2 N ammonia. Fig. 9 shows the elution pattern.

Thyroxine is clearly separated from the hydrolysis products. The recovery of the radioactivity is only 52%.

Fig. 9 : Separation of ^{131}I-thyroxine from papain hydrolysis products on BioGel P-6 , 1.8 x 25 cm. column



7.6. Separation of $^{82}Br^-$ from hydrolysis products by means of cation exchange chromatography AG 50W-X12

One ml serum with added $^{82}Br^-$ was hydrolyzed by papain for 18 hours. The enzyme was inactivated by adjusting the pH to 2.2 with 3 M acetic acid. The mixture was counted for radioactivity (on 3×3 inch NaI(Tl) scintillation detector with single channel analyzer: 6032 ± 80 cpm), placed on top of an AG 50W-X12, 0.9 × 23 cm, column and washed in with a few drops of 3 M acetic acid. The column was eluted with distilled water and the effluent collected in 2 ml fractions which were checked for radioactivity and ninhydrin reaction. Bromide was not retarded on the column and emerged with the front whereas the hydrolysis products were bound to the column. Table 12 shown the first 20 ml effluent comprising the bromide.

Table 12. Analysis of the effluent from AG 50W-X12 column

Effluent ml	$^{82}Br^-$ cpm		ninhydrin reaction (see Appendix 7.17)
2	2		negative
4	-		negative
6	837	5677 ± 80	negative
8	4970	cpm	negative
10	20		negative
12	-		negative
14	3		negative
16	-		negative
18	6		negative
20	-		negative

The recovery of the ^{82}Br placed on the column is about 95%. The fractions containing the ^{82}Br are free from ninhydrin positive compounds. This result makes the procedure useful for $^{82}Br^-$ separation from hydrolysis products.

7.7. *In vitro* ^{82}Br uptake by pronase

Twenty mg pronase were dissolved in 3 ml 0.15 M ammonium acetate pH 6.9 buffer, and approximately 4 µg ^{82}Br-labelled bromide were added. The mixture was kept at 40°C for 20 hours and desalted on Bio-Gel P-2, 1.7 × 18 cm, column in the way described in Chapter 3.1. The collected fractions were counted for ^{82}Br activity using a 3×3 inch NaI(Tl) scintillation detector coupled to a single channel analyzer. There was no measurable activity in the protein fractions. An uptake of 0.03% was the estimated detection limit when taking into account the background of 175 ± 5 cpm, the radioactivity of 80,000 cpm in the ionic fraction and assuming that 25 cpm would have been recognized as significant. Therefore, no more than 0.03% of the total Br^- were incorporated into pronase during the experiment.

7.8. Preparation of prolidase

Although pork kidney is the best known source for prolidase, the enzyme is present only in small amounts. This requires handling large amounts of material. The purification procedure of DAVIS and SMITH[51] was followed exactly here:

> Step 1. Preparation of the acetone powder: fresh frozen pork kidney (10 kg) is ground in a mechanical meat grinder and then treated in portions with an equal volume (10 liters) of 53.3% ethanol at -5°C in a Waring blendor. Four liters of 95% ethanol at -20°C are added to bring the ethanol concentration to approximately 40%, and chloroform (65 ml per kg of tissue) is added to remove hemoglobin. The insoluble material is removed in portions by centrifugation at 0 to 5°C.
>
> Acetone at -20°C is added to the supernatant fluid (250 ml per liter). After one hour in the cold, the precipitate is removed by filtration on a Büchner funnel with the aid of celite. To the clear, yellow filtrate more acetone is added (750 ml per liter), and the precipitate is allowed to settle overnight at 2°C. The clear supernatant fluid is siphoned off and discarded. The precipitate is collected by centrifugation washed on the Büchner funnel with acetone, washed repeatedly with acetone and then with ether and dried in the air.

The yield obtained was 23 g. The powder was used immediately for the second step.

> Step 2. Treatment with Mn^{++}, ammonium sulfate, and acetone. The acetone-dried powder is extracted with 390 ml of 0.02 M $MnCl_2$ at 40°C for one hour. The mixture is centrifuged, the clear, dark brown centrifugate (pH 6.0 - 6.5) is cooled to 0°C, and solid ammonium sulfate is added to bring the solution to 50% saturation (312 g per liter). After one hour at 2°C, the precipitate is collected by centrifugation, dissolved in a minimal amount of water, and dialyzed until free of sulfate.
>
> Solid $MnCl_2$ is added to the dialyzed solution to give a concentration of 0.002 M, which brings the solution to pH 5.4 to 5.6. The solution is kept at 40°C for 20 minutes. The inactive precipitate is removed by centrifugation and discarded. The solution is cooled to 0°C in an alcohol-dry ice bath and the temperature is gradually lowered to about -10°C, as acetone, pre-cooled to -20°C, is slowly added in a ratio of 30 ml of acetone for each 70 ml. After 1 hour, the precipitate is collected by centrifugation, suspended in a small amount of water, and stored frozen.

The enzymatic activity of this product was estimated according to ref. 86. In two separate preparations with C_1 = 23 and C_1 = 15 were obtained. For use in the hydrolysis an aliquot of the enzyme was thawed and dissolved in water. The protein content and activity of this solution was estimated.

7.9. Preparation and identification of 3-bromo-L-tyrosine

The synthesis first reported by ZEYNECK[70] was chosen.

L-Tyrosine (7.2 g) was suspended in 24 ml of formic acid and 0.9 ml bromine slowly added. During the bromination the reaction mixture was ice cooled and stirred. The hydrobromide of the brominated amino acid separates after a few hours. The precipitate was collected on a filter and re-dissolved in 14 ml 3 M hydrochloric acid and heated to nearly boiling. The solution was evaporated at 40°C and 15 mm Hg using a rotary evaporator. The residue was dissolved in water and neutralized with ammonia. Monobromotyrosine precipitates as grey sandy crystals which were dried over P_4O_{10}. Ten g were obtained, corresponding to a yield of 92%.

After re-crystallization from hot water the product exhibited the following properties:

Decomposition point: 244-246°C Lit. 249°C[70]

Analysis $C_9H_{10}O_3NBr \cdot H_2O$ (277)

Calc.: 28.7% Br, 5.04% N

Found: 28.4% Br, 5.2% N

Mass spectrum:

M^+ = 259 Both peaks showed almost equal intensity.

$M^+ + 2 = 261$

A small peak at 300 was also present.

7.10. Preparation and identification of 3,5-dibromo-L-tyrosine

As a convenient synthesis, direct bromination of L-tyrosine was chosen[74,75].

Thirty grams of powdered L-tyrosine were suspended in approximately 100 ml glacial acetic acid. While stirring a solution of 54 g bromine in approximately 150 ml glacial acetic acid was slowly added to the suspension. Heating to 80-90°C completed the reaction. While cooling, the sparingly soluble hydrobromide of dibromotyrosine separated from solution. The precipitate was filtered off, dissolved in water and the solution neutralized with ammonia. White needles occurred which were usually re-crystallized from water. The obtained product exhibited the following properties:

Decomposition point: 241°C Lit. 230°C[74,75]

Analysis $C_9H_9O_3NBr_2 \cdot 2H_2O$ (375.04)

Calc.: 28.8% C, 3.49% H, 3.74% N, 42.4% Br

Found: 28.5% C, 3.2% H, 3.9% N, 42.3% Br

Mass spectrum:

M^+ = 341

$M^+ + 2$ = 343

$M^+ + 4$ = 345

No higher peaks were observed.

7.11. Calibration of the amino acid separation column AG 50W-X8, 1.4 × 24 cm, with [^{82}Br]3,5-dibromo-L-tyrosine and [^{82}Br]3-bromo-L-tyrosine

In order to simulate the amino acid composition of a real hydrolyzate, an artificial mixture of 14 amino acids was prepared. The amounts chosen would represent approximately 1 μM = 100 mg of hydrolyzed serum proteins. Five ml of diluted acetic acid pH 2 to 3 dissolved the amino acids. The mixture was applied to the column and washed in with a few drops of the same acetic acid. The column was first eluted with the 0.1 M sodium citrate pH 5 and 142 ml effluent collected. This fraction comprised all non-basic amino acids. The basic amino acids remained on the column. They were now eluted with 0.1 M sodium phosphate pH 6.8. The effluent was collected in fractions which were checked for radioactivity, UV-absorption at 280 nm and ninhydrin reaction (see Appendices 7.17 and 7.18).

Table 13 shows the composition of the amino acid mixtures, Figs. 10 and 11 the analysis of the effluent in the region where the first basic amino acids emerge from the column. In the calibration run 2 only the UV-absorption was used to examine the effluent. This is justified since the elution pattern obtained from the UV-absorption and ninhydrin colour did not show differences.

Table 13. Composition of amino acid mixtures used for calibrations on an AG 50W-X8, 1.4 × 24 cm, column. Amounts in mg.

Amino acid	Calibration	
	1 (Fig. 10)	2 (Fig. 11)
Valine	6	7
Glutamic acid	9	-
Cystine	5	4
Tryptophan	7	8
Tyrosine	6	5
Leucine	6	7
Serine	5	3
Lysine HCl	10	6
Histidine HCl	5	10
Phenylalanine	5	6
Glycine	12	5
Alanine	6	6
[^{82}Br]3,5-dibromo-L-tyrosine	28	5
[^{82}Br]3-bromo-L-tyrosine	-	20
Total	110	93

Fig.10: Calibration of an AG 50W-X 8, 1.4 × 24 cm, column with ^{82}Br labelled 3,5-dibromo-L-tyrosine

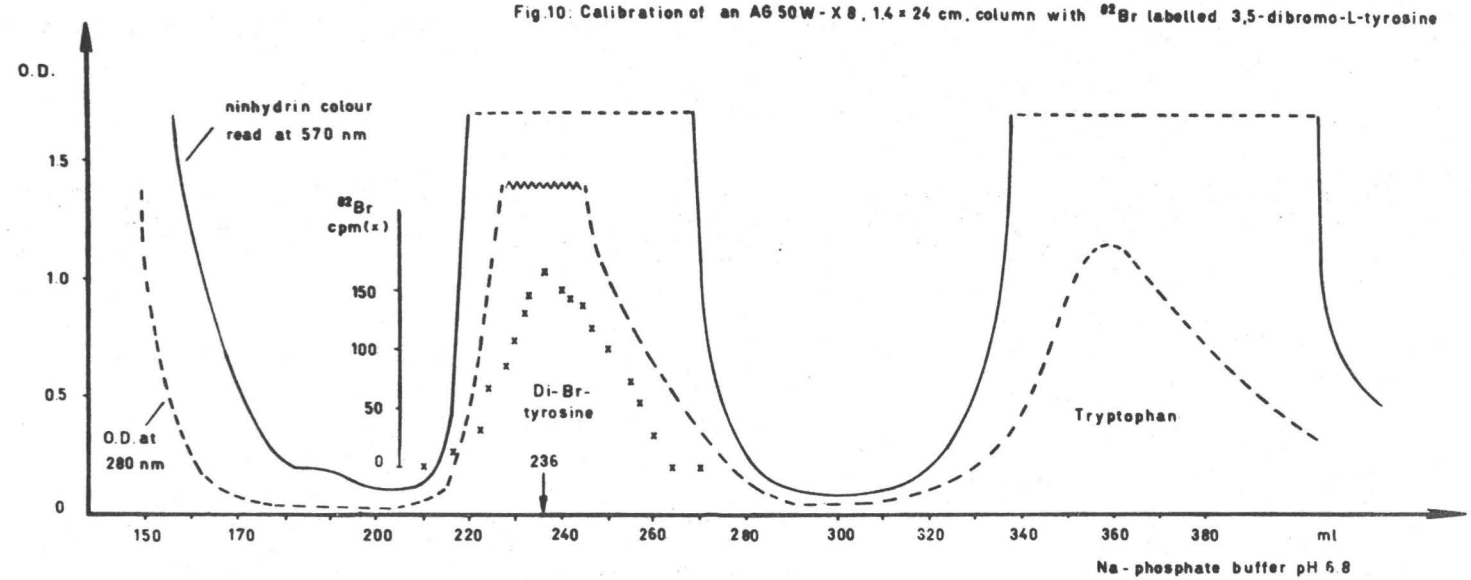
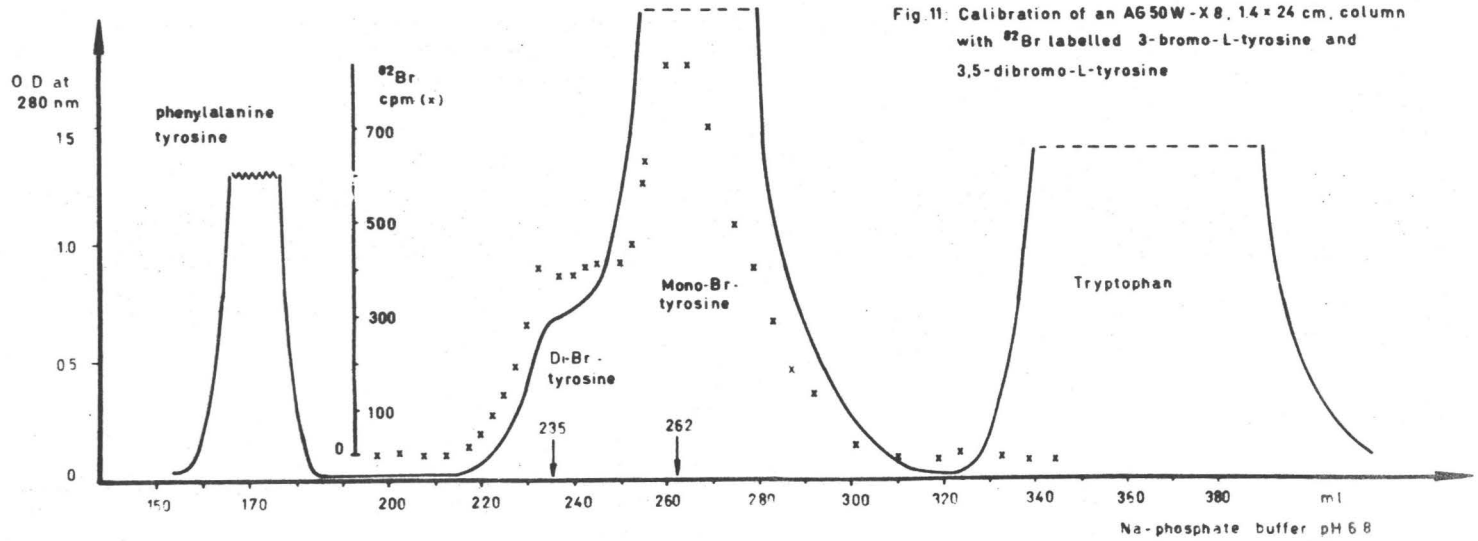


Fig.11: Calibration of an AG 50W-X 8, 1.4 × 24 cm, column with ^{82}Br labelled 3-bromo-L-tyrosine and 3,5-dibromo-L-tyrosine



7.12. Preparation of [^{82}Br]3,5-dibromo-L-tyrosine

The first bromination procedure for tyrosine using ^{82}Br was outlined by YAGI et al.[76]. He used a method by ROSENMUNT et al.[77] which was already criticized by ZEYNECK[70]. A crystalline [^{82}Br]3,5-dibromo-L-tyrosine was never obtained. The synthesis described below is essentially ZEYNECK's method (see Appendix 7.10) which was slightly modified to introduce ^{82}Br. It yields a crystallized product with a high specific activity.

A potassium bromide crystal (10 mg) was neutron irradiated for 10 hours at a flux of about 10^{13} neutrons $cm^{-2}sec^{-1}$. After a "cooling period" of one day the crystal was placed in a 100 ml round bottom distillation flask. Inactive potassium bromide functioning as a carrier, and 30 ml chromic-sulfuric acid were added. A distillation head as shown in ref. (84) was connected to the flask. The distillation flask was now heated with an open flame to about 150°C and an air stream was passed through the apparatus. ^{82}Bromide is oxidized to ^{82}bromine which was collected in a test tube containing 11 g bromine in 12 ml glacial acetic acid at 0-4°C. Losses of radioactive bromine were found to be negligible. The radioactive bromine solution was now slowly and under stirring added to a suspension of 6 g L-tyrosine in 20 ml glacial acetic acid. Heating to 80-90°C completed the reaction. When cooling the sparingly soluble hydrobromide of the brominated amino acid precipitated and was filtered off, washing with acetic acid and re-dissolved in 5 ml water. This solution was neutralized with conc. ammonia (35 ml) and allowed to stand at room temperature for 3 hours, later at 4°C. Nice long needles separated from solution which were collected in a filter,

washed with cold water and dried at 50°C in air for 2 hours and over P_4O_{10} in a desiccator for several hours. [^{82}Br]3,5-Dibromo-L-tyrosine was obtained in 50% yield, that is 5 g.

The dry product can be obtained in 12 hours. The specific activity was measured using a 3×3 inch NaI(Tl) well type scintillation detector coupled to a 512 channel analyzer. Based on the counts due to the 540 keV gamma line of ^{82}Br the product exhibited approximately 17,000 cpm/mg.

7.13. Preparation of [^{82}Br]3-bromo-L-tyrosine

The preparation of this amino acid as described by YAGI[76] did not yield a crystallized product. The slightly modified method ZEYNECK (see Appendix 7.9) was followed here.

Potassium bromide was neutron-irradiated (10 mg, 12 hours at 10 neutrons $cm^{-2}sec^{-1}$) and after a cooling period of one day $^{82}Br_2$ was liberated by an oxidation with chromic-sulfuric acid in the way described in Appendix 7.12. $^{82}Br_2$ was directly introduced into an ice cold suspension of 3.6 g L-tyrosine in formic acid. The bromination was completed with 1 ml bromine. The reaction mixture was worked up as described in Appendix 7.9. Sandy grey crystals were obtained. Yield and specific activity were not determined.

Decomposition point: 243-247°C Lit. 249°C[70]

Mass spectrum:

M = 259
$M^+ + 2 = 261$ Both peaks show almost equal intensity.

No higher peaks were observed.

7.14. Preparation of the p-toluenesulfonyl derivative of 3,5-dibromo-L-tyrosine

This derivative is described here for the first time. Its preparation was accomplished by a method outlined by SHRINER, FUSON and CURTIS[78]. About 1 g of the amino acid was dissolved in 20 ml 1 N sodium hydroxide, a solution of 2 g of p-toluenesulfonyl chloride in 25 ml ether was added, and the mixture vigorously stirred for 3 hours. The resulting suspension was acidified, and the salt went into solution. The p-toluenesulfonyl derivative then crystallized from the ether layer at 0°C and were removed by filtration. The product was re-crystallized from 75% aqueous ethanol. White needles were obtained.

M.P. 163-164°C

Analysis:

Calc.: 42.8% C, 3.25% H, 2.2% N

Found: 43.0% C, 3.47% H, 2.39% N

7.15. The formation of the sodium salt of p-toluenesulfonyl 3,5-dibromo-L-tyrosine. Yield determination.

[^{82}Br]3,5-Dibromo-L-tyrosine (4 mg, 4600 cpm) were "diluted" with 0.5 g of inactive 3,5-dibromo-L-tyrosine, and dissolved in 11 ml 1 N sodium hydroxide. A solution of 2 g p-toluenesulfonyl chloride in 22 ml ether was added and the mixture vigorously stirred for 3 hours. A white precipitate is soon formed which is the sparingly soluble sodium salt of the p-toluenesulfonyl-3,5-dibromo-L-tyrosine. Precipitate, aqueous solution and ether could be unambiguously separated by centrifugation. The aqueous solution and the precipitate, washed with 1 N sodium hydroxide, was counted for radioactivity and the following results were obtained:

Aqueous solution	820 ± 30 cpm
	3730 ± 60 cpm
	4550 ± 100 cpm

Eighty-one per cent of the recovered activity was in the precipitate.

7.16. The formation of the sodium salt of p-toluenesulfonyl-3,5-dibromo-L-tyrosine in the presence of ^{82}bromide

The procedure described in Appendix 7.14 was followed exactly. To the initial amino acid-NaOH solution approximately 100 µg $^{82}Br^-$ were added. 65,000 ± 250 cpm were obtained in a well type scintillation detector coupled to a single channel analyzer. The derivative was prepared and the formed precipitate was counted after washing it once with 1 N NaOH and contained 1400 ± 40 cpm.

The important result is that under preparative conditions leading to the derivative only approximately 2% of the $^{82}Br^-$ is co-precipitated.

7.17. Ninhydrin reaction

Ninhydrin reagent solution[47]: One g ninhydrin and 150 mg hydrindantin were dissolved in 37.5 ml methyl cellosolve. To this solution 12.5 ml of 4 N sodium acetate buffer pH 5.5 were added and the resulting reddish reagent was immediately transferred into a dark glass bottle. The solution is not stable. For this reason, not more than one experiment's supply was prepared at the time.

For the analysis of 2 ml effluent fractions from ion exchange chromatograms 0.5 ml of this ninhydrin solution was used. The buffer is strong enough so that no previous pH adjustment was necessary. After addition the test tube was shaken and kept in a boiling water bath for 15 minutes (accurately timed). The blue test tube content was now diluted with water to 20 ml and after cooling the light absorption at 570 nm was read using a BAUSCH & LOMB Spectronic 20.

7.18. Protein estimation using the UV-absorption

The method first outlined by WARBURG and CHRISTIAN[53] consists of two UV-absorption readings at 280 and 260 nm. With the empirical relationship given by LAYNE[86]

$$\text{mg protein/ml} = 1.55\ OD_{280\ nm} - 0.76\ OD_{260\ nm}$$

the protein concentration in an aqueous medium can be calculated.

The UV-spectrum of the protein - 0.15 M sodium acetate solution was scanned using the Unicam S.P. 800 automatic spectrometer (1 cm cell).

7.19. Protein estimation with FOLIN-CIOCALTEU reaction

Out of the combined fractions aliquots were taken, that contained 100-400 µg of protein. One ml of solution C was added. After 10 min 0.1 ml of solution D was added. The mixture was shaken immediately, allowed to stand for 30 min at room temperature and made up to 3 ml. The light absorption was read at 630 nm using the BAUSCH & LOMB Spectronic 20.

The relationship between protein concentration and absorption at 630 nm was established in a calibration curve using purified human albumin as protein reference. The method is not better than ± 10%.

7.20. Behaviour of [^{82}Br]3,5-dibromo-L-tyrosine on the anion exchange resin AG 2-X8

[^{82}Br]3,5-Dibromo-L-tyrosine (6 mg) was dissolved in 2 ml 0.2 N hydrochloric acid in a test tube and its radioactivity measured using the well type scintillation detector coupled to a single channel analyzer. 10,000 ± 100 cpm were counted. A 1% aqueous solution of picric acid was added and the mixture brought on top of a AG 2-X8, 1.6 × 6 cm, column. The column was eluted with 0.02 N hydrochloric acid and fractions were collected. The ^{82}Br radioactivity in the fractions was measured in comparable geometry. The following activity pattern was obtained:

Fraction Number	Elution Volume ml	^{82}Br cpm
1	2	-
2	5	-
3	8	210
4	12	3200
5	16	650
6	20	500
7	24	200
		4760 = 43% of the total

Only 43% of the ^{82}Br activity could be recovered in the effluent. The assumption that [^{82}Br]3,5-dibromo-L-tyrosine is contaminated with approximately 50% $^{82}Br^-$ which is held back by the resin is very unlikely. Recrystallized [^{82}Br]3,5-dibromo-L-tyrosine was used. It is, then, concluded that the [^{82}Br]3,5-dibromo-L-tyrosine recovery is by no means complete.

The fate of the lost radioactivity has not been investigated.

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