# THE BINDING OF $^{125}$I-3,5,3'-TRIIODO-L-THYRONINE AND $^{125}$I-L-THYROXINE TO THE PLASMA PROTEINS OF THE BROOK TROUT, SALVELINUS FONTINALIS (MITCHILL)

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Neil Ward Falkner

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ABSTRACT

1. Binding of $^{125}$I-3,5,3'-triiodo-L-thyronine (T3*) and $^{125}$I-L-thyroxine (T4*) to plasma proteins was studied in brook trout (Salvelinus fontinalis) acclimated at 10 C.

2. By precipitation with trichloroacetic acid 94.20-95.33% of T3* and 94.15-96.36% of T4* was protein-bound following incubation of the hormones with fish plasma. Using equilibrium dialysis 99.57-99.67% of T3* and 98.56-99.36% of T4* was protein-bound. The extent of binding was independent of added hormone concentration over a range of 0.05-5.0 µg/ml.

3. Owing to low binding affinities for thyronines by fish plasma proteins, cellulose polyacetate and paper media were unacceptable for electrophoretic identification of specific thyronine-binding protein fractions. Electrophoresis of plasma-radiothyronine mixtures on a 7.5% acrylamide gel using a tris-HCl-glycine buffer at pH 8.1 indicated that T3* was associated with beta-globulin-like, albumin-like, and prealbumin-like fractions while T4* was associated with beta-globulin-like and prealbumin-like fractions. The same plasma protein fractions bound T3* and T4* over added thyronine concentrations of 0.05-5.0 µg/ml.

4. Following intraperitoneal injection of tracer levels of T3* and T4*, acrylamide gel electrophoresis of fish plasma indicated T4* and T3* binding by beta-globulin-like, albumin-like and prealbumin-like

fractions. Following injection of radioiodide ($^{125}I^-$) and acrylamide gel electrophoresis of trout plasma, endogenous radioiodocompounds coincided with beta-globulin-like, albumin-like and prealbumin-like fractions.

5. As determined with the thyroxine-by-column method, normal thyronine levels in the plasma of brook trout were found to be 0.005-0.009 µg/ml (mean, 0.007 µg/ml).

6. While large amounts of T3* and T4* are bound mainly by albumin-like and prealbumin-like fractions, at more physiological thyronine levels (0.007 µg/ml) beta-globulin-like fractions are involved to a much greater extent in the binding of thyroid hormones. At higher thyronine concentrations prealbumin- and albumin-like fractions may serve to take up excess hormone with little resulting change in the circulating free thyronine levels.

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# INTRODUCTION

Primarily as a result of studies on mammals, it has been determined that 3,5,3'-triiodo-L-thyronine (T3) and L-thyroxine (T4), hormones produced by the thyroid gland, are secreted into the circulatory system and there exist in the free and protein-bound states (Lybeck, 1957; Robbins and Rall, 1957; Ingbar, 1960; Ingbar and Frienkel, 1960; Robbins and Rall, 1960; Tata, 1960; Stirling and Tabachnick, 1961; Tata, 1962; Rall *et al.*, 1964; Salvatore *et al.*, 1966).

In humans, three major plasma protein fractions normally associate with the thyroid hormones. These have been identified according to electrophoretic mobility as prealbumin, albumin and interalpha (thyroxine-binding globulin) fractions (Deiss *et al.*, 1952, 1953; Gordon *et al.*, 1952; Lybeck, 1957; Ingbar, 1958; Tata, 1964; Salvatore *et al.*, 1966).

There are few investigations on the binding of thyroid hormones to the plasma proteins of fish. Leloup and Fontaine (1960) state that Tata (unpublished) demonstrated the existence of a thyroxine-binding plasma protein in the plaice (*Pleuronectes platessa*) and brown trout (*Salmo trutta*). Leloup (1961a, 1961b) described the presence of thyroxine-binding plasma protein fractions in the Atlantic salmon (*Salmo salar*), the shad (*Alosa alosa*), the eel (*Anguilla anguilla*), the lungfish (*Protopterus annectens*), the ammocoete larva of the lamprey (*Lampetra planeri*), the mullet, the hagfish, the sea perch and the conger eel (no species given). These studies, along with those of Salvatore *et al.* (1959) on the ammocoete larva of the lamprey (*Petromyzon planeri*); Farer *et al.* (1962) on the snapper (*Lutianu vaigiensis*), the jack fish

(Caranx sexascuatus) and the tuna (Gymnosarda nuda); Refetoff et al. (1969) on the trout (no species given); and Tanabe et al. (1969) on the eel (Anguilla japonica), the gibel (Carrassius giblio longsdorfi) and the globefish (Fugu niphobles) have resulted in a rather inconsistent picture regarding this phenomenon. Nearly all plasma protein fractions have been described as thyroxine binders, but it is impossible to determine if observed differences are species specific or the result of variation in electrophoretic technique. Investigations on fish have been restricted to T4-binding even though T3 seems prominent in the circulation of rainbow trout (Salmo gairdneri) (Jacoby and Hickman, 1966) and plaice (Pleuronectes platessa) (Osborne and Simpson, 1969).

Most information regarding thyroid hormone binding to plasma proteins and the relationship of this binding to the overall picture of thyroid function has been compiled from experiments conducted on mammals (reviewed by: Sterling, 1964b; Tata, 1964; Lemarchand-Beraud et al., 1964; Salvatore et al., 1966; Osorio, 1967). The low level of potentially metabolically active free hormone is normally maintained within narrow limits by the buffering capacity of the plasma proteins. Factors altering the binding properties seriously influence the interpretation of thyroid function. Furthermore, any method depending on plasma-protein binding of the hormone to determine thyroid function (e.g., anion exchange resin or protein precipitation) is based on the assumption that most of the hormone is in the protein-bound state. In view of this and the few and conflicting data available for fishes the present study was undertaken to obtain information regarding the binding of T3 and T4 by the plasma proteins of the brook trout (Salvelinus fontinalis).

Specific objectives were: (1) to determine the extent of T3* and T4* binding with the plasma proteins; (2) to develop a method for electrophoretically identifying plasma protein fractions binding these hormones; (3) to investigate the influence of various electrophoretic media on the interpretation of results; and (4) to assess the effect of hormone concentration on the binding phenomenon.

# LITERATURE REVIEW

## 1. Protein-Chemical Complexes

The association of substances with proteins in the form of protein-chemical complexes has been known for some time. This is indicated by the work of Starling (1896), who discovered the regulated transport of water by fixation to the plasma proteins, and Sørenson (1909), who noted the formation of protein-ion complexes. Comprehensive reviews have been written by Goldstein (1949), Klotz (1953) and Scatchard et al. (1954). Several recent reviews have emphasized the interaction of a multitude of blood constituents with the plasma proteins: Bennhold (1966) on the transport of dyestuffs and iron; Guillot (1966) on bonds between proteins and carried molecules; Cohen et al. (1966) on the use of radioactive isotopes in investigating transport phenomena by the plasma proteins; Vannotti and Scazziga (1966) on the importance of the transport functions of the plasma proteins; Polonovski (1966) on the role of plasma proteins in the transport of lipids; Salvatore et al. (1966) on the interaction of thyroid hormones and plasma proteins; Froesh et al. (1966) on the nature and biological properties of various forms of insulin-like activity in the blood; Desgrez (1966) on the role of plasma proteins in corticosteroid transfer; Raoul (1966) on the transport of vitamins in the blood; Latner (1966) on the binding of circulating enzymes by plasma proteins; Lathe et al. (1966) on bilirubin transport by plasma protein; and Brodie (1966) on the pharmacological and clinical implications of drug transport. Table I summarizes the chemical interactions with protein electrophoretic fractions.

TABLE I

SURVEY OF ELECTROPHORETIC PLASMA PROTEIN FRACTIONS THAT BIND SUBSTANCES[a]

| SPECIES | BOUND SUBSTANCE | PLASMA PROTEINS G | B | $A_2$ | $A_1$ | A | PA | REFERENCE |
|---|---|---|---|---|---|---|---|---|
| Homo sapiens (man) | $H_2O$ | + | + | + | + | + | | Bennhold (1966)* |
| | $Ca^{++}$ | | | | | + | | |
| | $Cu^{++}$ | | | + | | + | | |
| | $Zn^{++}$ | | + | + | | + | | |
| Salmo gairdneri (rainbow trout) | $^{131}I^-$ | | | | | + | | Leloup & Fontaine (1960) |
| Salmo salar (Atlantic salmon) | $^{131}I^-$ | | | | + | | | |
| Esox lucius (northern pike) | $^{125}I^-$ | | | | | + | | Huang and Hickman (1968) |
| Coregonus clupeaformis (lake whitefish) | $^{125}I^-$ | | | | | + | | |
| Salmo gairdneri (rainbow trout) | $^{125}I^-$ | | | | | + | | |
| Thymallus arcticus (arctic grayling) | $^{125}I^-$ | | | | | + | | |
| Homo sapiens (man) | $^{51}Cr^{++}$ | | $+_m$ | + | + | | | Cohen (1966)* |
| | $Fe^{3+}$ | | + | + | | | | Bennhold (1966)* |
| | Phospholipids | | | | | | | |
| | lecithins | | + | + | + | | | Polonovski (1966)* |
| | sphingomyelins | | + | + | + | | | |
| | lysolecithins | | + | + | $+_m$ | | | |
| | Cholesterol | | + | + | + | | | |
| | Glycolipids | | + | + | + | | | |
| | Triglycerides | | + | | | | | |
| | Fatty Acids | | + | + | + | $+_m$ | | |
| | ILA[b] | + | + | + | + | | | Froesch et al. (1966)* |
| | Insulin | | | + | | | | |
| | Corticosteroids | | | | + | + | | Desgrez (1966)* |

TABLE I (continued)

| SPECIES | BOUND SUBSTANCE | PLASMA PROTEINS G | B | $A_2$ | $A_1$ | A | PA | REFERENCE |
|---|---|---|---|---|---|---|---|---|
| Homo sapiens (man) | Estrone | | | | | + | | Bennhold (1966)* |
| | Estradiol | | | | + | + | | |
| | Progesterone | | | | + | + | | |
| | Testosterone | | | | + | + | | |
| | Aldosterone | | | | + | + | | |
| | Corticosterone | | | | + | + | | |
| | Hydrocortisone | | | | + | + | | |
| | L-thyroxine | | | | +[c] | + | + | Tata (1964) |
| | 3,5,3'-triiodo-L-thyronine | | | | + | + | | |
| | 3,3',5-triiodo-L-thyronine | | | | + | | + | |
| | 3,5-diiodo-L-thyronine | | | | + | | | |
| | 3',5'-diiodo-DL-thyronine | | | | + | | + | |
| | 3,5,3',5'-tetraiodothyroacetic acid | | | | + | | + | |
| | 3,5,3'-triiodothyroacetic acid | | | | + | | + | |
| | Bilirubin | | + | + | + | + | | Lathe et al. (1966)* |
| | Bile acids | | | | | + | | Bennhold (1966)* |
| | Haematin | | | | | + | | |
| | Porphyrins | | | | | + | | |
| | Haemoglobin | | | + | | | | |
| | Uric Acid | | | | | + | | |
| | Acetylcholine | | | | | + | | |
| | Adensoine | | | | | + | | |
| | Carotenes | | + | + | + | + | | Raoul (1960)* |
| Cow (no species given) | Carotenes | + | + | + | + | + | | Raoul (1960)* |
| Homo sapiens (man) | Vitamin A | | + | + | | | | Bennhold (1966)* |
| | Vitamin $B_{12}$ | $+_m$ | + | | + | | | Raoul (1966)* |
| | Vitamin C | | | | | + | | Bennhold (1966)* |
| | Vitamin D | | | + | + | | | Raoul (1966)* |
| | Vitamin E | | + | | | | | |
| | Vitamin K | | | + | | | | Bennhold (1966)* |
| | Trypsin | | | + | | | | Latner (1966)* |
| | Esterases | | | + | | + | + | |
| | Cholinesterase | | | | | + | | Bennhold (1966)* |
| | Tetracycline | | | | | + | | Bennhold (1966)* |
| | Barbiturates | | | | | + | | |
| | Chloramphenicol | | | | | + | | |
| | Digitoxin | | | | | + | | |
| | Penicillin | | | | | + | | |
| | Salicylates | | | | | + | | |

TABLE I (continued)

| SPECIES | BOUND SUBSTANCE | PLASMA PROTEINS G | B | $A_2$ | $A_1$ | A | PA | REFERENCE |
|---|---|---|---|---|---|---|---|---|
| Homo sapiens (man) | Sulfonamides | | | | | + | | Bennhold (1966)* |
| | Streptomycin | | | | | + | | |
| | Congo Red | | + | + | | + | | |
| | Acid Dyes | | | + | | + | | |

Symbols used:

+ . . . . . position of binding substance in relation to plasma protein fraction

G . . . . . gamma globulin fraction

B . . . . . beta globulin fraction

$A_1$, $A_2$, . . alpha$_1$ and alpha$_2$ globulins

A . . . . . albumin fraction

PA . . . . prealbumin fraction

m . . . . . protein fraction with which the major portion of the binding substance is associated.

[a]Thyroid hormone binding to fish plasma proteins is summarized in Figures 2 and 3, and Table IV.

[b]Substance with insulin-like activity.

[c]Thyroxine or thyroxine-like compounds are here bound to the thyroxine-binding globulin (TBG) which migrates between the alpha$_1$ and alpha$_2$ globulins.

*Data from Desgrez and Traverse (1966).

The term "binding" has been used to indicate the association between a complexing chemical and some part of the protein molecule. In the formation of a specific protein-chemical complex, additional non-protein substances may assist in the formation of the bond or act as intermediaries between the protein and complexing chemical.

## 2. Methods for Investigating Protein-Chemical Complexes

Techniques for studying protein-chemical complexes have been outlined by Klotz (1953) and Cohen et al. (1966). Those relating to the present study used a radioactive isotope as the complexing chemical or as a labelling component of the complexing chemical. The complex was later isolated by Sephadex-gel filtration, ion-exchange chromatography or protein precipitation with trichloroacetic acid or neutral salts. The plasma protein fractions forming such complexes have been identified by a variety of methods (Klotz, 1953; Cohen et al. 1966), but the research of Gordon et al. (1952) is particularly pertinent to the present study. These authors, by employing paper electrophoresis, obtained the first specific information regarding thyroxine-binding to human-serum-protein fractions. Sterling et al. (1962) and Oppenheimer et al. (1963) used equilibrium dialysis to study the binding of T4 to human serum proteins. Similar techniques were employed by Huang and Hickman (1968) in their investigation of iodide binding to fish plasma proteins.

## 3. Thyroid Hormones in the Circulatory System

Rall (1950), while studying iodocompounds in the blood and urine of man, identified T4 in human plasma. At about the same time Gross and Pitt-Rivers (1951) identified T3 in human plasma.

Tata (1964) acknowledged that T3 and T4 constitute the bulk of organic iodine in the blood of many mammals, as well as some birds, amphibians and reptiles. L-thyroxine and T3 have been reported in the circulatory system of a number of fish species (Table II). The chemical structure of these hormones is shown in Figure 1.

a. Structural Determinants of Thyroid-Hormone Binding to Plasma Proteins. Sterling (1964a,b), using thyroxine analogues, found the following structural properties important in the binding of thyroxine to human serum albumin: (1) the diphenyl ether structure; (2) the presence of iodine atoms; (3) the presence of a dissociated (anionic) phenolic hydroxyl group; and (4) the alanine side chain with its carboxyl group. As a result of this knowledge he proposed a model for the binding locus on the protein molecule (Sterling, 1964a,b) which stressed the importance of free cationic groups on the protein molecule. He suggested that such cationic groups could interact with the phenolate and carboxyl groups of thyroxine. Between these cationic groups one might expect aromatic amino acids of the protein to be attracted to the diphenyl ether structure of thyroxine by means of hydrogen bonding or van der Waals' forces (Sterling, 1964a). Comparable interactions would appear possible between T3 and plasma proteins.

b. Thyronine-Binding to Human Plasma Proteins. This subject has been reviewed extensively by Robbins and Rall (1957), Sterling (1964b), Tata (1964), Lemarchand-Beraud et al. (1964), Salvatore et al. (1966), and Osorio (1967). Therefore, only aspects of particular importance to the present investigation are considered.

TABLE II

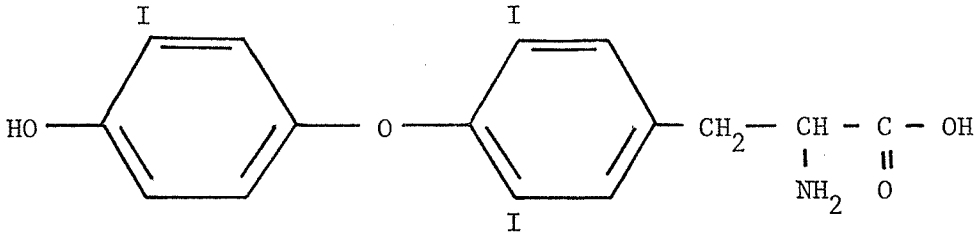
OCCURRENCE OF IODOAMINOACIDS
IN FISH BLOOD

| SPECIES | T4 | T3 | IT | SOURCE |
|---|---|---|---|---|
| Lampetra planeri (lamprey) | + | + | | Leloup (1955) |
| Periophthalmus koelreteri (mudskipper) | + | + | + | Leloup (1956) |
| Protopterus annectens (lungfish) | + | + | | Leloup (1958) |
| Umbra limi (mud minnow) | + | + | | Berg et al. (1959) |
| Eptatretus stoutii (hagfish) | + | | | Tong et al. (1961) |
| Carassius auratus (goldfish) | + | | | Chavin and Bouwman (1965) |
| Salmo gairdneri (rainbow trout) | + | + | + | Jacoby and Hickman (1966) |
| Pleuronectes platessa (plaice) | + | + | | Osborne and Simpson (1969) |

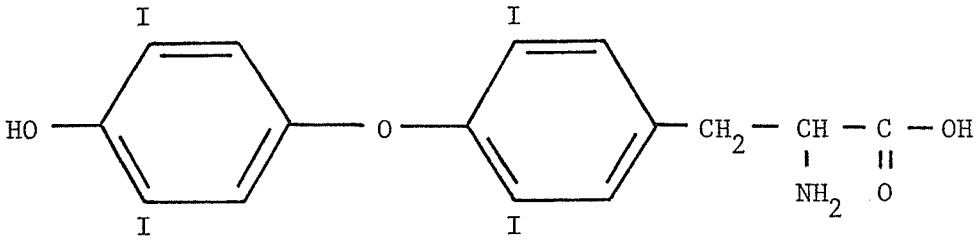
Symbols used: IT . . . . . iodotyrosines

\+ . . . . . indicates presence of substance.

Figure 1. Chemical structure of 3,5,3'-triiodo-L-thyronine and 3,5,3',5'-tetraiodo-L-thyronine (L-thyroxine).



3,5,3'-triiodo-L-thyronine



3,5,3',5'-tetraiodo-L-thyronine (L-thyroxine)

In humans most of the circulating thyroid hormone is in the form of T4, the iodide of which accounts for 90% of the organically bound iodine. Small amounts of T3 and trace amounts of 3,3',5'-triiodo-L-thyronine (reverse triiodothyronine), monoiodotyrosine, diiodotyrosine and other compounds are liberated by the thyroid gland. Most circulating T4 is bound to the plasma proteins by reversible non-covalent binding. Less than 0.1% (0.004 µg/100 ml) is free (Ganong, 1967).

Since Gordon et al. (1952) first identified T4 in the albumin and alpha globulin regions and since Ingbar (1958) noted that prealbumin also bound T4, numerous electrophoretic techniques have been used in the identification of plasma protein fractions binding thyroid hormones. However, the results in general have been the same implicating prealbumin, albumin and thyroxine-binding globulin of the alpha globulin region. Exceptions were noted by Christensen (1960), who observed two areas of radioactivity in the albumin region when plasma which had been previously incubated with T4* was subjected to starch gel electrophoresis; and Launay (1966), who found some binding of T4* to the gamma globulin fraction when human plasma was electrophoresed on cellulose polyacetate.

Each of the three major protein fractions binding thyroid hormones has a different affinity (type of binding site) and a different maximum binding capacity (number of binding sites) for the hormones (Table III). Abnormal protein-binding has been reported in a number of physiological and pathological conditions (Clark, 1967).

c. Thyroxine-Binding to Fish Plasma Proteins. Electrophoretic studies of fish plasma proteins (Salvatore et al., 1959; Leloup and Fontaine, 1960; Leloup, 1961a,b; Farer et al., 1962; Tanabe et al., 1969)

TABLE III

CHARACTERISTICS OF THE MAJOR THYROXINE-BINDING SITES OF THYROXINE-BINDING GLOBULIN (TBG), THYROXINE-BINDING PREALBUMIN (TBPA), AND SERUM ALBUMIN (A) IN HUMAN SERUM

| PROTEIN | CHARACTERISTICS | |
|---|---|---|
| | RELATIVE BINDING OF DERIVATIVES | AFFINITY AND CAPACITY IN WHOLE SERUM |
| TBG | $T_4 > T_3$ | High affinity[a], low capacity[b] |
| TBPA | $T_4 > T_3$; $T_3 \simeq 0$ | Moderately high affinity, high capacity |
| A | Both about the same | Low affinity, very high capacity |

Data from Tata (1964)

[a]Indicative of the types of binding sites.

[b]Indicative of the number of binding sites.

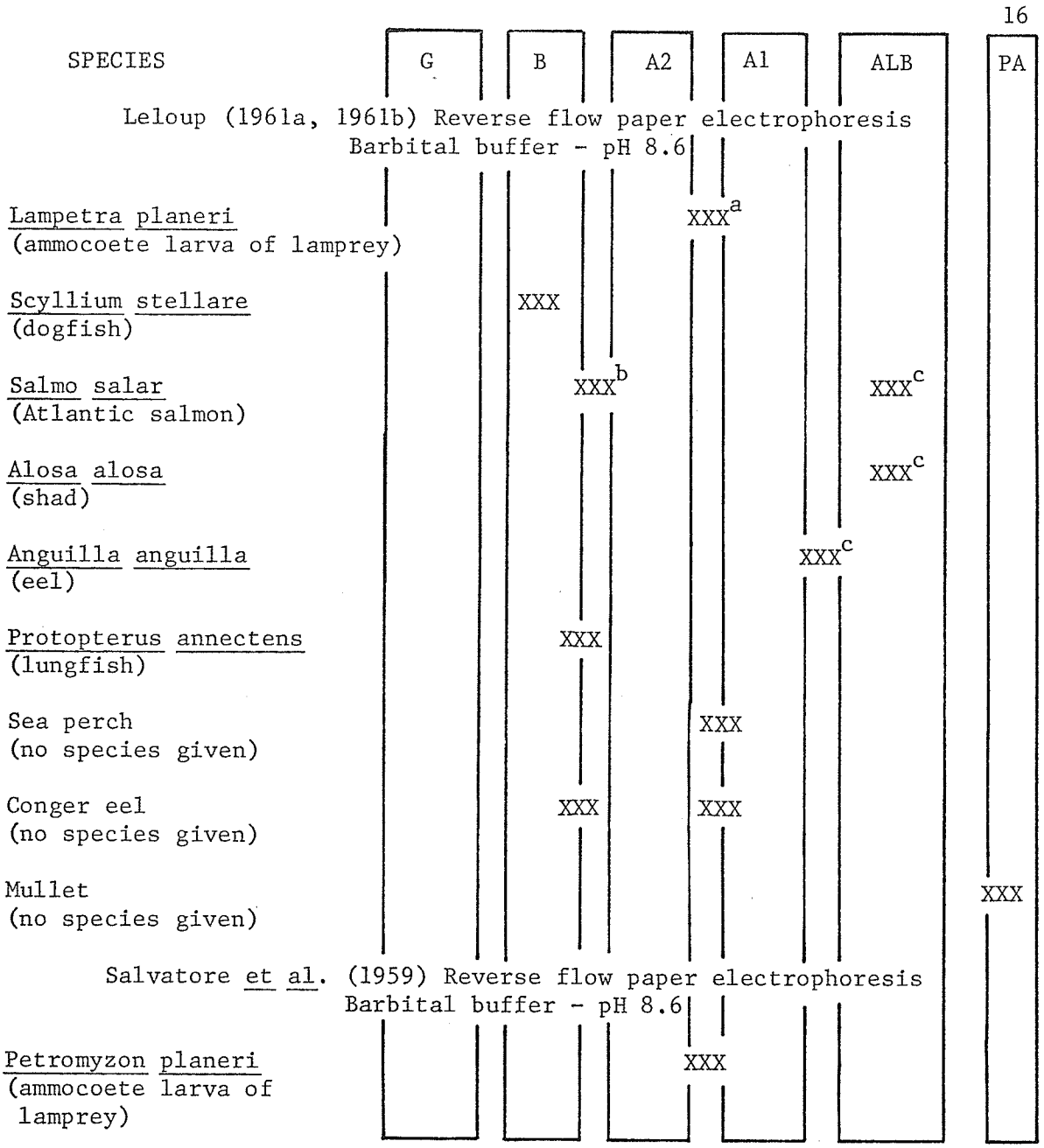
suggest the binding of T4 by all protein fractions except gamma globulin. Postalbumin, albumin and prealbumin fractions seem most frequently involved (Figures 2 and 3; Table IV). Although differences are apparent between species it is difficult to tell if these are real or simply the result of variation in electrophoretic technique. Furthermore, these studies assume binding to be indicated simply by positional similarities in the location of plasma protein fractions and T4* following electrophoresis. Since no dialysis experiments were conducted to verify binding, this seems a questionable assumption. The binding of T3 to fish plasma proteins has not been investigated using electrophoretic techniques. As a result of these facts it is particularly difficult to arrive at generalizations regarding the binding of thyroid hormones to fish plasma proteins.

d. Thyronine-Binding to Plasma Proteins of Other Vertebrates. The binding of T4 to the plasma proteins of vertebrates other than humans and fishes has been examined by Farer et al. (1962) and Tanabe et al. (1969). Table V summarizes the occurrence of thyroxine-binding proteins in eight species indicating interspecies variation along with variation related to electrophoretic technique. Tanabe et al. (1969) suggest that thyroxine-binding alpha globulin (thyroxine-binding globulin) is restricted to higher mammals such as primates, ungulates and some carnivores. However, this theory conflicts with the results of Salvatore et al. (1959) and Leloup (1961a,b) who describe TBG-like proteins in Petromyzon planeri Lampetra planeri, the sea perch and the conger eel (Figure 2).

In the few mammals studied, most of the circulating T3 is bound by thyroxine-binding globulin. A thyroxine-binding globulin-like protein

fraction has not been identified in birds and T3 along with T4 both exhibit equal affinities for albumin-like and prealbumin-like components (Tata, 1964).

Figure 2. Protein association of radioactivity in plasma-radiothyroxine mixture (0.015-0.1 μg/ml) after paper electrophoresis. Location of T4* indicated by "X's". Positions shown are relative to an electrophoretic separation of human serum with fractions indicated by rectangles.




[a]Electrophoresis of plasma 46 hours after injection with $^{131}I^-$. All other species were analyzed after incubation of plasma with T4*.

[b]T4* was present in this region only when the plasma was subjected to starch gel electrophoresis. This region of radioactivity was not apparent with paper electrophoresis.

[c]No change in the fractions to which T4* was bound occurred over T4 concentrations ranging from 1.5-10.0 μg/ml plasma.

Abbreviations used: G, gamma globulin; B, beta globulin; A2, alpha-2-globulin; A1, alpha-1-globulin; ALB, albumin; PA, prealbumin.

Figure 3. Protein association of radioactivity in serum-radiothyroxine mixtures (0.01-0.5 μg/ml) after starch gel electrophoresis. Location of T4* indicated by "X's". Positions shown are relative to an electrophoretic separation of human serum with fractions being named prealbumin, albumin and postalbumin. Electrophoresis was carried out using starch gel medium and veronal (i.e., barbital) buffer, pH 8.6. Data from Farer et al., (1962).

| SPECIES | SERUM PROTEIN FRACTION | | | | | |
|---|---|---|---|---|---|---|
| | POSTALBUMIN | | ALBUMIN | PREALBUMIN | | |
| Lutianu vaigiensis (snapper) | X | XXX | | X | | X |
| | X | XXX | | X | | X |
| Caranx sexfasciatus (jack fish) | X | XXX | | | X | |
| | X | XXX | | | X | |
| Gymnosarda nuda (tuna) | X | XXX | | | | |
| | X | XXX | | | | |
| Homo sapiens[a] (man) | | XX | XXX | X | | X |
| | | XX | XXX | X | | X |

[a]Included for comparison.

TABLE IV

PROTEIN ASSOCIATION OF RADIOACTIVITY IN PLASMA-RADIOTHYROXINE (5.0 μg/100 ml) MIXTURE AFTER CELLULOSE POLYACETATE ELECTROPHORESIS[a]

| SPECIES | PROTEIN FRACTION | | |
|---|---|---|---|
| | POSTALBUMIN | ALBUMIN | PREALBUMIN |
| Anguilla japonica (eel) | 12.9 | 50.8 | 19.8 |
| Carrassius giblio longsdorfi (gibel) | 17.8 | 56.9 | 10.8 |
| Fugu niphobles (globefish) | 18.9 | 42.5 | 6.8 |

[a]Location of the radioactivity is relative to an electrophoretic separation of human plasma with fractions being classified as prealbumin, albumin and postalbumin. Radioactivity is expressed as a percent of the total on the strip. Electrophoresis was carried out using cellulose acetate medium with barbital buffer, pH 8.6. Data from Tanabe et al. (1969).

TABLE V

PROTEIN ASSOCIATION OF RADIOACTIVITY IN SERUM-RADIOTHYROXINE (0.01-0.5 μg/ml) MIXTURE FOLLOWING ELECTROPHORESIS

| SPECIES | PAPER[a] | PAPER[b] | STARCH GEL[c] |
|---|---|---|---|
| Macaca mulatta (Rhesus monkey) | AG;A;PA | AG;A;PA | POA;$A_2$;$A_1$;PA |
| Sus scrofa (swine) | AG;A | AG;A | POA;Ab |
| Ovis sp. (sheep) | AG;A | | |
| Felis catus (cat) | AG;A | | |
| Callorhinus ursinus (Alaskan fur seal) | AG;A | AG;A | POA;Ab |
| Marmota caligata (marmot) | AG;A another globulin (slower) | AG;A another globulin (slower) | POA;Ab |
| Columba livia (pigeon) | A;PA | | |
| Meleagris gallopavo (turkey) | | A;PA | A;PA; two bands in globulin region |

Symbols used:
- PA . . . . . prealbumin region
- A . . . . . albumin region
- $A_1$ and $A_2$ . two separate bands occurring in the albumin region after electrophoresis on starch gel medium. $A_1$ is the faster migrating band and $A_2$ the slower.
- AG . . . . . alpha globulin region
- Aa . . . . . leading edge of albumin region
- Ab . . . . . trailing edge of albumin region
- POA . . . . . postalbumin region

Regions are relative to an electrophoretic separation of human serum.

[a]Reverse flow electrophoresis, barbital buffer, pH 8.6.

[b]Reverse flow electrophoresis, ammonium carbonate buffer, pH 8.6.

[c]Vertical starch gel electrophoresis, borate buffer, pH 8.6.

Data from Farer et al. (1962).

## MATERIALS AND METHODS

### 1. Fish Maintenance

One-year-old brook trout, from common brood stock maintained by the Ontario Department of Lands and Forests (Dorion Hatchery), were periodically brought to the laboratory from the Province of Manitoba trout hatchery at West Hawk Lake. Mean lengths and weights of the fish are given in Table VI. They were held in the laboratory under a natural photoperiod in a 560-liter fiberglass holding tank supplied with continuously running, dechlorinated tap water, the temperature of which varied from 3 C in April to 17 C in August (seasonal extremes).

Groups of up to 40 experimental fishes were taken from the holding tank and placed in 200-liter fiberglass tanks supplied with constantly flowing, dechlorinated tap water at 10 ± 0.5 C. The experimental tanks were enclosed with light-proof wooden covers housing a 40-Watt daylight fluorescent tube 25 cm from the water surface. Photoperiod (12 hour light - 12 hour dark) was controlled automatically (Carter Portable Time Control, James B. Carter Ltd., Winnipeg).

Food was offered on alternate days at a ration of approximately 2 g of food per fish per week. It consisted of a frozen homogenate of beef liver and ocean perch fillets (10:1 by weight) with gelatin added to facilitate dispersal when added to water. The total iodide content[1] was approximately 6.6 µg per 100 g of food.

Periodic outbreaks of fungus were prevented by adding 40 µl of one per cent (w/v) aqueous malachite green per liter of tank water.

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[1] Dried food was ground and digested with perchloric acid. Iodide content was measured by ceric ion reduction.

TABLE VI

GENERAL INFORMATION PERTAINING TO FISH

| FISH GROUP[a] | ANIMALS PER GROUP | MEAN WEIGHT (g) | MEAN FORK LENGTH (cm) |
|---|---|---|---|
| 1 | 10 | 70.5 | 18.2 |
| 2 | 10 | 65.1 | 18.2 |
| 3 | 10 | 117.5 | 21.9 |
| 4 | 10 | 83.4 | 21.0 |
| 5 | 10 | 98.0 | 21.5 |
| 6 | 10 | 78.9 | 19.6 |
| 7 | 10 | 116.6 | 21.6 |
| 8 | 10 | 82.6 | 20.8 |
| 9 | 10 | 102.2 | 20.9 |
| 10 | 10 | 93.9 | 21.5 |
| 11 | 10 | 123.2 | 22.4 |
| 12 | 10 | 92.5 | 21.2 |
| 13 | 20 | 118.4 | 21.9 |

[a]Each fish group represents the animals used to obtain one pooled plasma sample.

Clearance of the dye within two hours was assured by adjustment of the water flow to 100 liters per hour. Acclimation was carried out for three weeks and therefore corresponded to acclimation periods used by Brett (1946) and Anthony (1961) for similar temperature shifts with the goldfish (Carassius auratus).

2. Blood Collection

When initially removed from the tank, acclimated fish were lightly anaesthetized with tricaine methanesulphonate (MS-222, 0.05 g/liter). Each fish was further anaesthetized with MS-222 (0.3 g/liter) as used by Schiffman and Fromm (1959) and the caudal peduncle transected. Blood from the caudal artery was collected in disposable aluminium weighing dishes containing a drop of heparin solution (1 ml = 1000 USP units) to prevent clotting. Individual blood samples were placed in 1.5-ml conical plastic centrifuge tubes and centrifuged at 15,000 x g for five minutes. Plasma thus obtained was pooled, subdivided into smaller samples and stored in 2-ml plastic beakers at -22 C. Pooled human plasma samples were obtained from the Winnipeg General Hospital and stored as described for fish plasma. The storage period never exceeded 8 weeks.

Haematocrits were routinely determined for individual blood samples. A capillary tube (75 x 0.8 mm) was applied to a drop of freshly drawn blood and the capillary action resulted in the formation of a blood column within the tube. One end of the tube was sealed with a pliable vinyl plastic (Critoseal, Biological Research, Inc.) and the tube centrifuged at 15,000 x g for five minutes.

3. Total Protein Determination by Biuret Method

One hundred µl of a pooled-plasma sample was placed in a 10 x 1.2 cm cuvette and to this was added 2.4 ml of Biuret reagent (Biuret Reagent Tablets, Cambridge Chemical Products, Inc., Dearborn, Michigan). A standard was prepared in an identical manner with the exception that the plasma sample was replaced by Lab Trol (Dade Reagents, Inc.). After 30 minutes the color reaction was stopped by adding 0.6 ml of sodium sulfite to each cuvette. The optical density of the sample and standard were obtained with a Bausch and Lomb spectrophotometer (Spectronic 20) at a wavelength of 555 mµ. Total protein was calculated as follows.

$$\text{Total Protein in g/100 ml} = \frac{\text{Optical Density of Unknown}}{\text{Optical Density of Standard}} \times \text{Protein in Standard (g/100 ml)}$$

4. Total Thyronine Concentration

Since excessive amounts of inorganic iodine, iodoproteins or iodotyrosines are known to influence the results obtained with methods normally used in determining thyronine concentrations (Bio-Science Laboratories, 1969), and since the concentration of inorganic iodine is particularly high in the brook trout (509.0±46.0 µg/100ml; Dorey, 1970) the thyroxine-by-column method was chosen. This method is uninfluenced by high inorganic iodine (up to a minimum of 1000 µg/100ml), iodoprotein or iodotyrosine concentrations.

Analyses were carried out by Bio-Science Laboratories, Van Nuys, California, using their application of the thyroxine-by-column method. In this method, plasma samples are made alkaline with sodium hydroxide

solution and poured onto anion-exchange columns (Dowex-1). Thyroxine and related compounds, plasma proteins and plasma iodide are trapped in the column. Sodium acetate in isopropyl alcohol is then added to the column. This solution removes plasma proteins, carbonate and bicarbonate ions. Removal of these ions prevents the evolution of carbon dioxide gas and subsequent disruption of the column when acid is added. Column washes of increasing concentrations of acetic acid are used to remove monoiodotyrosines and diiodotyrosines. Finally, T3 and T4 are eluted with a column wash of 50% (v/v) acetic acid. Thyronine iodine is colourimetrically measured at 420 mμ with arsenious acid and ceric ammonium sulphate.

Three 4-ml aliquots of a plasma pool from 20 fishes were analyzed.

5. Radioactive Materials

Radioactive hormones (T3* and T4*; specific activity, 16.5 - 135.0 mCi/mg) were supplied by Mallinckrodt Nuclear, St. Louis, Missouri, and radioiodide by Atomic Energy of Canada, Ottawa. Both T3* and T4* were routinely analyzed for the presence of iodide ($^{125}I^-$). The methods used for this estimation were: descending paper chromatography butanol:acetic acid:water (4:2:1, v/v); ascending thin-layer chromatography on MN-Polygram Silica Gel (S/UV254; Machery-Nagel, Duren, Germany) with a butanol:ethanol:6N ammonia (50:40:10, v/v); or paper electrophoresis with Gelman High Resolution Buffer (pH 8.6) for 12 minutes at 300 volts and 12 milliamperes (2 ma/strip). Chromatograms and electrophoretic strips were cross sectioned and the radioactivity present in each segment determined by counting for one minute. The results obtained were plotted on graph paper and the iodide content expressed as a percentage of the total radioactivity comprising iodide and thyronine peaks.

Radioactivity was measured by placing samples in test tubes (15 x 1.5 cm) or flat-bottomed counting tubes (14 x 1.4 cm) and inserting in a well-type scintillation detector (Nuclear Chicago) having a thallium-activated sodium-iodide crystal (2¼"x2¼"). This detector was connected to an analyzer-scaler (Nuclear Chicago, Model 8725).

6. Preparation of Thyronine-Plasma Mixtures

Radioactive hormone (T3* or T4*) in 50% propylene glycol was added to plasma so the added hormone concentration was 0.05 µg/ml. If higher added-hormone concentrations were required the necessary amount of the sodium salt of cold T3 or T4 was used. Normally the final volume of the hormone-plasma solution was approximately 40 times greater than the volume of the added hormone solution.

The cold hormone was prepared by dissolving T3 or T4 in 1 ml of 4% aqueous propylene glycol (v/v) solution containing sodium hydroxide at a concentration of 0.1 N. This was then diluted to 0.002 N with 4% propylene glycol.

Unless otherwise specified, plasma thus prepared was incubated for two hours, at 10 C, with constant agitation, in total darkness (Metabolic Water Bath Shaker, Model G77, New Brunswick Scientific Co.).

7. Thyronine-Binding by Trichloroacetic Acid Precipitation

Trichloroacetic acid precipitation of fish plasma proteins has been used by a number of investigators (Hickman, 1961; Eales, 1963; Huang and Hickman, 1968; Dorey, 1970) to determine the amount of radioactive or non-radioactive thyroid hormone in the circulation. Addition of plasma-thyroid-hormone complex to trichloroacetic acid results in

precipitation of the proteins and destruction of the plasma iodide-protein bond (Leloup and Fontaine, 1960). The plasma-thyroid-hormone bond is apparently unaffected. Thus if plasma is incubated with T4* and added to trichloroacetic acid one would expect the added hormone to appear as radioactivity in association with the precipitate. Any free $^{125}I^-$ would appear in the supernatant.

An experiment (see Results) was carried out to determine the most reliable trichloroacetic acid-plasma protein precipitation technique and to assess the effect of incubation temperature. As a result of this experiment the routine procedure outlined in Figure 4 was adopted.

The fraction of the total radioactivity bound to plasma protein was calculated according to the following equation.

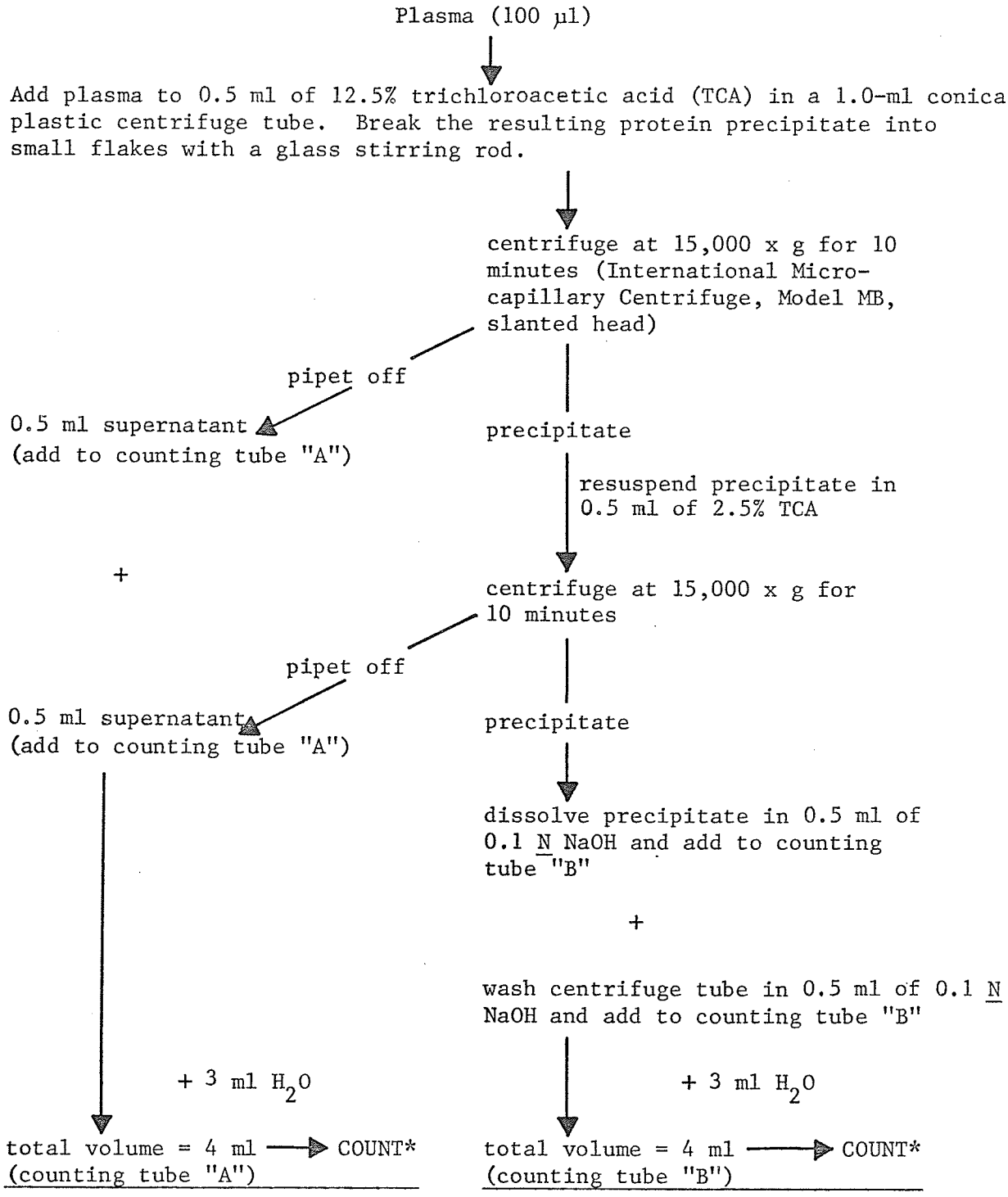
$$\text{Fraction Bound} = \frac{\text{Bound Count/Min (counting tube "B")}}{\text{Total Count/Min (counting tube "A"+"B")}}$$

In most instances this was expressed as a percentage by multiplying by 100.

8. Thyronine-Binding by Equilibrium Dialysis

Equilibrium dialysis of plasma incubated with thyroid hormones was carried out similar to the method described by Huang and Hickman (1968) for iodide dialysis against plasma proteins. The dialysis tubing (diameter 0.64 cm, Union Carbide) used had an average pore size of 24 A and contained cellulose, glycerine, water and a small amount of sulphur. Prior to use the tubing was soaked for 24 hours in three changes of distilled water. Sections were then cut measuring 9 cm (in length). The end of each section was twisted, turned back in the shape of a "U"

Figure 4. Flow chart of trichloroacetic acid protein-precipitation technique.




*Radioactivity recorded as counts per minute. Minimum total count 1000.

and tied with rayon string. The individual sections were placed in 13 x 1.4 cm siliconized test tubes containing 3 ml of Hickman's fish saline (Hoar and Hickman, 1967). A 250-µl sample of a radiohormone-plasma mixture was placed inside each section of the dialysis tubing. Equilibrium was established within 8 hours, but dialysis was continued for a total of 24 hours, at 10 C, with constant agitation, in total darkness (Metabolic Water Bath Shaker, Model G77, New Brunswick Scientific Co., Inc.). Separate 100 µl samples were then taken from inside and outside the dialysis bag and added to counting tubes containing 3.9 ml of water. Each tube was counted to a minimum of 1000 counts. The percentage of the added hormone bound to the plasma proteins was calculated as follows.

$$\text{Percentage of Added Hormone Bound to Plasma Proteins} = \frac{\text{Plasma-Hormone Sample (CPM)} - \text{Saline Sample (CPM)}}{\text{Plasma-Hormone Sample (CPM)}} \times 100$$

9. Cellulose Polyacetate Electrophoresis

Electrophoresis using cellulose polyacetate medium was carried out in a Gelman horizontal electrophoresis cell with eight cellulose polyacetate strips (Sepraphore III Cellulose Polyacetate Support Medium, 2.5 x 17.0 cm). The anode and cathode buffer chambers were each filled with 250 ml of buffer. Cellulose polyacetate strips were marked along the edge at 0.5-cm intervals, soaked in buffer, placed on a sheet of Parafilm and blotted gently. A 2-ul sample (containing 0.004-0.006 µCi) of previously incubated plasma-hormone mixture, or radioactive hormone alone (in aqueous saturated sucrose solution) was applied to each strip

with a Gelman electrophoresis sample applicator consisting of two parallel wires approximately 1 mm apart. Electrophoresis was carried out using constant current, at 10 C, in total darkness. The duration of the run and conditions of electrophoresis are given in Table XV.

Following electrophoresis all strips were stained for five minutes in a solution of 0.2% by weight of Ponceau S, 7.5% by weight trichloroacetic acid, and 7.5% by weight sulfosalicylic acid (Nerenberg, 1966). To prevent proteins from being washed-off in the staining solution, a 7.5%-trichloroacetic acid solution was used rather than the usually recommended 3% (Nerenberg, 1966). The stained strips were cleared with successive changes of 5% (v/v) acetic acid and rinsed with distilled water. Preliminary experiments indicated no difference in the location of radioactivity between strips subjected to staining and clearing procedures and those not subjected to such treatment. Individual strips were removed from the water rinse, placed on glass microscope slides (11 x 25 cm), and lightly blotted. Each was then wet-scanned at 575 mμ with a Gelscan Scanner (logarithmic response recorder). Scanner settings used were: defining slit, 0.125 cm; scan width, 1.0 cm; scan speed, fast; chart speed, 200 mm/min; millivolt control setting, 50. Since the scanning speed of the strip and the chart speed of the recorder were not identical the resulting electrophoretogram gave a protein fraction migration distance 2.2 times that of the strip.

The strips were sectioned at the previously marked 0.5-cm intervals and each section counted for one minute. Radioactivity was expressed as a percentage of a standard or as a percentage of the total radioactivity on the strip. That radioactivity in excess of 1% on replicate

strips was averaged and plotted in relation to the densitometric scan of the plasma protein fractions. Percentages of less than 1% were not plotted unless occurring consistently at a specific location or between radioactive peaks. Standards were prepared by applying to each of two 1 x 2.5-cm sections of cellulose polyacetate a sample identical to that placed on each strip.

10. Paper Electrophoresis

Paper electrophoresis was conducted using a Shandon horizontal paper electrophoresis cell (Kohn Tank, 25 x 24 cm) in conjunction with six 3.5 x 20.2-cm strips of Whatman number one chromatography paper. Each strip was marked on the edge at 0.5-cm intervals. The location for sample application was similarly marked. Anode and cathode chambers of the cell were each filled with 325 ml of buffer. The strips were dipped in buffer, placed on a Parafilm sheet and lightly blotted. A 5 µl sample of incubated radiothyronine mixture (containing 0.010-0.014 µCi) or a 5 µl sample (containing 0.010-0.014 µCi) of radioactive hormone in aqueous saturated sucrose solution was applied to each strip as described for cellulose polyacetate electrophoresis. Electrophoresis was carried out using constant voltage, at 10 C, in total darkness. The duration of the run and milliampere variation are given in Table XV.

Following electrophoresis one strip was stained as described for cellulose polyacetate, cleared in successive changes of 5% (v/v) acetic acid, rinsed with distilled water, and oven dried at 80 C. The strip was then scanned at 575 mµ with a Gelscan scanner adjusted as follows: defining slit, 0.2 cm; scan width, 1.0 cm; scan speed, fast; chart speed, 200 mm/min; millivolt setting, 50.

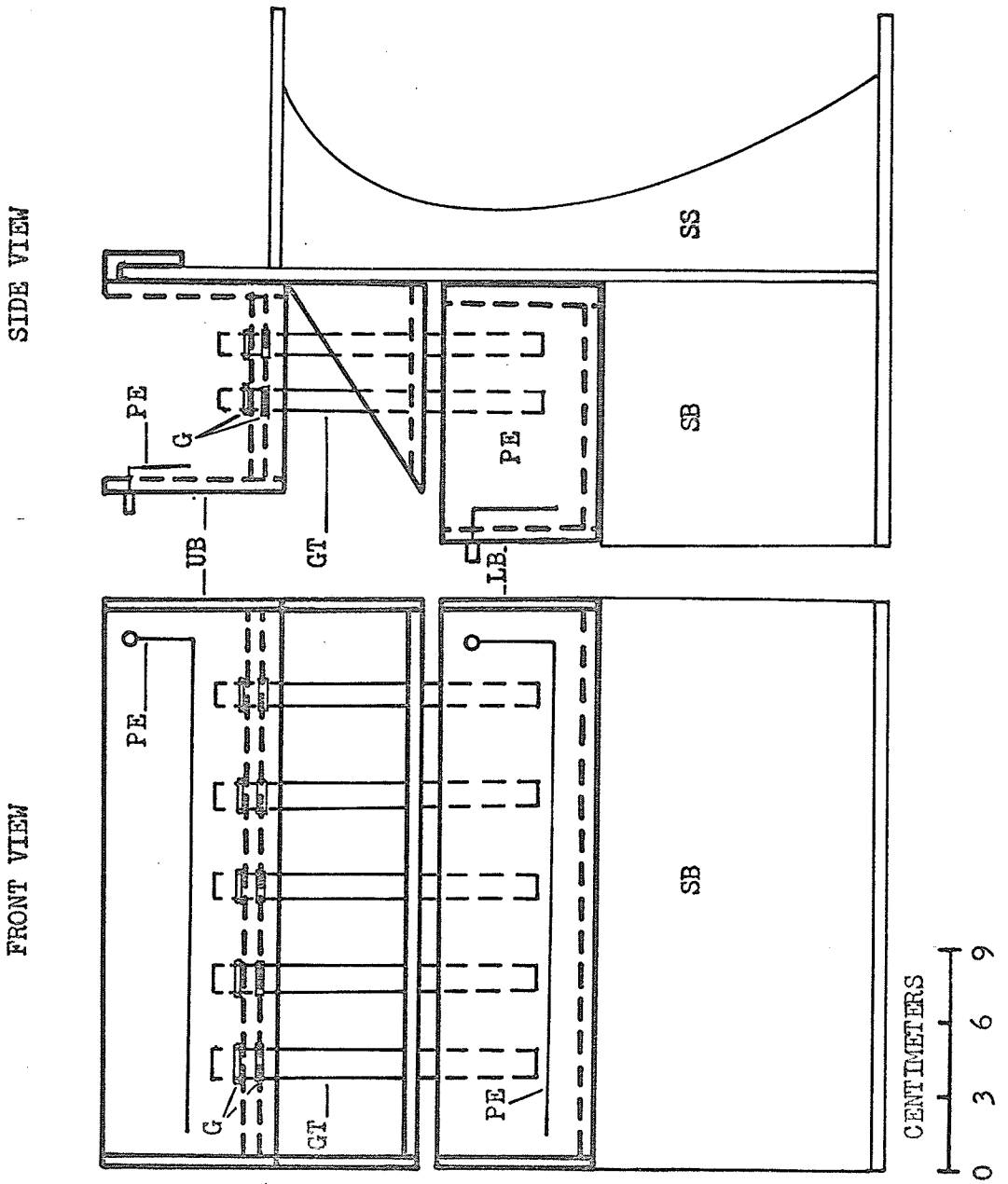
The unstained strips were sectioned at 0.5-cm intervals and each section counted for one minute. The radioactivity was expressed as a percentage of a standard prepared by applying to each of two 1.0 x 3.5-cm sections of Whatman number one chromatography paper a sample identical to that placed on each strip. The radioactivity thus recorded was plotted as described for cellulose polyacetate.

11. Acrylamide Gel Electrophoresis

Front and side elevations of the acrylamide gel electrophoresis equipment are shown in Figure 5. The stand and lower electrode chamber were supplied as part of a vertical starch-gel electrophoresis system (Buchler Instruments, Inc. Fort Lee, N. J.). The upper electrode chamber was constructed from Plexiglass sheeting (0.6-cm thickness). Two sections of Plexiglass were sandwiched together to form the base of the upper electrode chamber. Ten matching holes (diameter, 1.11 cm) were drilled through each of the Plexiglass sections. The holes of sides in apposition were appropriately countersunk to permit the positioning of synthetic rubber, corrosion-resisting grommets (1.0 cm inside diameter, 1.4 cm outside diameter). Each electrode chamber was supplied with a platinum electrode.

Glass tubing (0.8 cm inside diameter, 1.1 cm outside diameter) was cut into 13.8-cm lengths and the ends bevelled with a grinding stone. A number of tubes were then sealed on one end (bottom) by insertion into red rubber Vacutainer caps (Becton, Dickinson and Co., Parsippany, N. J.). Each tube was placed in an upright position and 12 ml of water pipeted into the interior. Ten tubes were selected in which the heights of the

Figure 5. Front and side elevations of acrylamide gel electrophoresis equipment (G, grommets; GT, glass tube; LB, lower buffer chamber; PE, platinum electrode; SB, support block; SS, support stand; UB, upper buffer chamber).


SIDE VIEW
FRONT VIEW
SS
SB
SB
PE
G
UB
GT
LB
PE
PE
G
GT
PE
CENTIMETERS
0 3 6 9

menisci coincided. These tubes were diamond-marked at the base of the menisci and used as a matched set of tubes for acrylamide electrophoresis.

Prior to use the glass tubes were thoroughly cleaned and rinsed in a 1:200 (v/v) aqueous solution of Photoflo (Canadian Kodak Co.). After oven drying at 90 C one end of each tube was sealed by insertion into a Vacutainer cap. The uncapped end of each tube was then pushed through the grommets in the base of the upper electrode chamber. A thin film of siliconized stopcock grease (Silicone Lubricant, Dow Corning Corp., Midland, Mich.) was applied around that portion of the tubes situated in the electrode chamber and another grommet slipped over each unsealed end. The whole system was placed inside a refrigerator and cooled to 5 C. Using a 10-ml continuous pipetor (automatic syringe), individual tubes were filled to the diamond mark with acrylamide gel solution (Table VII). Immediately thereafter 0.2 ml of water was layered on top of the gel solution in each tube with a length of polyethylene tubing forced over the needle on a disposable 1-ml tuberculin syringe. The barrel of the tuberculin syringe was in turn connected to a micro-pipet filler (Adams Suction Apparatus, Clay Adams Division of Becton, Dickinson and Co., Parsippany, N. J.) by a 2.5-cm section of rubber tubing.

After gelation the apparatus was removed from the refrigerator and the water layer poured off the gels. Any remaining water was removed by lightly touching the gel surface with a piece of absorbent tissue. The Vacutainer caps were removed and the top of each gel aligned with the upper edge of the electrode chamber base. An inverted test tube was used to push the rubber grommets into contact with the floor of the electrode chamber. This seal prevented leakage from the upper buffer chamber.

TABLE VII

ACRYLAMIDE GEL COMPOSITION

| CONSTITUENT | CONCENTRATION[a] | VOLUME (ml) |
|---|---|---|
| Acrylamide Monomer* | 15.0% | 75 |
| N,N' methylenebisacrylamide* | 2.2% | 13 |
| Water | | 19 |
| Tetramethylenediamine* | 0.28% | 19 |
| Buffer (concentration of buffer constituents given in Table XIV is the concentration in 150 ml, i.e., the final volume) | | 19 |
| Ammonium persulfate[b] | 12.00% | 1 |
| | GEL TOTAL VOLUME..... | 150 |

*Eastman Chemicals.

[a]Concentration given is the concentration in a prepared solution which was mixed with other constituents as indicated to a total volume of 150 ml.

[b]Catalyst solidifying gel. Added just prior to pouring gels.

Data similar to that given by Nerenberg (1966).

Buffer was first layered on the gel surfaces in the glass tubes. This prevented air-bubble formation between the gel surface and the open end of the tubes. Upper and lower buffer chambers were each filled with 500 ml of buffer and the apparatus returned to the refrigerator.

A separate sample was prepared for application to each gel. This was done by placing a 2-ml disposable beaker (Dispo Beaker, Canadian Laboratory Supplies) with a cone-shaped bottom into the open end of a 13 x 1.1-cm test tube. A ridge around the outer edge of the beaker prevented total entry into the test tube. Saturated aqueous sucrose solution, followed by a plasma-radioactive-hormone mixture or radioactive hormone was added to each beaker. A crystal of bromphenol blue (marker dye) was placed in beakers containing plasma samples for application to gels that would eventually be stained. No such marker dye was added to samples destined for application to gels used in localizing radioactivity as it was feared that this dye might interfere with formation of the protein-hormone complex (normal procedure for any particular electrophoretic run was to stain half of the total number of gels for protein fraction localization and to section half for radioactivity localization). Individual beakers were covered with Parafilm drawn down over the upper end of the test tube. The sucrose and plasma-hormone or sucrose and hormone components were mixed by placing the test tube in a Vortex mixer (Delux Mixer Scientific Products).

Individual samples were drawn into glass tubes (0.4 cm inside diameter, 0.5 cm outside diameter) pulled to a fine point (12 cm total length). Each tube in turn was connected to an Accropet pipet filler (Manostat Corporation) by a length of polyethylene tubing. The fine point

of the glass tube was gently slipped through the surface of the buffer in the upper chamber and applied against the inner wall of the gel tube. The sample was then slowly released onto the upper surface of the gel.

Electrophoresis was carried out with constant current at 10 C, in total darkness. The duration of the run and voltage variations are given in Table XV.

Electrophoresis continued until the marker dyes had reached a predetermined point 9 cm from the origin. All of the gels were then removed from the tubes by ringing the end of each tube between the gel and the tube wall with a 20-gauge hypodermic needle connected to the outer casing of a disposable tuberculin syringe. The syringe was connected to running tap water by a length of rubber tubing.

Those gels destined for staining were immediately transferred to test tubes (15 x 1.5 cm) containing a solution of 12.5% trichloroacetic acid (w/v) and 0.04% Coomassie blue (w/v). After staining for 15 hours the gels were individually destained in two changes (one hour each) of 5% acetic acid:ethanol (9:3, v/v). Gels tended to take up the staining solution and thereby increase in diameter and length. This particular destaining procedure returned the gels to their original size. Prior to scanning, each gel was submerged in a 1% Photoflo solution and returned to its original glass tube. Tubes containing the gels were scanned at 575 mμ using a carrier similar to, but larger than, the one normally supplied with the Gelscan unit for scanning disc electrophoretic separations. Scanner settings used were: defining slit, 0.05 cm; scan width, 0.7 cm; scan speed, fast; chart speed, 200 mm/min; millivolt setting, 100. The resulting electrophoretograms showed a protein fraction displacement 2.35 times that of the gel.

Gels to be sectioned for radioactivity determinations were placed individually on a slicing platform consisting of two pocket combs (length, 13 cm) aligned parallel to each other with a wooden block (0.9 x 1.0 x 13 cm) glued between. The gel position was secured by inserting thin plastic strips between the teeth of the combs at either end of the gel. The slicing platform had been previously marked at 0.5-cm intervals. A stainless steel razor blade periodically dipped in concentrated Photoflo was used to section each gel into 0.5-cm segments. Individual sections were placed in glass counting tubes and counted for one minute. Radioactivity per section was expressed as a percentage of the radioactivity applied to the gel by counting duplicate standards of the applied amount in 0.86 ml of water. This volume corresponded in geometry to that of the gel segments. Results obtained for corresponding sections of replicate gels were averaged and radioactivity in excess of one per cent of the standard plotted in relation to the median densitometric scan. Values of less than one per cent were plotted only if consistently occurring in a specific migratory position or between radioactive peaks.

12. Verification of Hormone Binding to Specific Plasma Protein Fractions in Acrylamide Gel

The binding of T3* and T4* to specific plasma protein fractions in the gel was verified by equilibrium dialysis. Fish plasma-T3* mixture or fish plasma-T4* mixture (both at 0.05 μg/ml) was applied to each of four acrylamide gels. At the same time samples of free T3* or free T4* in aqueous saturated sucrose solution (controls, 0.05 μg/ml) were applied to another four gels in the same electrophoretic system. Electrophoresis

was conducted using the tris-HCl-glycine buffer described in Table XIV, according to conditions described in Table XV. Following electrophoresis the gels were cut into 0.5-cm sections. The central 1-cm section was taken from the major radioactive peak of each gel and placed inside individual 10-cm lengths of dialysis tubing (diameter 2.0 cm; Union Carbide). This tubing was treated identical to and had the same composition as that previously described (see, Thyronine-Binding by Equilibrium Dialysis). Buffer identical to that used in the electrophoretic gel was placed inside (5 ml) and outside (20 ml) the dialysis membrane. The outer container for each dialysis bag consisted of a 50-ml siliconized glass beaker. These beakers were sealed with Parafilm and incubated at 10 C, with constant agitation, in total darkness, for 40 hours in the case of fish plasma or corresponding controls. Equilibrium was established within 24 hours. At the end of this period the radioactivity remaining in the gel was determined by addition of each segment to a test tube (14 x 1.4 cm) and counting to a total of 1000 counts. Duplicate samples were taken from the solutions inside and outside the dialysis tubing, placed in flat-bottomed counting tubes, and counted to 1000 counts. The radioactivity inside (including that in the gel segments) and outside the dialysis membrane was then converted to counts per minute (CPM) per millilitre and the following dialysis index calculated.

$$\text{Dialysis Index} = \frac{\text{CPM/ml Inside} - \text{CPM/ml Outside}}{\text{CPM/ml Inside}}$$

The higher the index the greater has been the retention of radioactivity (radiothyronine) within the dialysis bag. It was assumed that if the proteins in the bag were binding radiothyronines this would be reflected

in a dialysis index higher than control sections where no proteins were present. The objective of this experiment was to determine if greater retention of radioactivity occurred when proteins were present in the gel. It was not used to obtain an exact quantitative measure of thyronine binding by a particular plasma protein fraction.

13. Injection Procedures

Immediately prior to injection the animals were anaesthetized in MS-222 (0.05 g/liter). The radioactive material (T3*, T4* in 50% propylene glycol or $Na^{125}I$ in aqueous solution) was injected in a volume of 0.1 ml using a one-inch, 26-gauge needle attached to a 1-ml tuberculin syringe. The tip of the needle was inserted parallel to and just below the lateral line at a point corresponding to the anterior edge of the dorsal fin. The injection was intraperitoneal.

# RESULTS

## 1. Total Protein

Total protein concentrations for the pooled plasma samples are given in Table VIII. Bartlett's Test (Snedecor and Cochoran, 1967) for homogeneity of variance resulted in acceptance of the null hypothesis that the variances were equal ($P > 0.5$). A one-way analysis of variance gave a significant F value (137.39; $P < 0.01$) showing that some of the means differed. Tukey's Fixed Range Test (Snedecor and Cochoran, 1967) indicated those means which differed.

## 2. Haematocrits

Mean haematocrit values obtained for the different fish groups are given in Table VIII. Although small differences do appear between the groups they show little relationship to the total protein concentration.

## 3. Plasma Thyronine Levels

Bio-Science Laboratories (Van Nuys, California) determined the plasma thyronine concentrations using the thyroxine-by-column method. Results obtained for the three subsamples of the single plasma pool were: 0.8, 0.9 and 0.5 µg/100 ml (mean: 0.73 µg/100 ml). These values were approximately one-tenth that normally found for human plasma.

## 4. Variation in Trichloroacetic Acid Protein-Precipitation Method

A series of experiments was conducted to determine the most reliable method of precipitating protein-bound radioiodine using trichloroacetic acid. The variations in the method already outlined in

TABLE VIII

HAEMATOCRIT AND TOTAL PROTEIN VALUES
FOR POOLED PLASMA SAMPLES

| FISH GROUP | MEAN HAEMATOCRIT % | MEAN TOTAL PROTEIN g/100 ml | EQUALITY LINES AT FIVE PER CENT LEVEL[a] (Total Protein) |
|---|---|---|---|
| 4 | 40.8 ± 1.7[b] | 3.68 ± 0.11[c] | |
| 5 | 40.7 ± 2.6 | 3.77 ± 0.13 | |
| 1 | 39.4 ± 2.3 | 3.83 ± 0.17 | |
| 2 | 40.0 ± 2.6 | 3.86 ± 0.26 | |
| 10 | 40.2 ± 4.4 | 4.28 ± 0.29 | |
| 11 | 37.9 ± 3.6 | 4.70 ± 0.28 | |
| 6 | 35.5 ± 2.7 | 4.72 ± 0.19 | |
| 3 | 39.2 ± 2.5 | 5.00 ± 0.30 | |
| 8 | 42.1 ± 2.6 | 5.77 ± 0.23 | |
| 12 | 39.1 ± 3.8 | 5.78 ± 0.32 | |
| 9 | 48.4 ± 4.0 | 6.27 ± 0.28 | |
| 7 | 40.9 ± 3.6 | 6.31 ± 0.20 | |

Bartlett's Test for Homogeneity of Variances (Total Protein)

Chi square calculated . . . . . 7.74 ($P > 0.5$)
Degrees freedom . . . . . 11

One-Way ANOVA Summary (Total Protein)

| | | |
|---|---|---|
| Treatment Sum of Squares . . . . . | 53.444 | (degrees freedom = 11) |
| Error Sum of Squares . . . . . | 1.715 | (degrees freedom = 55) |
| Total Sum of Squares . . . . . | 55.159 | |
| Mean Square Treatment . . . . . | 5.344 | |
| Mean Square Error . . . . . | 0.038 | |
| F calculated . . . . . | 137.38 | ($P < 0.01$) |

[a]Any two means not within the boundary of the same vertical line are significantly different at the 5% level.

[b]Ninety-five per cent confidence interval: mean based on values from 10 fish.

[c]Ninety-five per cent confidence interval: mean based on 5 replicates from each plasma pool.

Figure 4 (method III) are summarized in Table IX. Using these methods the fraction of the total radioactivity bound to plasma proteins was determined for 8 replicates of both fish and human plasma incubated with T4* (0.05 μg/ml; radioiodide content 5% of total radioactivity) at temperatures of 10, 20 and 36 C. The effect of either one or two washes with 2.5% trichloroacetic acid was examined in the 20-C group.

A factoral analysis (Snedecor and Cochoran, 1967) was conducted on the results obtained when the precipitate was washed once with 2.5% trichloroacetic acid (Tables X and XI). Here, the degree of binding has been reported as a fraction of the total radioactivity rather than a percentage. This was necessary to facilitate the statistical analysis. The data were tested for homogeneity of variances by use of Bartlett's Test. The chi square calculated was 93.96 ($P<0.005$); therefore, the null hypothesis that the variances were equal was rejected. A transformation for the data was obtained using Taylor's Power Law as outlined by Southwood (1966). The transformation was accomplished by the conversion of each value to the tenth power. Bartlett's Test was performed on the transformed data and the null hypothesis for homogeneity of variances accepted (chi square calculated = 4.08; $P>0.995$).

All the main factors, incubation temperature, precipitation technique and blood type (i.e., species) had a significant effect on the results ($P<0.01$). There was interaction between incubation temperature and precipitation technique, and incubation temperature and blood type. Interaction was also apparent between all the main factors. No interaction was observed between precipitation technique and blood type.

TABLE IX

VARIATION IN TRICHLOROACETIC ACID PROTEIN-PRECIPITATION METHOD

| METHOD | VOLUME OF INITIAL 12.5% TCA PRECIPITANT (ml) | CENTRIFUGATION x g | VOLUME OF 2.5% TCA WASH (ml) | NUMBER OF 2.5% TCA WASHES | VOLUME OF 0.1 N NaOH TO DISSOLVE PPT (ml) | VOLUME OF 0.1 N NaOH TO WASH CENTRIFUGE TUBE (ml) |
|---|---|---|---|---|---|---|
| I | 2 | 1800 | 2 | 1 | 2 | 2 |
| 2 WASHES* | 2 | 1800 | 2 | 2 | 2 | 2 |
| II | 2 | 4500 | 2 | 1 | 2 | 2 |
| 2 WASHES* | 2 | 4500 | 2 | 2 | 2 | 2 |
| III | 0.5 | 15000 | 0.5 | 1 | 0.5 | 0.5 |
| 2 WASHES | 0.5 | 15000 | 0.5 | 2 | 0.5 | 0.5 |

Volume used for counting in all cases was 4 ml. If necessary, water was added to counting tube to obtain this volume. Total minimum count 1000.

*Four-ml volume of supernatant counted and count/min/6 ml obtained by: count/min for 4 ml x 1.5.

TABLE X

SUMMARY OF TRICHLOROACETIC ACID PROTEIN-PRECIPITATION ANALYSIS

| INCUBATION TEMPERATURE | FRACTION OF TOTAL RADIOACTIVITY ASSOCIATED WITH THE PLASMA PROTEINS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PRECIPITATION TECHNIQUE I | | | | PRECIPITATION TECHNIQUE II | | | | PRECIPITATION TECHNIQUE III | | | |
| | HUMAN | | FISH | | HUMAN | | FISH | | HUMAN | | FISH | |
| | NUMBER OF WASHES | | | | NUMBER OF WASHES | | | | NUMBER OF WASHES | | | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 10 C | 0.736 | | 0.732 | | 0.878 | | 0.868 | | 0.922 | | 0.928 | |
| | 0.756 | | 0.757 | | 0.884 | | 0.884 | | 0.932 | | 0.948 | |
| | 0.772 | | 0.777 | | 0.889 | | 0.898 | | 0.940 | | 0.941 | |
| 20 C | 0.737 | 0.678 | 0.690 | 0.603 | 0.825 | 0.770 | 0.844 | 0.762 | 0.932 | 0.910 | 0.931 | 0.920 |
| | 0.781 | 0.697 | 0.741 | 0.642 | 0.845 | 0.806 | 0.874 | 0.814 | 0.939 | 0.924 | 0.940 | 0.930 |
| | 0.810 | 0.713 | 0.771 | 0.668 | 0.861 | 0.832 | 0.897 | 0.846 | 0.945 | 0.935 | 0.946 | 0.940 |
| 36 C | 0.752 | | 0.646 | | 0.872 | | 0.647 | | 0.936 | | 0.922 | |
| | 0.758 | | 0.687 | | 0.882 | | 0.665 | | 0.942 | | 0.930 | |
| | 0.764 | | 0.715 | | 0.891 | | 0.679 | | 0.948 | | 0.938 | |

Each group of three values represents the mean (middle value), and upper and lower limits of the 95% confidence interval. In obtaining these values the raw data were first converted to the tenth power (transformation required for homogeneity of variances in factorial analysis). Means and confidence intervals were computed for the transformed values. The means and confidence intervals were then transformed back and recorded as above. Precipitation technique III (1 wash) was the method of choice because the results showed little variability and close agreement with the 5% inorganic radioiodide content determined by chromatography of the T4* sample ( i.e., all organic radioiodide was bound to plasma proteins ).

TABLE XI

SUMMARY OF FACTORIAL ANALYSIS FOR PLASMA PROTEIN PRECIPITATION

| SOURCE OF VARIATION | DF* | SUM OF SQUARES | MEAN SQUARE | "F" CALCULATED | |
|---|---|---|---|---|---|
| Replications | 7 | 0.0116 | | | |
| Treatments | 17 | 5.7225 | 0.3366 | 181.95 | P <0.01 |
| Temperature | 2 | 0.0590 | 0.0295 | 15.95 | P <0.01 |
| Precipitation Technique | 2 | 5.2146 | 2.6073 | 1409.35 | P <0.01 |
| Blood Type (Human or Fish) | 1 | 0.0430 | 0.0430 | 23.26 | P <0.01 |
| Temperature-Precipitation | 4 | 0.1109 | 0.0277 | 14.99 | P <0.01 |
| Temperature-Blood | 2 | 0.1433 | 0.0716 | 38.72 | P <0.01 |
| Precipitation-Blood | 2 | 0.0159 | 0.0079 | 4.29 | P >0.05 |
| Temp-Pptn-Bld | 4 | 0.2490 | 0.0622 | 33.64 | P <0.01 |
| Error | 119 | 0.2209 | 0.0018 | | |

*Degrees freedom

As a result of this experiment, precipitation technique III (1 wash) was selected as the most reliable of those examined. This method was routinely used for estimating thyronine-binding by plasma proteins.

5. Comparison of Radiothyronine-Plasma-Protein Binding by Dialysis and Precipitation

The reliability of the trichloroacetic acid precipitation method for assessing binding of thyronines was checked by equilibrium dialysis. T3* or T4*, at various specific activities, was incubated with fish plasma at 10 C to provide added hormone concentrations of 0.05, 0.275, 0.5, 2.75 and 5.0 µg/ml. At each concentration four replicate determinations were made with the protein precipitation technique and four with the equilibrium dialysis method.

Duplicate samples of T3* and T4* were analyzed by paper chromatography. Less than 1% of the radioactivity in T4* was inorganic. However, 11.2% of the T3* radioactivity was inorganic and a correction for this high inorganic radioiodide content was necessary.

The binding of iodide to the plasma proteins of the brook trout was estimated by substituting $Na^{125}I$ (0.10 µCi) for the radioactive hormone in the dialysis procedure previously described. The mean of four replicates indicated that 90.92% of the radioiodide was associated with the plasma proteins. This compared well with results obtained by Huang and Hickman (1968) on Salmo gairdneri. Each percentage of hormone bound to plasma proteins in the T3* precipitation analysis was increased by a factor of 0.909 x 11.15 (per cent $^{125}I^-$ bound to plasma protein/100 x per

cent $^{125}I^-$ present in the T3* sample). This permitted a comparison of precipitation and dialysis results.

The results are summarized in Table XII. A one-way analysis of variance was conducted to evaluate the effect of added-hormone concentration within each precipitation or dialysis group. Bartlett's Test was used to assess the homogeneity of variances within each precipitation or dialysis group. No effect of added hormone concentration was apparent, but in each case the precipitation technique gave a lower protein-bound percentage.

6. Electrophoresis

To identify the fish plasma protein fractions binding thyroid hormones and to analyze the effect of hormone concentration on this phenomenon, plasma-protein-radiohormone mixtures were electrophoresed on several media in combination with different buffer systems.

Thyronine-binding properties of fish and human plasma proteins were compared. T3* and T4* in aqueous saturated sucrose solution or $^{125}I^-$ in aqueous solution were separately electrophoresed in the absence of plasma proteins to establish the location of the unbound component. In all systems any radioiodide present migrated ahead of the plasma proteins. Table XIII provides an index for the electrophoretic analyses. Buffers used in the various systems are described in Table XIV, and the conditions of electrophoresis are outlined in Table XV.

A photograph and densitometric scan of a representative electrophoretic separation of fish plasma proteins on acrylamide gel are provided in Figure 6.

TABLE XII

SUMMARY OF DIALYSIS-PROTEIN PRECIPITATION STUDY OF THYRONINE-BINDING TO TROUT PLASMA PROTEINS

| HORMONE CONC ug/ml | T4* % BOUND (TCA PPTN) | T4* % BOUND (DIALYSIS) | T3* % BOUND (TCA PPTN) | T3* % BOUND (DIALYSIS) |
|---|---|---|---|---|
| 0.05 | 95.33±0.65[a] | 98.56±1.43 | 96.36±2.70 | 99.67±0.13 |
| 0.275 | 94.65±1.46 | 99.35±0.71 | 94.95±1.69 | 99.56±0.04 |
| 0.5 | 94.45±1.56 | 99.11±0.80 | 94.70±0.91 | 99.57±0.08 |
| 2.75 | 94.75±0.71 | 99.16±0.97 | 95.41±0.80 | 99.57±0.12 |
| 5.0 | 94.20±1.55 | 99.18±0.82 | 94.15±1.55 | 99.58±0.09 |

Bartlett's Test for Homogeneity of Variances Within Precipitation or Dialysis Groups

| | | | | |
|---|---|---|---|---|
| Chi Square Calculated..... | 3.45 | 1.80 | 4.95 | 3.16 |
| Degrees Freedom | 4 | 4 | 4 | 4 |
| | (P > 0.500) | (P > 0.750) | (P > 0.250) | (P > 0.500) |

Summary of One-Way ANOVA Within Precipitation or Dialysis Groups

| | | | | |
|---|---|---|---|---|
| TSS (df=4) | 2.831 | 1.426 | 11.088 | 0.008 |
| ESS (df=16) | 9.352 | 5.682 | 16.596 | 0.037 |
| T'SS | 12.183 | 7.107 | 27.685 | 0.046 |
| MST | 0.708 | 0.356 | 2.772 | 0.002 |
| MSE | 0.623 | 0.379 | 1.106 | 0.002 |
| F | 1.135 | 0.941 | 2.505 | 0.833 |
| | (NS, 0.01) | (NS, 0.01) | (NS, 0.01) | (NS, 0.01) |

[a]Ninety-five per cent confidence interval about the mean.

Abbreviations Used:

TSS . . . . . treatment sum of squares
ESS . . . . . error sum of squares
T'SS . . . . . total sum of squares
MST . . . . . mean square treatment
MSE . . . . . mean square error
df . . . . . degrees freedom
NS . . . . . not significant at level indicated.

TABLE XIII

INDEX OF ELECTROPHORETIC ANALYSIS

| HORMONE | MEDIUM | PLASMA | HORMONE CONC (μg/ml) | FIGURE | BUFFER | pH | # STRIPS/ RUN | # STRIPS STAINED/ RUN | # STRIPS SECTIONED/ RUN |
|---|---|---|---|---|---|---|---|---|---|
| T3* | Cellulose poly-acetate | Human | 0.05 | 7a | T-E-B | 8.8 | 8 | 8 | 8 |
| | | Fish | 0.05 | 7b | T-E-B | | 8 | 8 | 8 |
| | Paper | Human | 0.05 | 8a | T-H | 7.4 | In each case five strips had plasma + T3*: one was stained and four sectioned. One strip had T3* and was sectioned. | | |
| | | Fish | 0.05 | 8b | T-H | 7.4 | | | |
| | | Human | 0.05 | 9a | T-H | 8.8 | | | |
| | | Fish | 0.05 | 9b | T-H | 8.8 | | | |
| | | Human | 0.05 | 10a | T-E-B | 8.8 | | | |
| | | Fish | 0.05 | 10b | T-E-B | 8.8 | | | |
| | Acrylamide Gel | Human | 0.05 | 11a | T-H | 8.8 | One run with a total of ten gels. Seven gels had fish plasma + T3*: three were stained and four sectioned. Two gels had human plasma: one was stained and one sectioned. One gel had T3* with no plasma and was sectioned. | | |
| | | Fish | 0.05 | 11b | T-H | 8.8 | | | |
| | | Human | 0.05 | 12a | T-H-G | 8.1 | 10 | 5 | 5 |
| | | Fish | 0.05 | 12b | T-H-G | 8.1 | 10 | 5 | 5 |
| | | Fish | 5.00 | 12c | T-H-G | 8.1 | 10 | 5 | 5 |
| T4* | Cellulose poly-acetate | Human | 0.05 | 13a | T-E-B | 8.8 | 8 | 4[a] | 8 |
| | | Fish | 0.05 | 13b | T-E-B | 8.8 | 8 | 4[a] | 8 |
| | | Fish | 0.50 | 13c | T-E-B | 8.8 | 8 | 4[a] | 8 |
| | | Fish | 5.00 | 13d | T-E-B | 8.8 | 8 | 4[a] | 8 |

TABLE XIII (continued)

| HORMONE | MEDIUM | PLASMA | HORMONE CONC (μg/ml) | FIGURE | BUFFER | pH | # STRIPS/ RUN | # STRIPS STAINED/ RUN | # STRIPS SECTIONED/ RUN |
|---|---|---|---|---|---|---|---|---|---|
| T4* | Paper | Human | 0.05 | 14a | T-H | 7.4 | In each case five strips had plasma + T4*: one was stained and four sectioned. One strip had T4* with no plasma and was sectioned. | | |
| | | Fish | 0.05 | 14b | T-H | 7.4 | | | |
| | | Human | 0.05 | 15a | T-H | 8.8 | | | |
| | | Fish | 0.05 | 15b | T-H | 8.8 | | | |
| | Acrylamide Gel | Human | 0.05 | 16a | T-H | 8.8 | One run with a total of ten gels. Seven gels had fish plasma + T4*: three were stained and four sectioned. One gel had T4* with no plasma and was sectioned. | | |
| | | Fish | 0.05 | 16b | T-H | 8.8 | | | |
| | | Human | 0.05 | 17a | T-H-G | 8.1 | 8 | 4 | 4 |
| | | Fish | 0.05 | 17b | T-H-G | 8.1 | 8 | 4 | 4 |
| | | Fish | 0.50 | 17c | T-H-G | 8.1 | 8 | 4 | 4 |
| | | Fish | 5.00 | 17d | T-H-G | 8.1 | 8 | 4 | 4 |

Abbreviations used: T-E-B, tris-EDTA-borate buffer; T-H, tris-HCl buffer; T-H-G, tris-HCl-glycine buffer; Tris, tris (hydroxymethyl) amino methane; EDTA, ethylene diamine tetraacetic acid (as free acid not sodium salt).

[a]Eight strips were used in each run. Of the eight strips four had T4* + plasma, and all were stained and sectioned. The remaining four strips had T4* with no plasma and all were sectioned.

TABLE XIV

BUFFERS USED FOR ELECTROPHORESIS

| BUFFER | PH | CONSTITUENTS | CONCENTRATION OF CONSTITUENTS |
|---|---|---|---|
| TRIS-HCL | 8.8 | TRIS | 0.279 M |
| | | HCL | 0.050 M |
| TRIS-HCL | 7.4 | TRIS | 0.059 M |
| | | HCL | 0.050 M |
| TRIS-EDTA-BORATE | 8.8 | TRIS | 0.420 M |
| | | EDTA | 0.020 M |
| | | BORIC ACID | 0.107 M |
| TRIS-HCL-GLYCINE (gel buffer) | 8.1 | TRIS | 0.0025 M |
| | | GLYCINE | 0.0316 M |
| (electrode buffer) | 7.9 | TRIS | 0.020 M |
| | | HCL | 0.005 M |
| | | GLYCINE | 0.250 M |

TRIS: tris (hydroxymethyl) amino methane

EDTA: ethylenediamine tetraacetic acid (as free acid, not sodium salt)

Preparation temperature of buffer solutions: 10 C

TABLE XV

CONDITIONS OF ELECTROPHORESIS FOR VARIOUS SYSTEMS

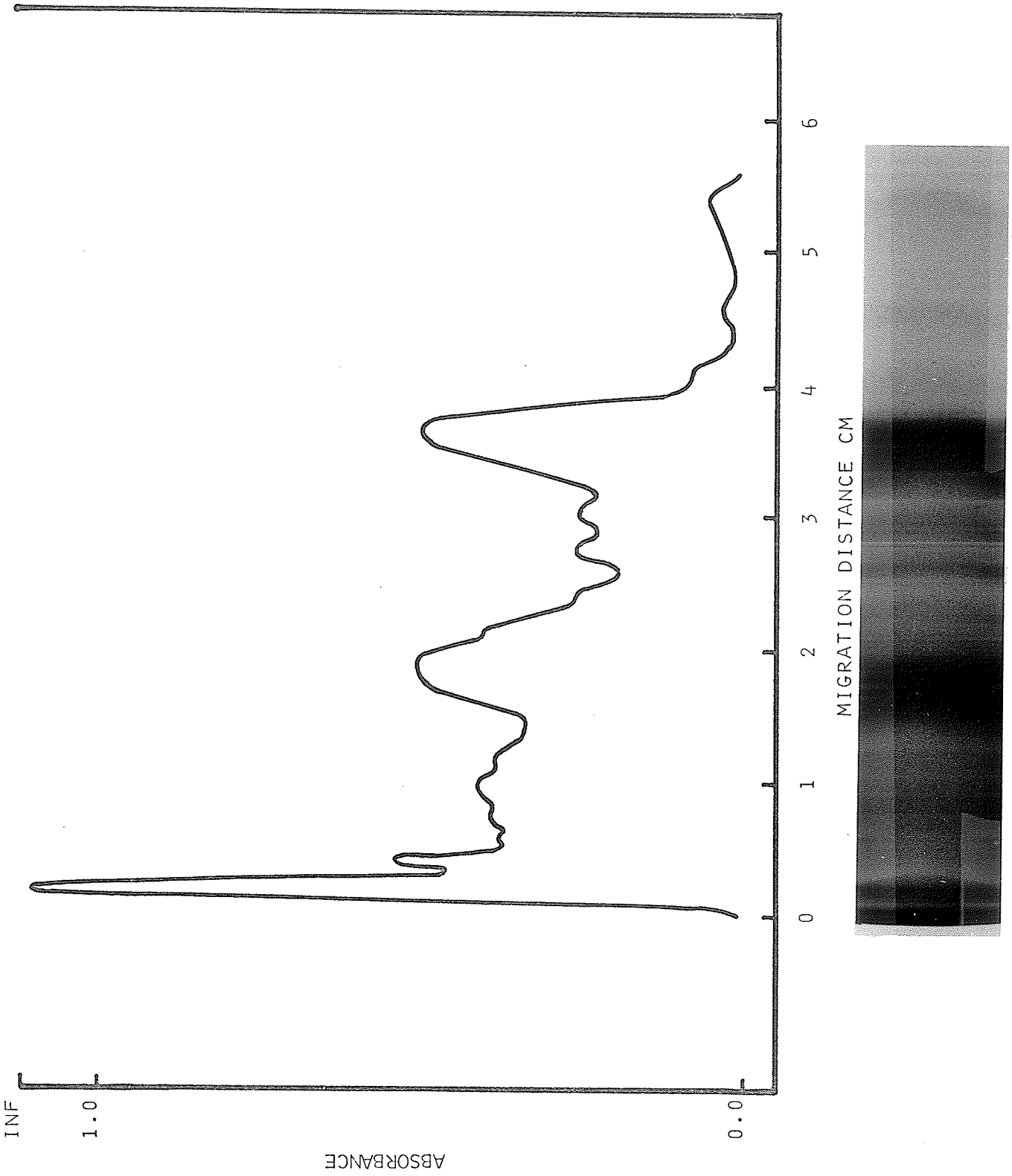
| MEDIUM | BUFFER | PH | START MA | START VOLTS | FINISH MA | FINISH VOLTS | ELECTROPHORESIS HOURS |
|---|---|---|---|---|---|---|---|
| Cellulose Polyacetate | T-E-B | 8.8 | 10.0 | 275 | 10.0 | 315 | 2.00 |
| Paper | T-H | 7.4 | 7.0 | 150 | 7.1 | 150 | 6.50 |
| | T-H | 8.8 | 6.0 | 150 | 6.2 | 150 | 6.50 |
| | T-E-B | 8.8 | 5.0 | 150 | 5.2 | 150 | 10.00 |
| Acrylamide Gel | T-H | 8.8 | 5.0[a] | 50 | 5.0 | 80 | 16.00 |
| | T-H-G | 8.1 | 3.5[a] | 200 | 3.5 | 400 | 1.75 |

Abbreviations used:
T-H . . . . . TRIS-HCL
T-E-B . . . . TRIS-EDTA-BORATE
T-H-G . . . . TRIS-HCL-GLYCINE
TRIS . . . . tris (hydroxymethyl) amino methane
EDTA . . . . ethylenediamine tetraacetic acid (as free acid not sodium salt).

Milliamperes and voltages unless otherwise specified represent totals for the system. Replicates given in Table XIII.

[a]Milliamperes per gel.

Figure 6. Photograph and densitometric scan of representative electrophoretic separation of fish plasma proteins on acrylamide gel. Tris-HCL-glycine buffer, pH 8.1.


INF
1.0
0.0
ABSORBANCE
0
1
2
3
4
5
6
MIGRATION DISTANCE CM

a. Triiodothyronine

1) Cellulose Polyacetate. The radioactivity remained near the origin when either fish or human plasma-T3* mixture was examined (Figure 7a,b). No association with particular protein bands was observed, but a comparison of results obtained in the presence and absence of plasma indicated displacement of T3* toward the anode in the former case. This displacement was greater with human plasma. This suggests that T3* has a moderate affinity for certain fish plasma protein fractions, a greater affinity for certain human plasma protein fractions, and an even greater affinity for cellulose polyacetate.

2) Paper. Paper electrophoresis of human plasma-T3* mixture at pH 7.4 (Figure 8a) indicated that most radioactivity corresponded to thyroxine-binding globulin. Some radioactivity occurred in the beta globulin-fibrinogen region and a smaller amount in the albumin region. At pH 8.8 (Figures 9a, 10a) most radioactivity was in the thyroxine-binding globulin area while lesser amounts appeared with albumin, fibrinogen and gamma globulin fractions. Free T3* (electrophoresed in the absence of plasma) remained on the origin at pH 7.4 and in the beta to gamma globulin regions at pH 8.8. Different buffer systems at the same pH (Figures 9a, 10a) altered the radioactivity of the albumin region. At pH 8.8 the tris-EDTA-borate buffer system had a greater proportion of hormone associated with the albumin fraction.

Electrophoresis performed on fish plasma-T3* mixture at pH 7.4 (Figure 8b) resulted in a broad band of radioactivity from albumin-like to gamma-globulin-like fractions. At pH 8.8 (Figures 9b, 10b) the radioactivity was located in a region extending from albumin-like through to

gamma-globulin-like fractions. In this latter instance, the main radioactive peak appeared in the postalbumin-like area regardless of the buffer system used. A smaller trailing peak was located in a position identical to that of hormone electrophoresed in the absence of plasma.

In summary, paper electrophoresis of human plasma protein-T3* mixture always resulted in a major peak of radioactivity in the thyroxine-binding globulin region. The distribution of radioactivity in albumin and fibrinogen to gamma globulin regions was influenced by the pH and composition of the buffer. The location of radioactivity after paper electrophoresis of fish plasma-T3* mixture indicated binding, but the distribution of radioactivity made it impossible to assign binding properties to any specific plasma protein fraction. The anodal movement of radioactivity in the presence and absence of plasma exceeded that observed for cellulose polyacetate medium, suggesting a greater affinity of T3* for the medium in the latter case.

3) Acrylamide Gel. Following electrophoresis of human plasma-T3* mixture on acrylamide gel at pH 8.8 and 8.1 the radioactivity was situated primarily in the postalbumin region (Figures 11a, 12a). Small amounts corresponded to albumin and prealbumin fractions. At the lower pH less hormone was associated with prealbumins. Free hormone occurred in the prealbumin position.

At a pH of 8.8 with fish plasma-T3* mixture (Figure 11b) the radioactivity corresponded to a very diffuse, leading prealbumin-like fraction. At a pH of 8.1 (Figures 12b,c) radioactivity was associated with the albumin-like and two slowest prealbumin-like fractions. A small,

but consistent amount of radioactivity appeared with proteins in the beta-globulin-like region. Increasing the added hormone concentration did not alter the position or distribution of radioactivity.

The location of radioactivity after electrophoresis of T3* in the presence of human plasma compared favourably to that found using paper electrophoresis with the greatest proportion of radioactivity in the postalbumin area. The position of radioactivity following electrophoresis of fish plasma-T3* mixture differed considerably from that obtained with paper medium, where the major peak was prealbumin-like, but consistency was exhibited with most of the radioactivity in prealbumin-like regions regardless of the buffer system.

b. Thyroxine

1) Cellulose Polyacetate. When human plasma-T4* mixture was electrophoresed on cellulose polyacetate medium at pH 8.8 (Figure 13a) most of the radioactivity was associated with the thyroxine-binding globulin, albumin and prealbumin proteins. Smaller amounts of radioactivity were found in the beta globulin and gamma globulin positions. Free hormone extended from the origin to the gamma globulin region.

With fish plasma-T4 mixture (Figure 13b) the hormone exhibited an affinity for the plasma proteins. This was indicated by anodal displacement of the radioactivity when plasma was present. However, the radioactivity generally extended from the origin to gamma-globulin-like area thus preventing identification of binding fractions. Increased hormone concentrations simply resulted in a spreading of the radioactive zone.

A comparison of the radioactivity position in the presence and absence of human plasma demonstrates that T4* has a higher affinity for

the plasma proteins or a lower affinity for the medium than does T3*. The increased migration of radioactivity in the presence of human plasma shows that the affinity for the hormone by human plasma proteins exceeds that of fish plasma proteins.

2) Paper. When T4* was electrophoresed with human plasma on paper medium at pH 7.4 (Figure 14a) the radioactivity was found mainly in the thyroxine-binding globulin region, but some corresponded to the albumin fraction. Free T4* remained close to the origin. The location of radioactivity changed at pH 8.8 in which case small amounts were situated in both prealbumin and albumin areas, nevertheless, most remained in the thyroxine-binding globulin region (Figure 15a). At pH 8.8 most radioactivity remained in the thyroxine-binding globulin region, but small amounts appeared in both prealbumin and albumin regions.

Fish plasma-T4* mixture electrophoresed at pH 7.4 and 8.8 (Figures 14b, 15b) suggested association of the hormone with albumin-like through to gamma-globulin-like fractions. At the lower pH most hormone was in the gamma-globulin-like region and a smaller portion occurred as a peak in a postalbumin-like position. With the higher pH, one major peak of radioactivity appeared in the postalbumin-like zone with a possible second peak in the gamma-globulin-like region. Radioactive hormone electrophoresed in the absence of plasma was situated in the beta- to gamma-globulin-like area.

The human plasma-T4* separation resembled that on cellulose polyacetate medium. The greatest proportion of radioactivity was in the thyroxine-binding globulin region, but the distribution varied with pH. Variability in the position of radioactivity following electrophoresis

of fish plasma T4* mixture prevented the description of specific fractions as protein binders, but binding was exhibited by the greater anodal displacement of radioactivity in the presence of plasma.

3) Acrylamide Gel. Human plasma-T4* mixture electrophoresed on acrylamide gel at pH 8.8 and 8.1 (Figures 16a, 17a) resulted in peaks of radioactivity at prealbumin, albumin and postalbumin positions. At pH 8.8 a greater proportion of the radioactivity was found in the post-albumin region and a small portion in the prealbumin region.

When fish plasma-T4* mixture was electrophoresed at pH 8.8 (Figure 16b) the radioactivity was found in a position corresponding to prealbumin-like fractions; however, the prealbumin-like band was so diffuse that assignment of binding properties to specific fractions proved difficult. Also, radioactivity was located in similar regions both in the presence and absence of plasma. At pH 8.1 (Figures 17b,c,d) a sharp radioactive peak was centered over the leading prealbumin-like fraction and extended to the second and third prealbumin-like fractions. A small amount of radioactivity was found in the beta-globulin-like region. The free hormone migrated far in advance of the plasma protein fractions and normally left the gel to enter the anode buffer chamber. Increasing the added hormone concentration hardly altered the binding phenomenon; however, a slight increase of radioactivity occurred in the albumin-like region at the highest hormone concentration.

In summary, electrophoresis of human plasma protein-T4* mixture gave results similar to those described by others (Table XXI): a major peak of radioactivity in the postalbumin zone and minor peaks in pre-albumin and albumin zones; variation in the distribution of radioactivity

according to the buffer system used. With fish plasma-T4* mixture the major radioactive peak corresponded to prealbumin-like fractions regardless of the buffer system used, and minor peaks of radioactivity appeared in the albumin-like and beta-globulin-like areas. Alteration of the added hormone concentration had little effect on binding.

## EXPLANATION OF TERMS AND ABBREVIATIONS USED IN FIGURES 7 TO 17

Recovery. The total amount of radioactivity recovered from electrophoretic strips or gels. This value is expressed as a percentage of the standard. The number of strips or gels used to obtain this is given in brackets.

Iodide. Radioiodide ($^{125}I^-$) present in the original T3* or T4* sample as a percentage of the total radioactivity (mean of two determinations).

Radioactive-Peak Percentages. In each figure the mean and 95% confidence interval are given for the radioactivity in individual peaks. Unless otherwise indicated, the radioactivity is expressed as a percentage of the standard. The radioactivity in a segment is considered to contribute to a peak if the percentage in the particular segment is equal to or greater than one per cent of the standard. Amounts of radioactivity less than one per cent were recorded with peaks only if consistently occurring. The number of electrophoretic strips or gels used to determine these percentages was the same as used for the calculation of recovery. Lines showing the position of radioactivity are governed by the same criteria. In each figure, the plot of radioactivity relative to plasma protein fractions is taken from electrophoretic strips from a single representative electrophoretic run; therefore, the number of observations may differ from that used for calculation of radioactive-peak percentages.

Symbols and Abbreviations. Symbols and abbreviations used: $\nabla$, origin; n, number of replicates; A, albumin; A1, alpha-1-globulin; A2, alpha-2-globulin; B, beta globulin; F, fribrinogen; G, gamma globulin; HP, haptoglobins; IG, immunoglobulins; PA, prealbumin; PALB, postalbumin.

Comparisons between Human and Fish Plasma Proteins. Comparisons between electrophoretic separations of human and fish plasma proteins are made on the basis of migration distance. There does not exist a fraction to fraction relationship between a densitometric scan of fish plasma proteins and a densitometric scan of human plasma proteins.

Figure 7a. Electrophoresis of human-plasma-T3* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 94.42% (n = 8); plasma-T3*, 95.55% (n = 8). Iodide: 2.49%. See page 60 for further clarification.

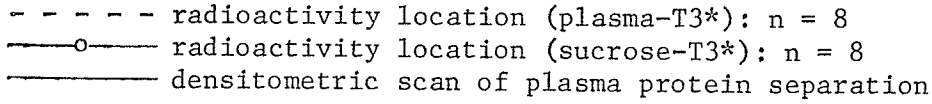
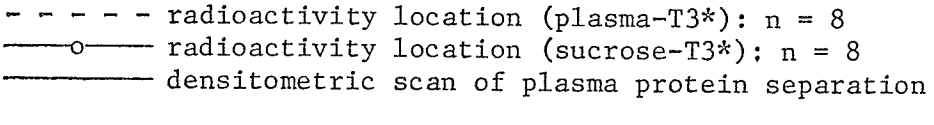
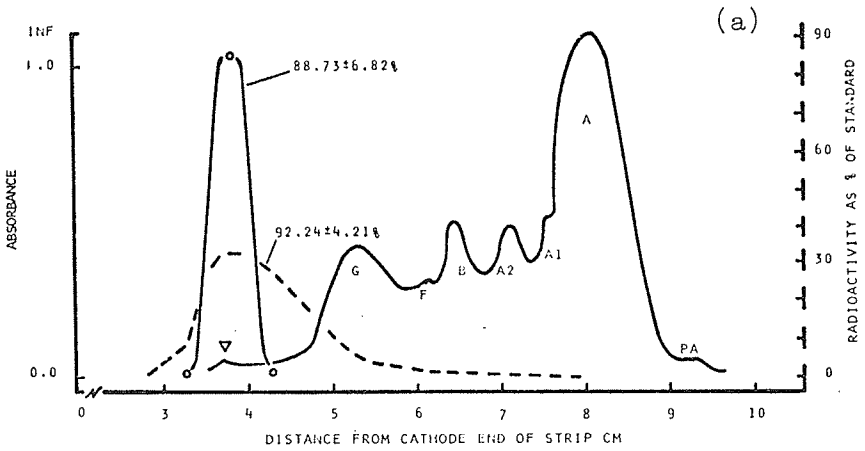



Figure 7b. Electrophoresis of fish-plasma-T3* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 93.10% (n = 8); plasma-T3*, 94.00% (n = 8). Iodide: 2.49%. See page 60 for further clarification.




(a)
INF
1.0
0.0
ABSORBANCE
88.73±6.82%
92.24±4.21%
G
F
B
A2
A1
A
PA
90
60
30
0
RADIOACTIVITY AS % OF STANDARD
0
3
4
5
6
7
8
9
10
DISTANCE FROM CATHODE END OF STRIP CM

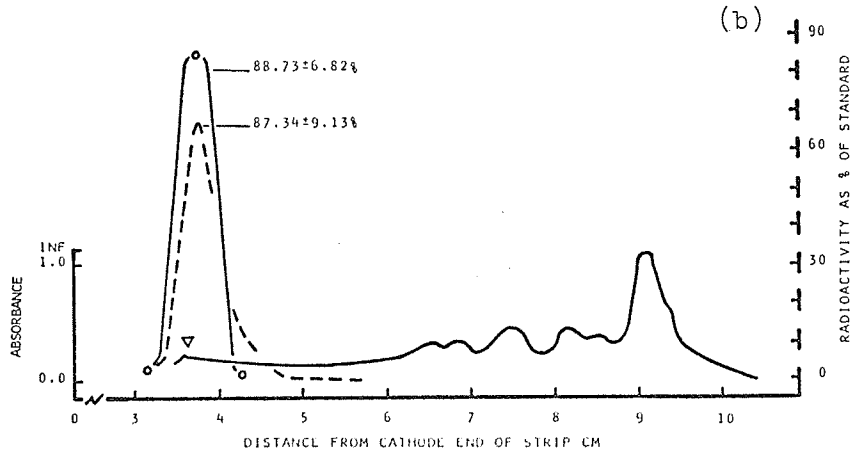
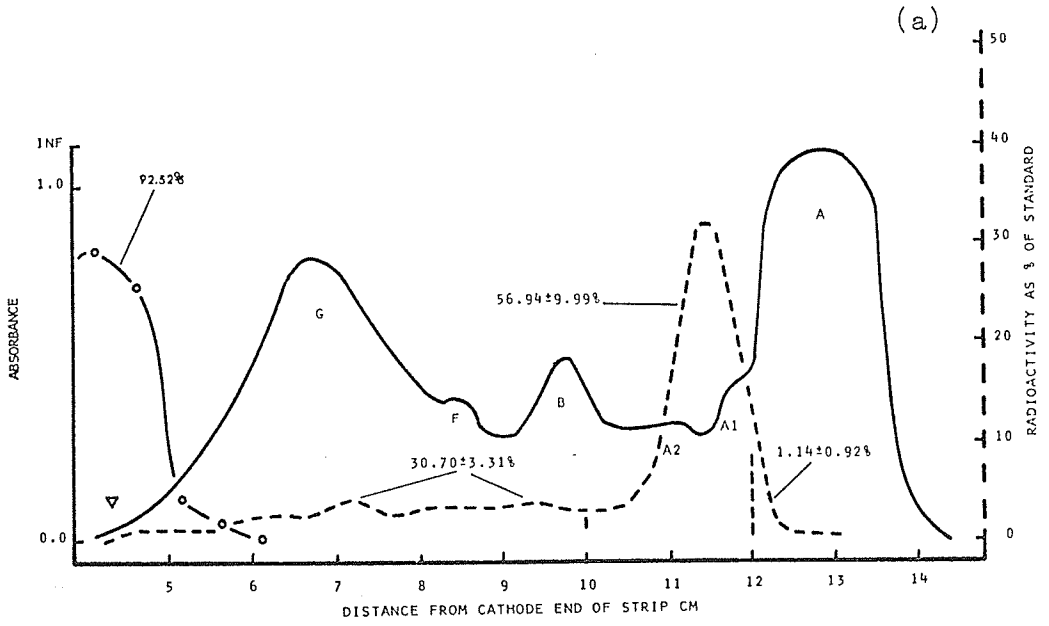

(b)
88.73±6.82%
87.34±9.13%
INF
1.0
0.0
ABSORBANCE
90
60
30
0
RADIOACTIVITY AS % OF STANDARD
0
3
4
5
6
7
8
9
10
DISTANCE FROM CATHODE END OF STRIP CM

Figure 8a. Electrophoresis of human-plasma-T3* mixture on paper medium.
Tris-HCl buffer, pH 7.4. Added T3* concentration: 0.05 µg/ml.
Recovery: sucrose-T3*, 95.00% (n = 1); plasma-T3*, 91.43% (n = 4).
Iodide: 2.49%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T3*): n = 4
——o—— radioactivity location (sucrose-T3*): n = 1
———— densitometric scan of plasma protein separation

Figure 8b. Electrophoresis of fish-plasma-T3* mixture on paper medium.
Tris-HCl buffer, pH 7.4. Added T3* concentration: 0.05 µg/ml.
Recovery: sucrose-T3*, 94.23% (n = 1); plasma-T3*, 94.04% (n = 4).
Iodide: 2.49%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T3*): n = 4
——o—— radioactivity location (sucrose-T3*): n = 1
———— densitometric scan of plasma protein separation


(a)
INF
1.0
0.0
ABSORBANCE
92.52%
56.94±9.99%
30.70±3.31%
1.14±0.92%
G
F
B
A2
A1
A
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

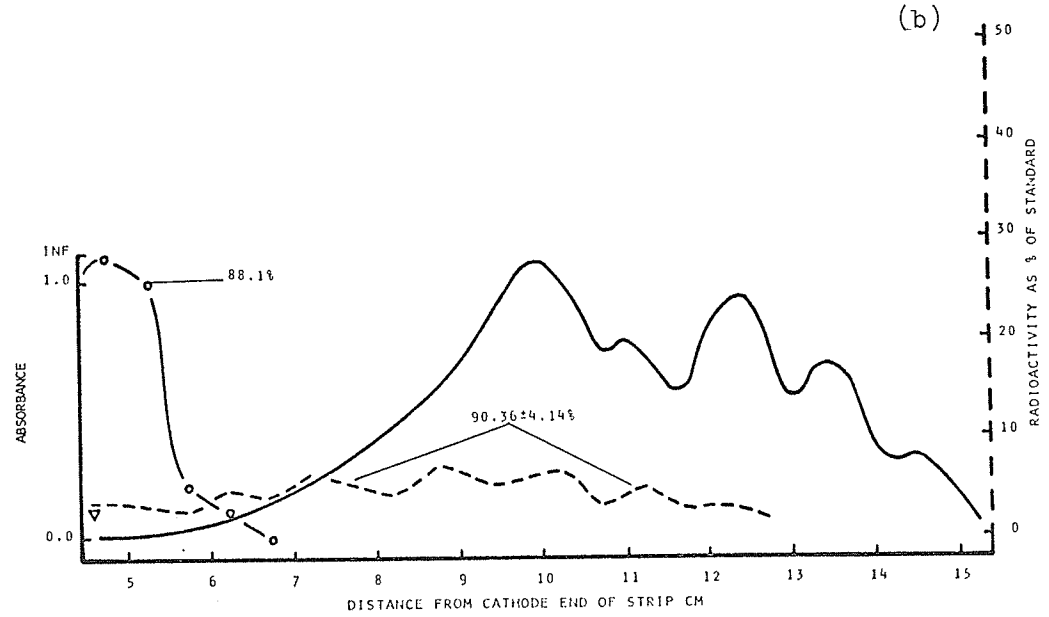

(b)
INF
1.0
0.0
ABSORBANCE
88.1%
90.36±4.14%
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
5
6
7
8
9
10
11
12
13
14
15
DISTANCE FROM CATHODE END OF STRIP CM

Figure 9a. Electrophoresis of human-plasma-T3* mixture on paper medium. Tris-HCl buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 94.96% (n = 1); plasma-T3*, 92.06% (n = 4). Iodide: 2.49%. See page 60 for further clarification.

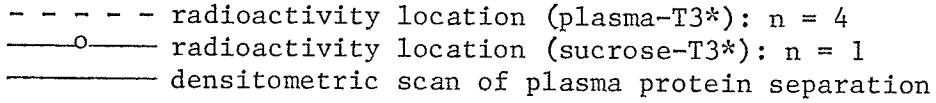
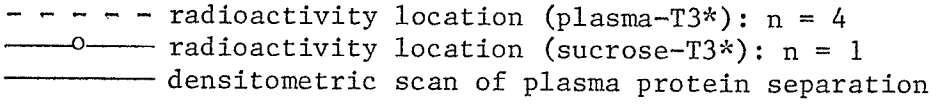
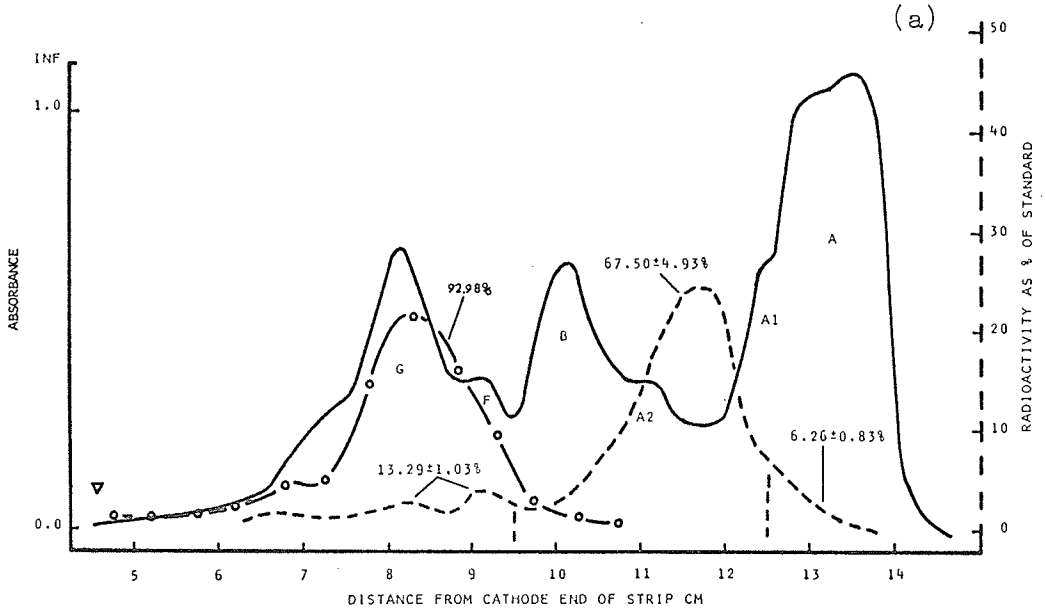



Figure 9b. Electrophoresis of fish-plasma-T3* mixture on paper medium. Tris-HCl buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 93.34% (n = 1); plasma-T3*, 89.59% (n = 4). Iodide: 2.49%. See page 60 for further clarification.




(a)
INF
1.0
0.0
ABSORBANCE
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
92.98%
67.50±4.93%
13.29±1.03%
6.20±0.83%
G
F
B
A2
A1
A
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

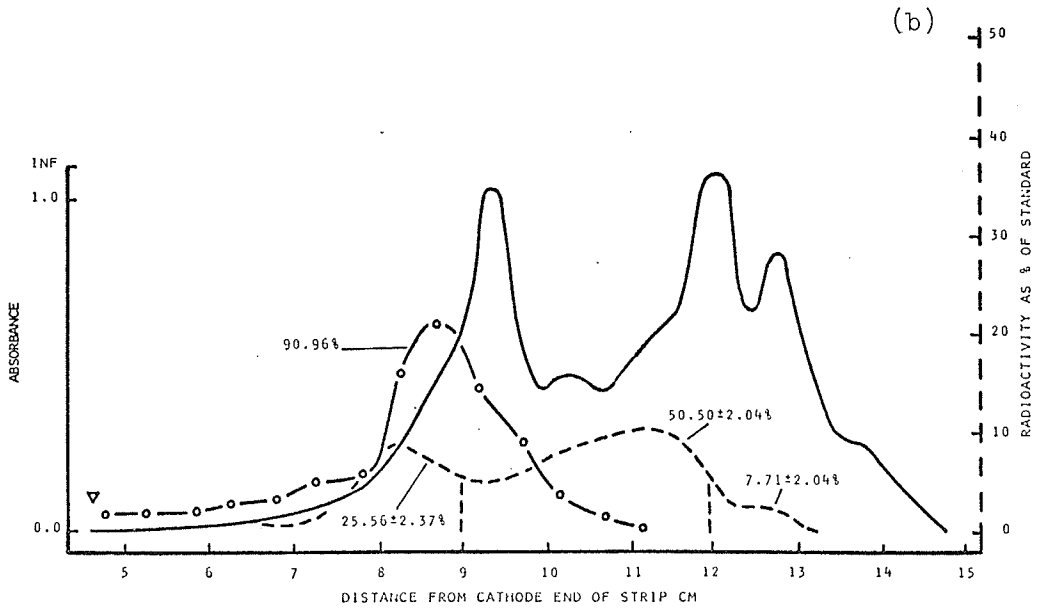

(b)
INF
1.0
0.0
ABSORBANCE
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
90.96%
50.50±2.04%
7.71±2.04%
25.56±2.37%
5
6
7
8
9
10
11
12
13
14
15
DISTANCE FROM CATHODE END OF STRIP CM

Figure 10a. Electrophoresis of human-plasma-T3* mixture on paper medium. Tris-EDTA-borate buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 97.07% (n = 1); plasma-T3*, 95.52% (n = 4). Iodide: 2.49%. See page 60 for further clarification.

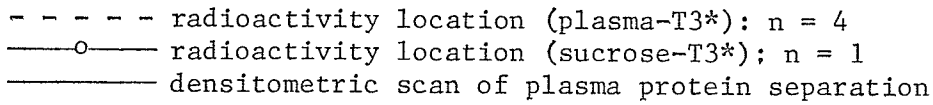
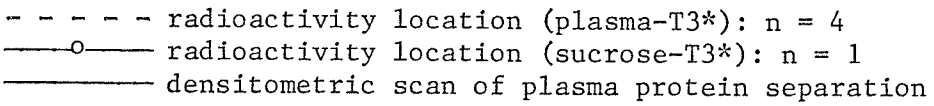
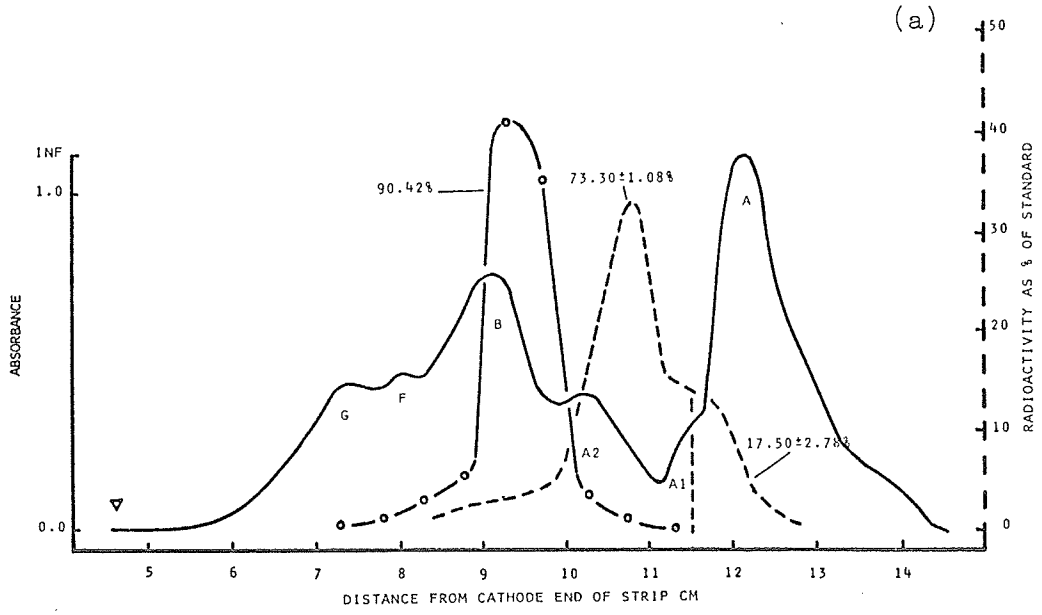



Figure 10b. Electrophoresis of fish-plasma-T3* mixture on paper medium. Tris-EDTA-borate buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 106.21% (n = 1); plasma-T3*, 96.27% (n = 4). Iodide: 2.31%. See page 60 for further clarification.




(a)
INF
1.0
0.0
ABSORBANCE
90.42%
73.30±1.08%
17.50±2.78%
A
B
F
G
A2
A1
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

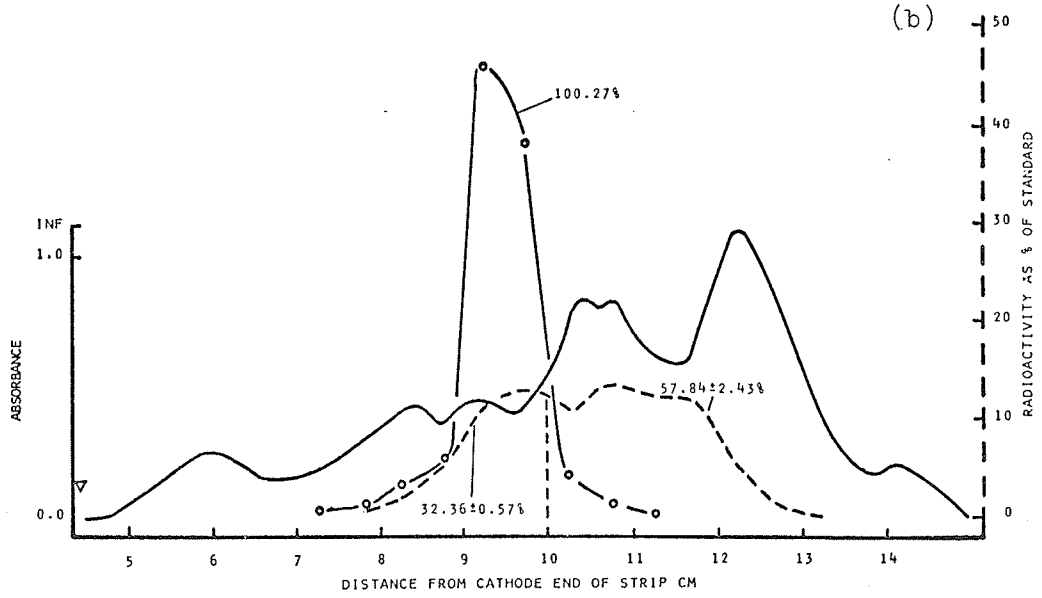

(b)
INF
1.0
0.0
ABSORBANCE
100.27%
57.84±2.43%
32.36±0.57%
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

Figure 11a. Electrophoresis of human-plasma-T3* mixture on acrylamide gel medium. Tris-HCl buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 96.30% (n = 1); plasma-T3*, 97.76% (n = 1). Iodide: 2.49%. See page 60 for further clarification.

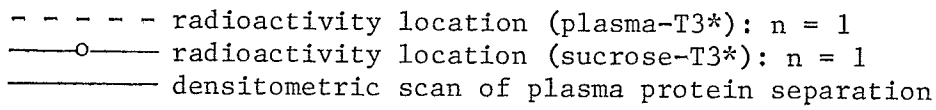
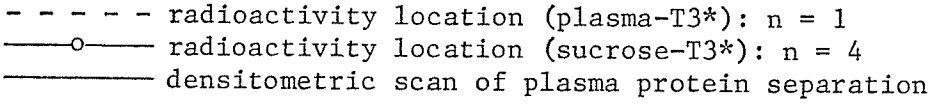
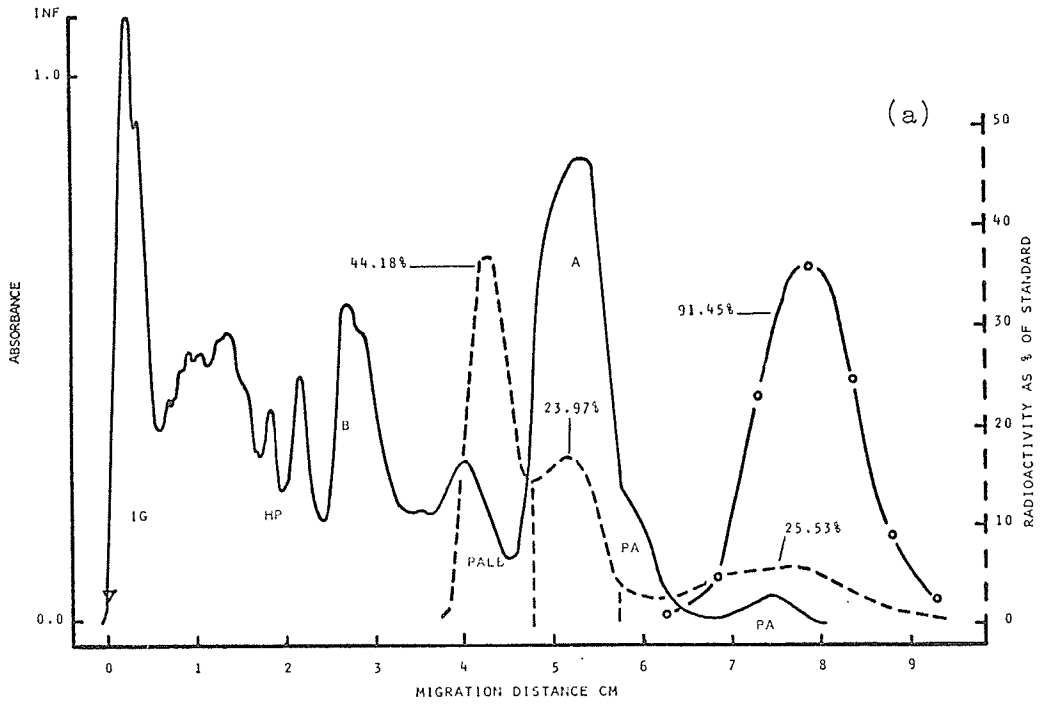



Figure 11b. Electrophoresis of fish-plasma-T3* mixture on acrylamide gel medium. Tris-HCl buffer, pH 8.8. Added T3* concentration: 0.05 µg/ml. Recovery: sucrose-T3*, 96.30% (n = 1); plasma-T3*, 97.76% (n = 4). Iodide: 2.49%. See page 60 for further clarification.




INF
1.0
0.0
ABSORBANCE
(a)
44.18%
A
91.45%
23.97%
B
IG
HP
PALB
PA
25.53%
PA
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
MIGRATION DISTANCE CM

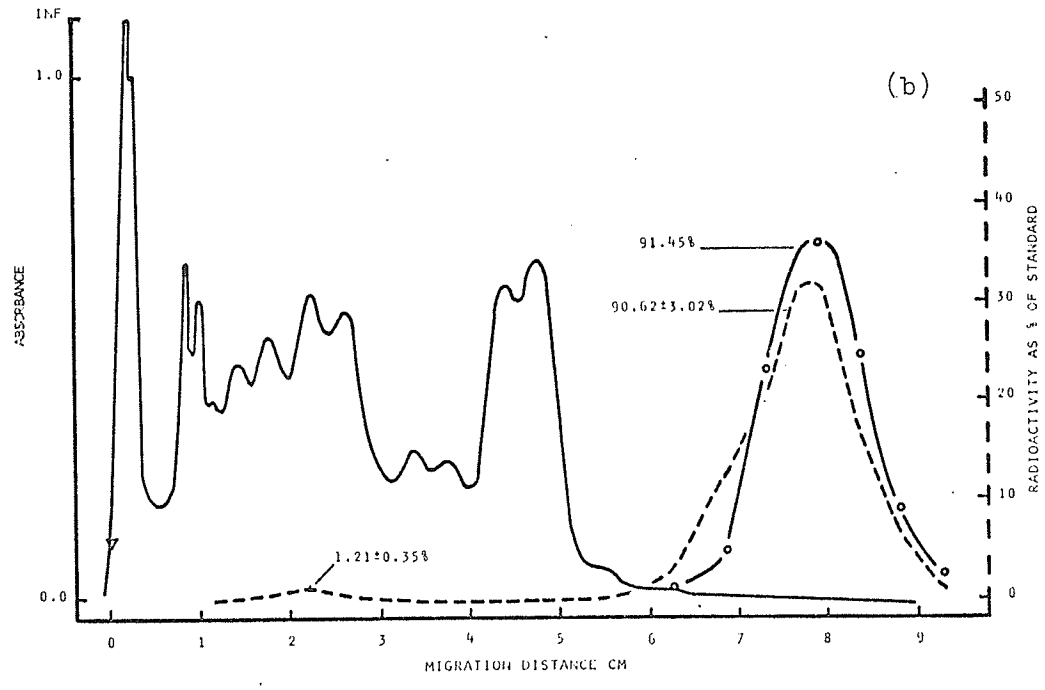

INF
1.0
0.0
ABSORBANCE
(b)
91.45%
90.62±3.02%
1.21±0.35%
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
MIGRATION DISTANCE CM

Figure 12a. Electrophoresis of human-plasma-T3* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T3* concentration: 0.05 μg/ml. Recovery: sucrose-T3*, 95.12% (n = 5); plasma-T3*, 95.08% (n = 5). Iodide: 2.49%. See page 60 for further clarification.

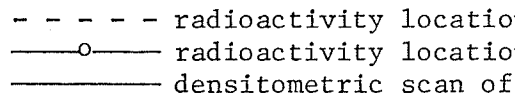



Figure 12b. Electrophoresis of fish-plasma-T3* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T3* concentration: 0.05 μg/ml. Recovery: sucrose-T3*, 95.12% (n = 5); plasma-T3*, 92.77% (n = 10). Iodide: 2.49%. See page 60 for further clarification.

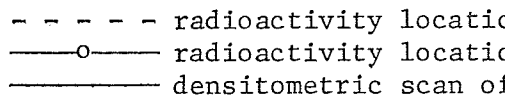
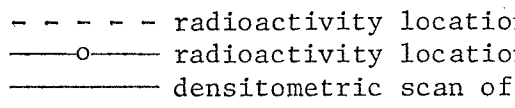
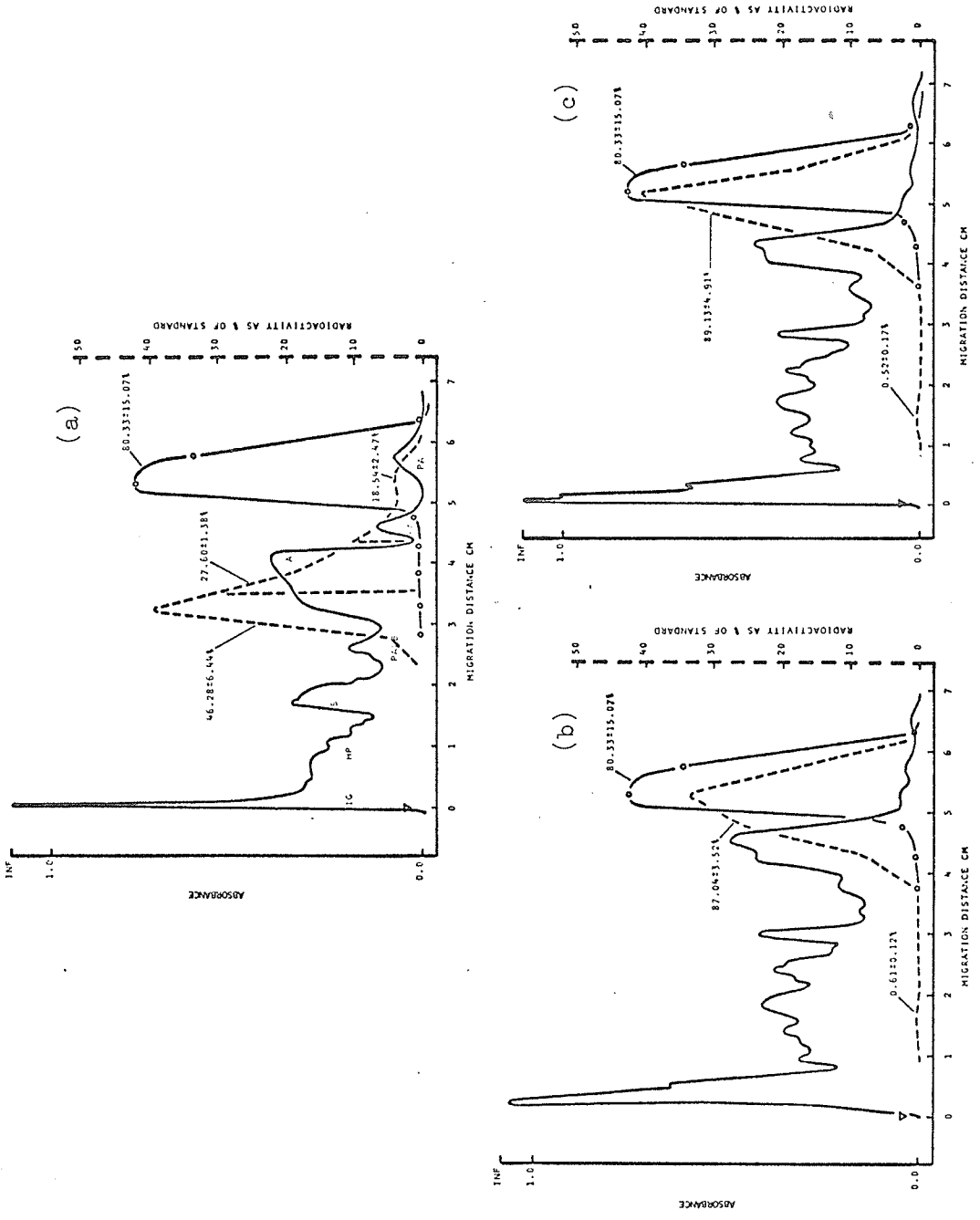



Figure 12c. Electrophoresis of fish-plasma-T3* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T3* concentration: 5.0 μg/ml. Recovery: sucrose-T3*, 95.12% (n = 5); plasma-T3*, 96.06% (n = 10). Iodide: 2.49%. See page 60 for further clarification.




(a)
(b)
(c)
ORIGIN
MP
S
A
P1
P2
80.33±15.07%
46.28±6.44%
27.60±1.38%
18.54±2.47%
87.04±3.52%
0.61±0.12%
89.13±4.91%
0.52±0.12%
ABSORBANCE
INF
1.0
0.0
RADIOACTIVITY AS % OF STANDARD
MIGRATION DISTANCE CM

Figure[a] 13a. Electrophoresis of human-plasma-T4* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T4* concentration: 0.05 ug/ml. Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 2
——o—— radioactivity location (sucrose-T4*): n = 4
———————— densitometric scan of plasma protein separation

Figure[a] 13b. Electrophoresis of fish-plasma-T4* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T4* concentration: 0.05 ug/ml. Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
——o—— radioactivity location (sucrose-T4*): n = 4
———————— densitometric scan of plasma protein separation

Figure[a] 13c. Electrophoresis of fish-plasma-T4* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T4* concentration: 0.5 ug/ml. Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
——o—— radioactivity location (sucrose-T4*): n = 4
———————— densitometric scan of plasma protein separation

Figure[a] 13d. Electrophoresis of fish-plasma-T4* mixture on cellulose polyacetate medium. Tris-EDTA-borate buffer, pH 8.8. Added T4* concentration: 5.0 ug/ml. Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
——o—— radioactivity location (sucrose-T4*): n = 4
———————— densitometric scan of plasma protein separation

[a]In each of these figures radioactivity is plotted as a percentage of the total on the strip. Therefore, recoveries are not given as a percentage of a standard. For the radioactive peaks "n" was the same as used for radioactivity location.

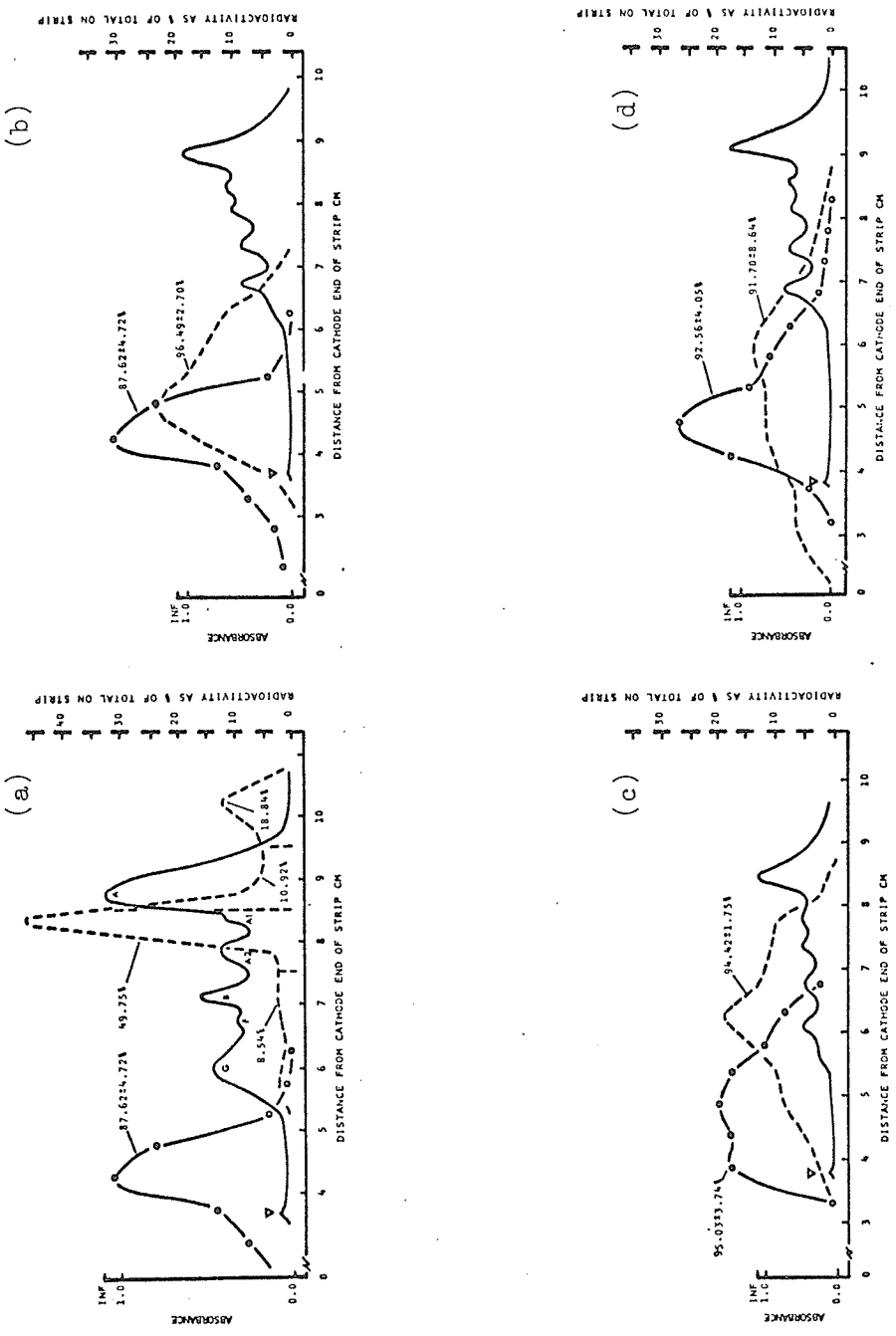

(a)
(b)
(c)
(d)
RADIOACTIVITY AS % OF TOTAL ON STRIP
DISTANCE FROM CATHODE END OF STRIP CM
ABSORBANCE
INF
1.0
0.0
87.6234.72%
49.75%
8.54%
18.84%
10.92%
96.4922.70%
92.5624.05%
91.7058.64%
95.0333.74%
94.4231.75%

Figure 14a. Electrophoresis of human-plasma-T4* mixture on paper medium. Tris-HCl buffer, pH 7.4. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 91.99% (n = 1); plasma-T4*, 91.56% (n = 4). See page 60 for further clarification.

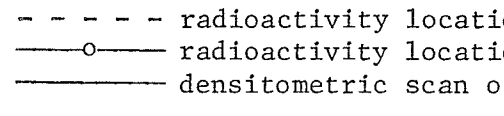
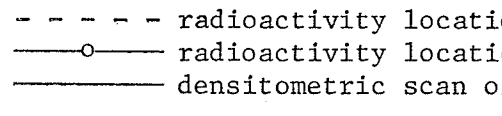
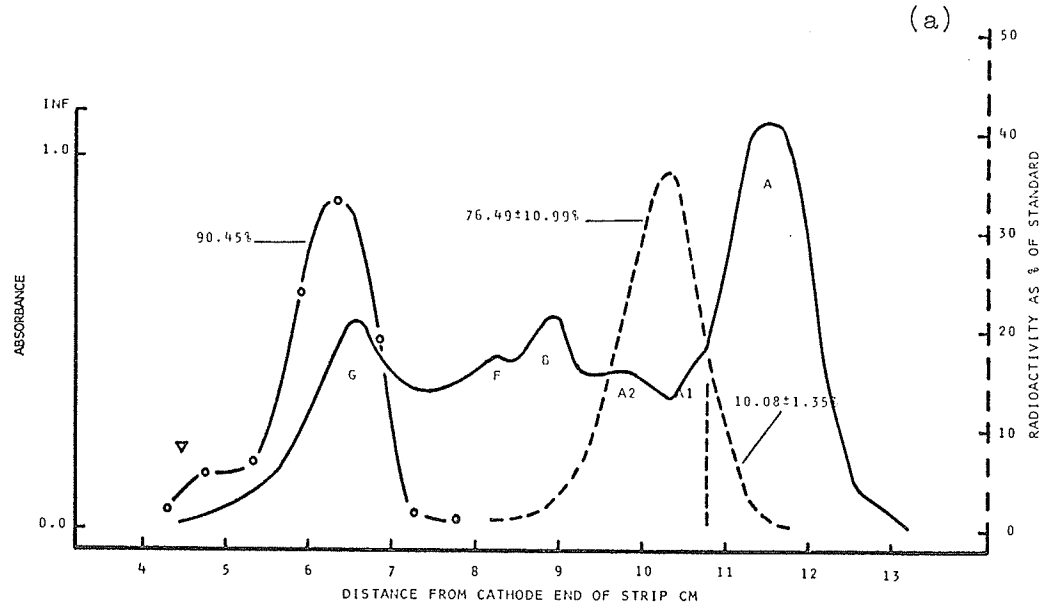



Figure 14b. Electrophoresis of fish-plasma-T4* mixture on paper medium. Tris-HCl buffer, pH 7.4. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 93.90% (n = 1); plasma-T4*, 93.36% (n = 4). Iodide: 2.31%. See page 60 for further clarification.




(a)
INF
1.0
0.0
ABSORBANCE
90.45%
76.49±10.99%
10.08±1.35%
G
F
G
A2
A1
A
4
5
6
7
8
9
10
11
12
13
DISTANCE FROM CATHODE END OF STRIP CM
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD

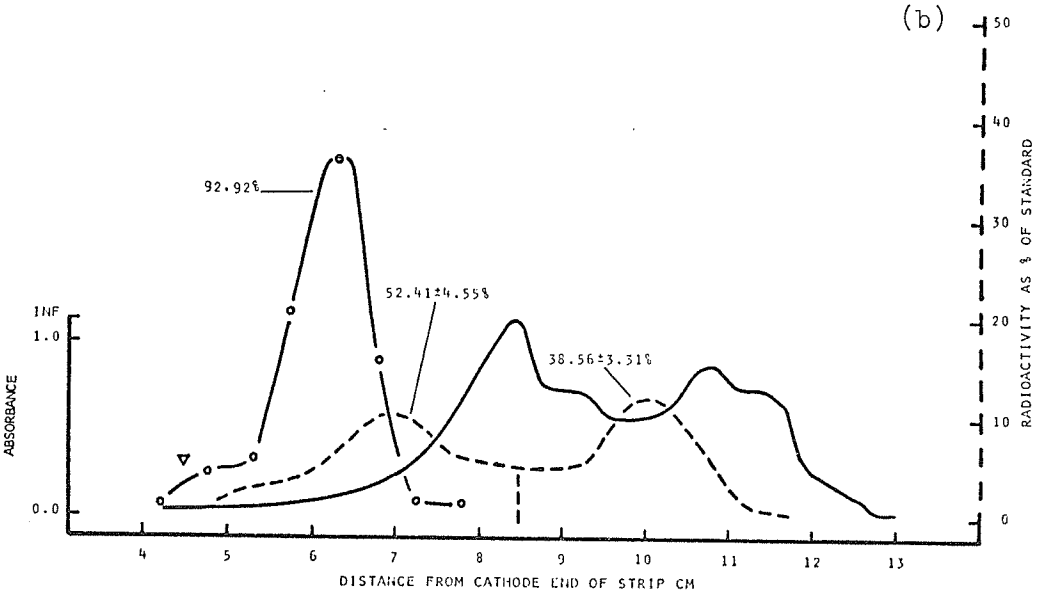

(b)
INF
1.0
0.0
ABSORBANCE
92.92%
52.41±4.55%
38.56±3.31%
4
5
6
7
8
9
10
11
12
13
DISTANCE FROM CATHODE END OF STRIP CM
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD

Figure 15a. Electrophoresis of human-plasma-T4* mixture on paper medium. Tris-HCl buffer, pH 8.8. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 93.37% (n = 1); plasma-T4*, 93.73% (n = 4). Iodide: 2.31%. See page 60 for further clarification.

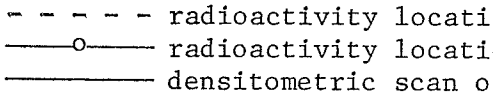
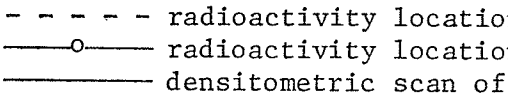
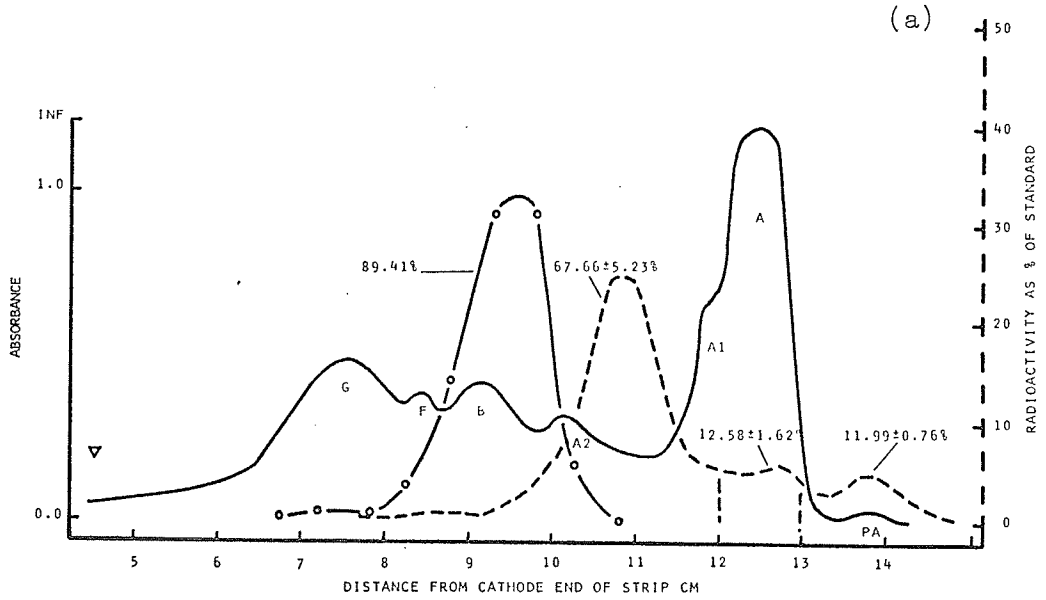



Figure 15b. Electrophoresis of fish-plasma-T4* mixture on paper medium. Tris-HCl buffer, pH 8.8. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 105.16% (n = 1); plasma-T4*, 99.95% (n = 4). Iodide: less than 1%. See page 60 for further clarification.




(a)
INF
1.0
0.0
ABSORBANCE
89.41%
67.66±5.23%
G
F
b
A2
A1
A
12.58±1.62%
11.99±0.76%
PA
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

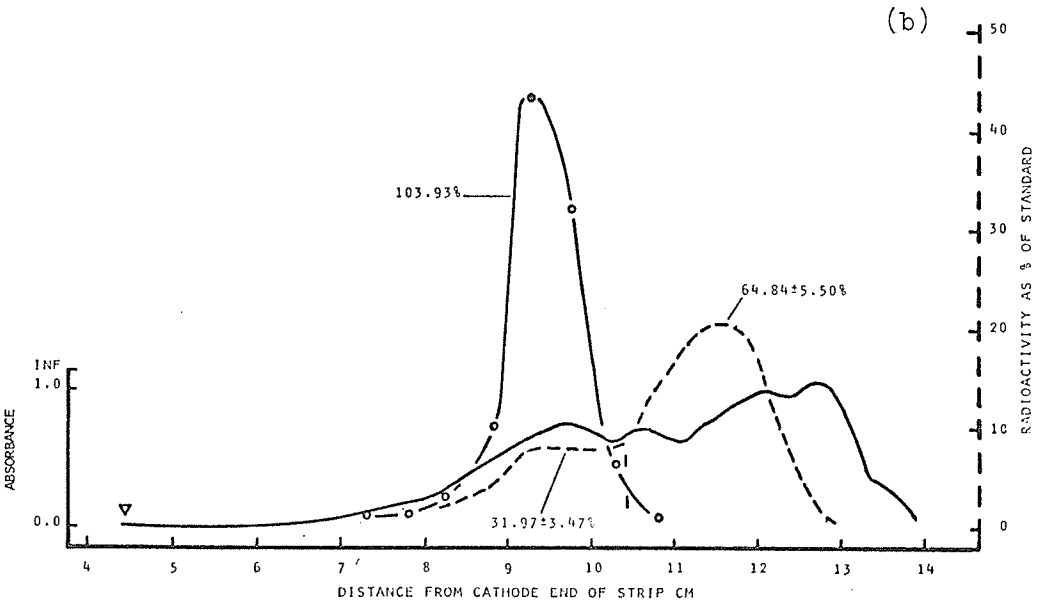

(b)
INF
1.0
0.0
ABSORBANCE
103.93%
64.84±5.50%
31.97±3.47%
50
40
30
20
10
0
RADIOACTIVITY AS % OF STANDARD
4
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM CATHODE END OF STRIP CM

Figure 16a. Electrophoresis of human-plasma-T4* mixture on acrylamide gel medium. Tris-HCl buffer, pH 8.8. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 93.70% (n = 1); plasma-T4*, 90.44% (n = 1). Iodide: 2.31%. See page 60 for further clarification.

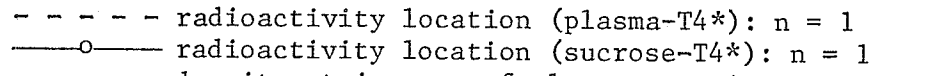
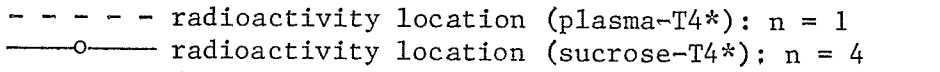
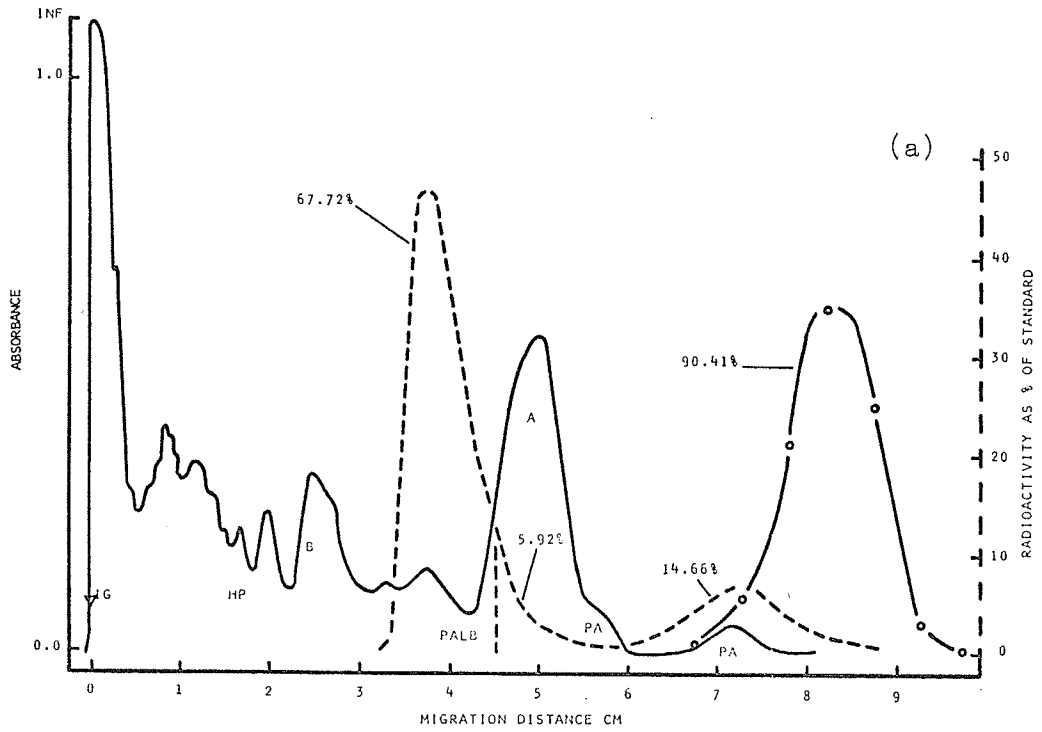
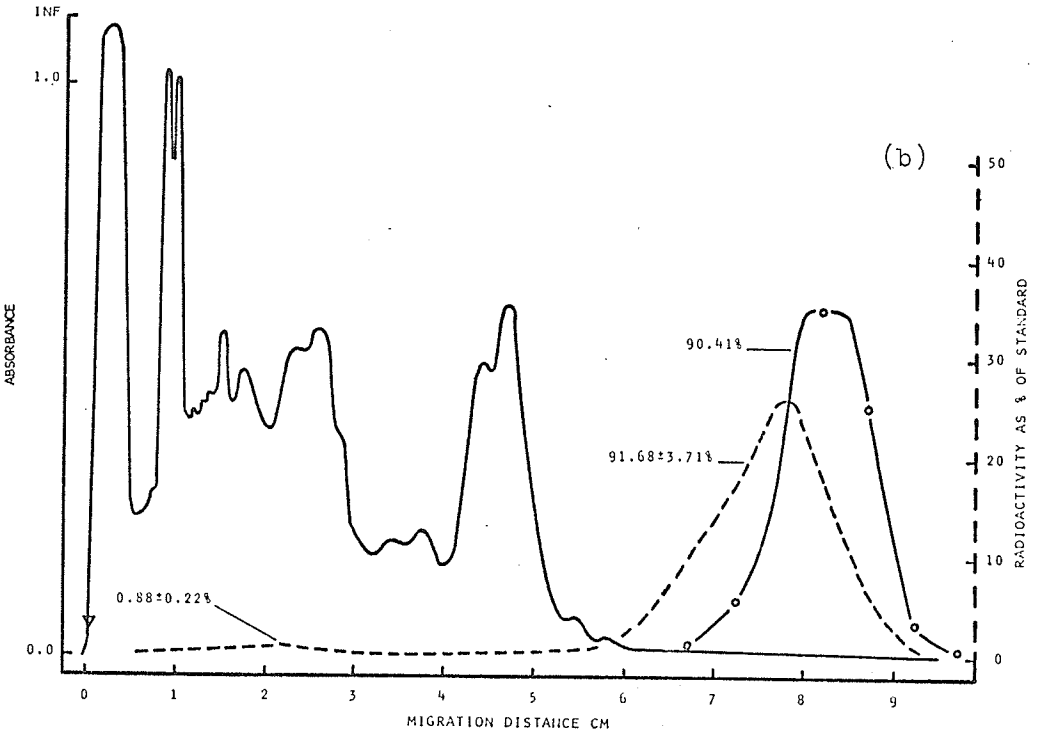



Figure 16b. Electrophoresis of fish-plasma-T4* mixture on acrylamide gel medium. Tris-HCl buffer, pH 8.8. Added T4* concentration: 0.05 µg/ml. Recovery: sucrose-T4*, 93.70% (n = 1); plasma-T4*, 90.44% (n = 4). Iodide: 2.31%. See page 60 for further clarification.




INF
1.0
0.0
ABSORBANCE
(a)
67.72%
90.41%
5.92%
14.66%
A
B
IG
HP
PALB
PA
PA
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
MIGRATION DISTANCE CM


INF
1.0
0.0
ABSORBANCE
(b)
90.41%
91.68±3.71%
0.88±0.22%
RADIOACTIVITY AS % OF STANDARD
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
MIGRATION DISTANCE CM

Figure[a] 17a. Electrophoresis of human-plasma-T4* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T4* concentration: 0.05 μg/ml. Recovery: plasma-T4*, 89.05% (n = 4). Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
———— densitometric scan of plasma protein separation

Figure[a] 17b. Electrophoresis of fish-plasma-T4* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T4* concentration: 0.05 μg/ml. Recovery: plasma-T4*, 85.20% (n = 16). Iodide: 6.81%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
———— densitometric scan of plasma protein separation

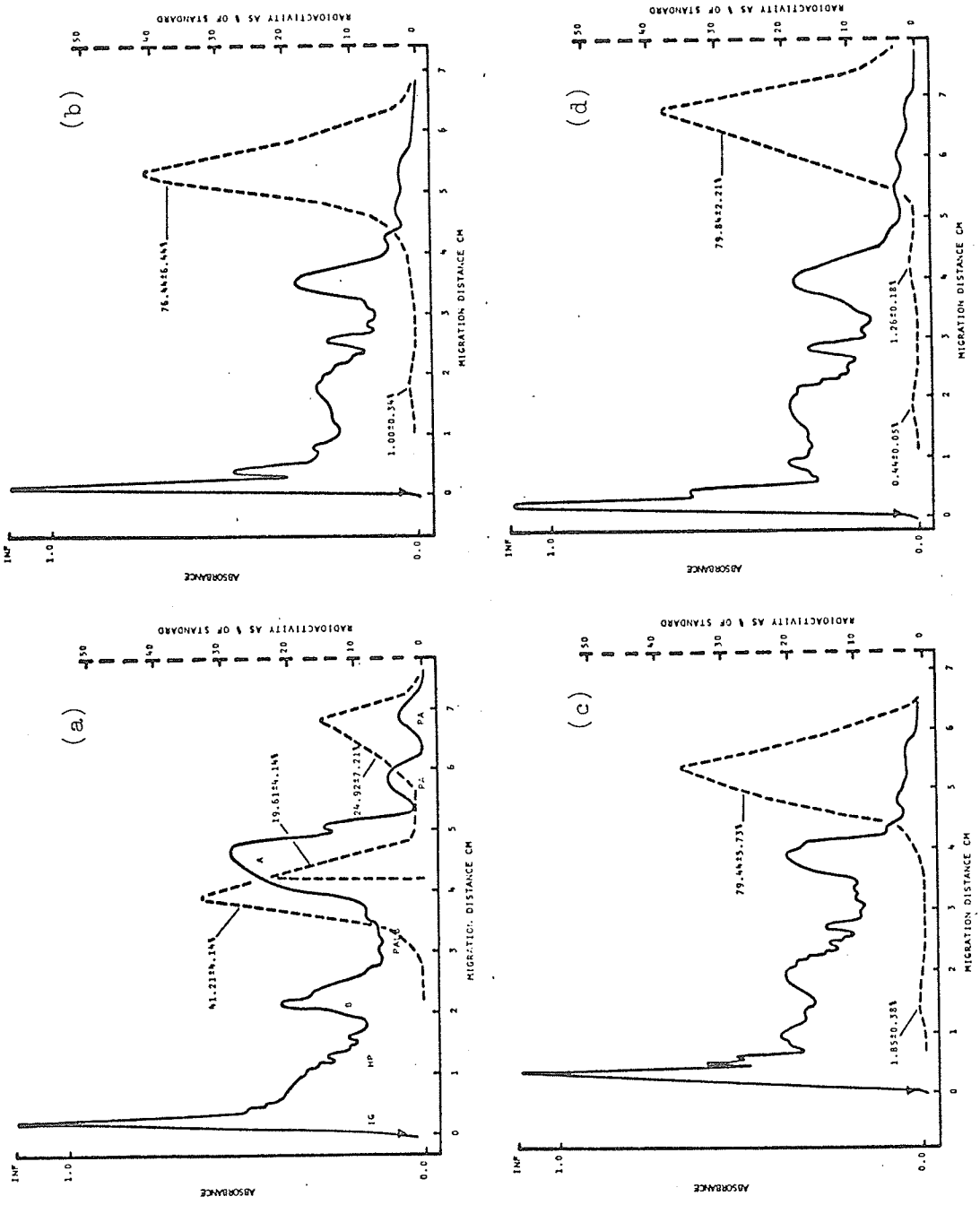
Figure[a] 17c. Electrophoresis of fish-plasma-T4* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T4* concentration: 0.5 μg/ml. Recovery: plasma-T4*, 88.22% (n = 16). Iodide: 6.81%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
———— densitometric scan of plasma protein separation

Figure[a] 17d. Electrophoresis of fish-plasma-T4* mixture on acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. Added T4* concentration: 5.0 μg/ml. Recovery: plasma-T4*, 88.58% (n = 16). Iodide: 8.44%. See page 60 for further clarification.

- - - - - radioactivity location (plasma-T4*): n = 4
———— densitometric scan of plasma protein separation

[a]In this buffer system sucrose-T4* mixture migrated off the gel.


(a)
(b)
(c)
(d)
ABSORBANCE
RADIOACTIVITY AS % OF STANDARD
MIGRATION DISTANCE CM
INF
1.0
0.0
IG
HP
A
PA
41.2±4.1%
19.6±4.1%
24.9±2.2%
76.4±6.4%
1.00±0.3%
79.4±3.7%
1.85±0.3%
79.8±2.2%
1.26±0.1%
0.44±0.0%

7. Gel Dialysis to Verify Thyronine Binding

It is difficult on the basis of electrophoresis alone to determine if coincidence of radiothyronines with a protein fraction represents binding of the radiohormone by that fraction. For this reason gel sections containing free hormone were compared by dialysis with those supposedly containing bound hormone. The results are shown in Table XVI.

When 100-µl samples of either plasma-radiothyronine mixture or radiothyronine-sucrose mixture were electrophoresed and the sections of gel containing radioactive peaks compared by dialysis, binding was shown for both T3* ($P<0.001$) and T4* ($P<0.05$). The dialysis index was more variable when no plasma was present.

8. Binding of Intraperitoneally-Injected T3* and T4*

Ten fish were injected with 5 µCi of T3* (specific activity 35.6 mCi/mg) and 10 with 5 µCi of T4* (specific activity 135 mCi/mg). They were killed 3 hours after injection and a pooled plasma sample collected for both groups. The percentage of the total radioactivity bound to plasma protein was assessed by trichloroacetic acid precipitation (Table XVII).

Ten replicates from each plasma pool were electrophoresed using acrylamide gel and tris-HCL-glycine buffer (Figures 18a,b). Nine of the ten gels carried large (50 µl) plasma samples, while the tenth carried a 15 µl sample. The latter gel and four others were stained in Coomassie blue. The remaining five were sectioned (0.5 cm) and each section counted for one minute or a total of 1000 counts in regions where radioactive peaks were located. Radioactivity was expressed as a percentage of a

TABLE XVI

GEL DIALYSIS TO VERIFY THYRONINE BINDING

| SAMPLE ELECTROPHORESED ON GEL | HOMOGENEITY OF VARIANCES F CALCULATED | df | MEAN DIALYSIS INDEX | t CALCULATED | df FOR "t" |
|---|---|---|---|---|---|
| T4 + FISH PLASMA (100 µl) | 270.73 (S, 0.025) | 3,3 | 0.681±0.008[b] | t' = 17.16[a] (P < 0.05) | 3 |
| T4 + SUCROSE CONTROL (100 µl) | | | 0.280±0.126 | | |
| T3 + FISH PLASMA (100 µl) | 1.33 (NS, 0.025) | 3,3 | 0.659±0.034 | 25.11 (P < 0.001) | 6 |
| T3 + SUCROSE CONTROL (100 µl) | | | 0.255±0.039 | | |

[a]The value calculated for the analysis of independent samples when $6_1^2 \neq 6_2^2$ (Snedecor and Cochran, 1967).

[b]Ninety-five per cent confidence interval about the mean.

Abbreviations used: df, degrees freedom; NS, not significant at level indicated; S, significant at level indicated.

TABLE XVII

PERCENTAGE OF TROUT-PLASMA RADIOACTIVITY PRECIPITATED WITH TRICHLOROACETIC ACID FOLLOWING INJECTION[a] OF T3* OR T4*

| FISH NUMBER | T3*[b] | T4*[b] |
|---|---|---|
| 1 | 65.2 | 82.2 |
| 2 | 76.3 | 74.3 |
| 3 | 68.8 | 78.9 |
| 4 | 67.3 | 73.6 |
| 5 | 61.5 | 60.3 |
| 6 | 67.9 | 72.1 |
| 7 | 65.7 | 76.5 |
| 8 | 75.4 | 69.5 |
| 9 | 74.4 | 67.7 |
| 10 | 65.9 | 74.9 |
| Mean . . . . . | 68.8 | 73.0 |
| Pooled plasma samples (mean of two determinations). . . | 69.9 | 72.2 |

[a]Ten fish were injected with T3* and ten with T4*. In each case the animals were numbered 1 to 10.

[b]Three hours after injection of T3* or T4*.

Figure 18a. Electrophoresis of plasma from T3* injected fish. Acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1.

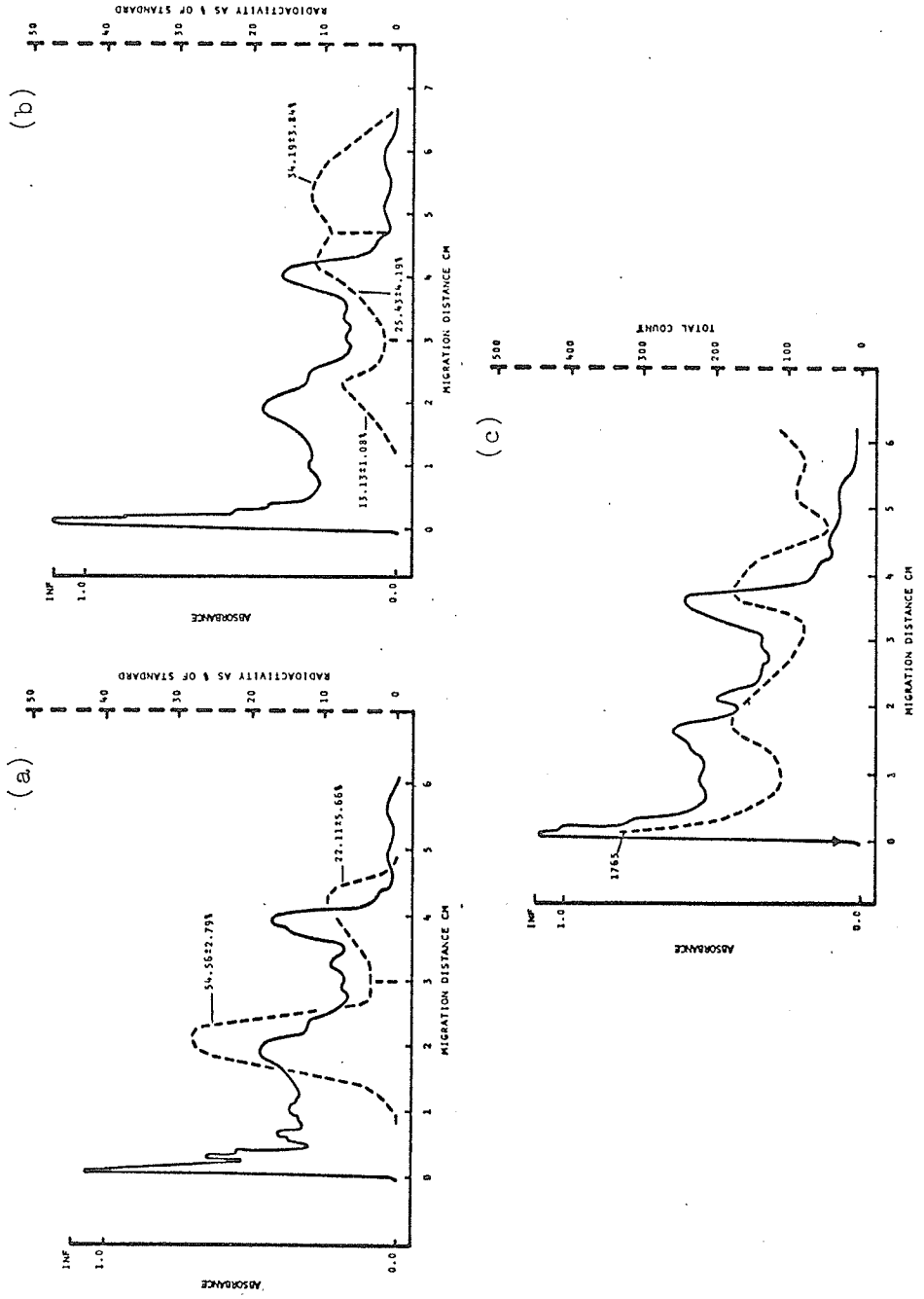
- - - - - radioactivity location: n = 5
——————— densitometric scan of plasma protein separation

Figure 18b. Electrophoresis of plasma from T4*-injected fish. Acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1.

- - - - - radioactivity location: n = 5
——————— densitometric scan of plasma protein separation

Figure 18c. Electrophoresis of plasma from $Na^{125}I$ -injected fish. Acrylamide gel medium. Tris-HCl-glycine buffer, pH 8.1. "Total count" refers to total counts recorded in a 20-minute period.

- - - - - radioactivity location: n = 5
——————— densitometric scan of plasma protein separation


(a)
RADIOACTIVITY AS % OF STANDARD
ABSORBANCE
MIGRATION DISTANCE CM
54.5642.795
22.1125.664
(b)
RADIOACTIVITY AS % OF STANDARD
ABSORBANCE
MIGRATION DISTANCE CM
34.1955.849
25.4324.191
15.1321.084
(c)
TOTAL COUNT
ABSORBANCE
MIGRATION DISTANCE CM
1765

standard and plotted relative to the median electrophoretic scan. In gels containing the large plasma samples certain bands were obscured by protein trailing from leading fractions. Difficulties in fraction identification were to some extent overcome by reference to the gel containing the 15-µl sample.

For T3*-injected fish the percentage of the total radioactivity bound to the plasma proteins ranged from 61.6-76.3 with a mean of 69.9 (Table XVII). For the pooled plasma sample 69.9% of the radioactivity was bound. Electrophoresis of the pooled plasma sample (Figure 18a) showed two peaks of radioactivity: a large peak in the beta-globulin-like region; and a smaller peak in the slowest prealbumin-like to albumin-like region.

For T4*-injected fish the percentage of the total radioactivity bound to plasma proteins ranged from 60.3-82.2 with a mean of 73.0 (Table XVII). In the pooled plasma sample 72.2% of the radioactivity was bound. Three radioactive peaks were identified following electrophoresis of the pooled plasma sample (Figure 18b): a broad peak overlying the two leading prealbumin-like fractions; a second peak overlying the third prealbumin-like fraction; and a third peak in the region of the beta-globulin-like protein fractions.

9. Chromatography of Pooled Plasma from Injected Fish

Thin-layer chromatography of samples from the pooled plasma of fish injected with T3* revealed T3* and $^{125}I^-$, whereas after T4* injection T3*, T4* and $^{125}I^-$ were identified (Table XVIII).

The percentage of thyronines present in both instances compared favourably with the value obtained for the bound fraction in trichloroacetic

TABLE XVIII

THIN-LAYER CHROMATOGRAPHY OF RADIOIODOCOMPOUNDS IN TROUT PLASMA FOLLOWING T3* OR T4* INJECTION[a]

| STRIP % | ORIGIN[b] % | %T4* | %T3* | TOTAL THYRONINE % | IODIDE % | REMAINDER[b] % |
|---|---|---|---|---|---|---|
| T4* INJECTED | | | | | | |
| Chloroform:Methanol:Concentrated Ammonia (50:25:2.5, v/v) | | | | | | |
| 1 | 3.2 | 55.9 | 14.4 | 70.3 | 25.4 | 1.1 |
| 2 | 3.9 | 58.8 | 12.1 | 70.9 | 21.2 | 4.0 |
| 3 | 4.4 | 55.5 | 14.4 | 70.0 | 23.2 | 2.5 |
| Butanol:Ethanol:6N Ammonia (50:40:10, v/v) | | | | | | |
| 1 | 2.8 | 57.6 | 13.5 | 71.1 | 18.5 | 7.6 |
| 2 | 2.5 | 61.2 | 12.3 | 73.5 | 20.5 | 3.5 |
| 3 | 2.3 | 59.8 | 14.0 | 73.8 | 20.9 | 3.0 |
| T3* INJECTED | | | | | | |
| Chloroform:Methanol:Concentrated Ammonia (50:25:2.5, v/v) | | | | | | |
| 1 | 2.1 | 1.6 | 67.1 | 68.6 | 24.7 | 4.5 |
| 2 | 3.4 | 2.2 | 64.7 | 66.9 | 21.0 | 8.8 |
| 3 | 2.2 | 2.0 | 76.0 | 78.0 | 16.9 | 2.8 |
| Butanol:Ethanol:6N Ammonia (50:40:10, v/v) | | | | | | |
| 1 | 3.2 | 2.2 | 66.1 | 68.3 | 22.4 | 6.1 |
| 2 | 2.6 | 3.3 | 65.1 | 68.4 | 21.2 | 7.7 |
| 3 | 1.5 | 2.3 | 68.7 | 71.0 | 23.2 | 4.3 |

[a]Determinations made by J. G. Eales.

[b]Percentage of the total radioactivity on the strip remaining at the origin or in unspecified areas (remainder).

acid analysis. These data are consistent with the hypothesis that both T3* and T4* are bound to plasma proteins.

10. Binding of Endogenously Produced Radioiodocompounds

Ten fish were injected with $Na^{125}I$ (10 μCi). Fifty days after injection the conversion ratio (percentage of the total radioactivity bound to plasma proteins) was determined for each fish by trichloroacetic acid precipitation of the plasma proteins (Table XIX). Plasma from the five fish with the highest conversion ratio were pooled and electrophoresed using acrylamide gel and tris-HCl-glycine buffer (Figure 18c). To each of nine gels was applied 100 μl of the pooled plasma sample. A 15 μl sample was applied to the tenth gel. Five of the gels (including the one carrying 15 μl of plasma) were stained and scanned, and the other five sectioned (0.5 cm). Each section was counted for 20 minutes due to the low level of radioactivity in the protein-bound fraction. The counts obtained for corresponding segments of replicate gels were averaged and plotted relative to the median densitometric scan.

The percentage of the total radioactivity bound to plasma proteins ranged from 1.8 to 5.1 with a mean of 2.9 (Table XIX). In the pooled plasma sample 2.7% of the total radioactivity was bound to plasma proteins. Three major radioactive peaks occurred following electrophoresis of the pooled plasma sample: in the leading prealbumin-like region; in the prealbumin-like to albumin-like region; and in the beta-globulin-like region (Figure 18c). Typical of results obtained when a high proportion of radioiodide was present, the origin retained a fairly large proportion of the radioactivity.

TABLE XIX

PERCENTAGE OF TROUT-PLASMA RADIOACTIVITY PRECIPITATED WITH TRICHLOROACETIC ACID FIFTY DAYS AFTER INJECTION OF $Na^{125}I$

| FISH NUMBER | PERCENTAGE OF RADIOACTIVITY PRECIPITATED |
|---|---|
| 1 | 1.8 |
| 2 | 5.1 |
| 3 | 2.5 |
| 4 | 2.2 |
| 5 | 2.5 |
| 6 | 2.3 |
| 7 | 2.9 |
| 8 | 4.2 |
| 9 | 2.2 |
| 10 | 3.4 |
| Mean . . . . . | 2.9 |
| Pooled plasma samples (mean of two determinations) . . . . . | 2.7 |

## 11. Variation in Relative Percentages of Fish-Plasma-Protein Fractions Binding Thyronines

In view of the variation in total protein for different plasma pools, it was deemed advisable to record relative percentages of the total protein (by densitometry) in fractions occurring at migratory positions corresponding to the radioactive peaks in the gel. This procedure was followed for analyses involving acrylamide gel with tris-HCl-glycine buffer (pH 8.1) since it was most commonly used. Table XX gives means and 95% confidence intervals for the relative percentages of the total protein in fractions from fish plasma which, following electrophoresis, corresponded in migration with radioactive peaks. These results were automatically provided by the integrator component of the densitometer. Little variation was observed between the pooled plasma samples.

TABLE XX

RELATIVE PERCENTAGES OF TOTAL PROTEIN IN FRACTIONS CORRESPONDING IN MIGRATION TO RADIOACTIVE PEAKS

| FISH GROUP | TOTAL PROTEIN gm/100ml | NO. OF GELS | PA1 % | PA2 % | PA3 % | ALBUMIN-LIKE % | BETA GLOBULIN-LIKE |
|---|---|---|---|---|---|---|---|
| 2 | | 4 | 2.34±0.45[a] | 2.97±0.40 | 2.48±0.41 | 15.81±2.49 | 12.42±3.36 |
| 2 | | 4 | 1.44±0.59 | 2.33±0.44 | 1.76±0.56 | 15.67±1.78 | 12.56±1.59 |
| 2 | | 4 | 1.59±0.34 | 2.44±0.22 | 1.90±0.54 | 16.63±2.32 | 14.39±2.67 |
| 2 | 3.86 | 4 | 1.66±0.25 | 2.21±0.09 | 2.55±1.63 | 15.09±1.14 | 15.11±0.39 |
| 2 | | 4 | 1.82±0.88 | 2.48±0.43 | 2.09±0.68 | 15.53±1.79 | 14.32±2.08 |
| 2 | | 4 | 1.76±0.21 | 2.25±0.54 | 2.09±0.47 | 17.96±1.35 | 13.23±2.15 |
| 2 | | 4 | 1.92±0.96 | 2.62±0.72 | 2.77±1.07 | 16.76±0.56 | 11.96±2.86 |
| 3 | | 5 | 1.53±0.78 | 1.85±0.48 | 1.97±0.40 | 15.61±1.52 | 13.92±1.39 |
| 3 | | 4 | 2.08±0.59 | 3.11±0.81 | 2.27±0.35 | 16.30±1.18 | 9.65±1.01 |
| 3 | | 4 | 2.27±0.68 | 2.58±1.90 | 2.66±1.59 | 15.31±3.52 | 10.72±1.40 |
| 3 | 5.00 | 4 | 1.14±0.28 | 1.98±0.25 | 1.71±0.72 | 16.45±2.29 | 13.50±2.99 |
| 3 | | 4 | 2.51±0.84 | 3.21±0.66 | 2.72±0.71 | 15.93±1.54 | 10.18±6.40 |
| 3 | | 4 | 2.13±0.50 | 3.24±0.60 | 3.15±0.79 | 16.35±2.49 | 13.44±1.55 |
| 4 | | 5 | 1.84±0.90 | 1.64±0.87 | 1.62±0.46 | 19.78±1.50 | 13.49±2.48 |
| 4 | 3.63 | 5 | 1.47±0.81 | 1,80±0.94 | 1.69±0.40 | 19.33±0.88 | 13.73±0.91 |
| 4 | | 5 | 1.13±0.86 | 1.42±0.88 | 2.02±0.71 | 18.73±2.77 | 11.32±2.70 |
| 10 | 4.28 | 5 | 1.91±1.02 | 1.83±1.10 | 1.98±1.15 | 15.07±1.33 | 19.69±2.40 |
| 11 | 4.70 | 5 | 1.49±0.98 | 1.24±0.72 | 1.52±0.50 | 14.80±2.60 | 15.13±1.28 |
| 12 | 5.78 | 5 | 1.76±0.28 | 2.15±0.51 | 1.48±0.31 | 17.06±2.08 | 15.91±3.37 |

[a]Means ±95% confidence interval.

Abbreviations used: PA1, fastest migrating prealbumin-like fraction; PA2, second fastest migrating prealbumin-like fraction; PA3, third fastest migrating prealbumin-like fraction.

# DISCUSSION

## 1. Total Plasma Protein

Field et al. (1943) reported a total plasma protein value of 3.46 g/100 ml for Salvelinus fontinalis and observations from 2.2 to 7.6 g/100 ml have been recorded for different species of fishes (reviewed by Fenwick, 1964). The results of these earlier investigations compare favourably with those obtained in the present study (range, 3.68-6.31 gm/100 ml; mean, 4.82 g/100 ml). The variation between pooled plasma samples is not unusual since the total protein of fish plasma changes with acclimation temperature, season, sex, age and nutritional status (reviewed by Fenwick, 1964). Furthermore, these differences in total plasma protein did not influence the relative percentage of the total protein present in fractions binding thyroid hormones and had no detectable influence on the binding phenomenon.

Alterations in the total plasma protein did not relate to changes in haematocrit as variation in one failed to correspond with variation in the other.

## 2. Radiothyronine-Protein Binding by Dialysis and Precipitation

At all added thyronine concentrations there were no differences in binding detected by trichloroacetic-acid precipitation or equilibrium dialysis. Thus both T3* and T4* were taken up by a protein fraction or fractions with high binding capacity.

The differences observed in thyronine binding between the precipitation and dialysis techniques were probably due to a loss of either

precipitate or hormone to the supernatant as a result of physical or chemical treatment. However, the differences were small (approximately 4%) indicating that the trichloroacetic acid precipitation technique is a reliable measure of plasma protein-thyroid hormone complex formation.

3. Electrophoretic Studies

a. Thyronine-Binding to Human Plasma Proteins

A summary of results from previous electrophoretic studies of thyronine binding to human plasma proteins is provided in Table XXI. These investigations along with those of Britton et al. (1965), Launay (1966) and Gordon and Coutsoftides (1969) show that thyroid hormone binding to the plasma proteins during electrophoresis may be altered by: the type of electrophoretic medium; the pH and ionic strength of the buffer; the added hormone concentration; and the temperature of the electrophoretic system. Hence, specific comparisons between this and other experiments are difficult to make and caution must be used in the interpretation of results.

Lack of hormone migration during electrophoresis of T3-plasma protein mixture on cellulose polyacetate medium (Figures 7a,b) implies the affinity of T3* is greater for the electrophoretic medium and less for the plasma proteins than is T4*. This probably reflects the greater affinity of T4* for human plasma proteins.

The human plasma protein-thyronine binding observed in this study generally agrees with that reported elsewhere (Table XXI). At the added hormone concentrations examined thyroxine-binding globulin is the major thyronine-binding protein fraction while albumin and prealbumin carry

TABLE XXI

SURVEY OF RESULTS OBTAINED FOR PROTEIN ASSOCIATION OF RADIOACTIVITY IN HUMAN-PLASMA-RADIOACTIVE-THYROID-HORMONE MIXTURE FOLLOWING ELECTROPHORESIS

| SOURCE | MEDIUM | BUFFER | HORMONE | ADDED HORMONE CONC ug/ml | PERCENTAGE OF TOTAL RADIOACTIVITY ON STRIP ASSOCIATED WITH PROTEIN FRACTIONS INDICATED PA | ALB | TBG |
|---|---|---|---|---|---|---|---|
| Mitchell et al. (1964) | paper | barbital (veronal) pH 8.6 | $T3^{131}I$ | 0.054 | | | 42.6±2.0 |
| Britton et al. (1965) | starch gel | borate pH 8.6 | $T3^{131}I$ | 0.012-0.018 | | 3.3 | 32.4±7.1 |
| | " | " | $T4^{131}I$ | " | 12.3±1.6 | 43.9±6.8 | 33.1±4.7 |
| Marshall & Levy (1966) | acrylamide gel | tris-borate pH 8.95 | $T4^{131}I$ | 0.142 | 22.0 Free T4 represented 34 per cent of total. | 13.0 | 18.0 |
| Lemarchand et al. (1964) | paper | tris-maleate pH 8.6 | $T4^{131}I$ | 0.06 | 34.0±5.3 | 19.1±3.1 | 46.8±4.1 |
| Myant & Osorio (1960) | paper | tris-NaOH maleate pH 7.6 | $T4^{131}I$ | 0.01 | | some | 90.0 |
| | | 8.6 | " | " | 43.0 | 8.0 | 56.0 |
| | | 9.2 | " | " | 48.0 | 9.0 | 40.0 |

TABLE XXI (continued)

| SOURCE | MEDIUM | BUFFER | HORMONE | ADDED HORMONE CONC µg/ml | PERCENTAGE OF TOTAL RADIOACTIVITY ON STRIP ASSOCIATED WITH PROTEIN FRACTIONS INDICATED PA | ALB | TBG |
|---|---|---|---|---|---|---|---|
| Lutz et al. (1969) | agar gel | sodium phosphate pH 7.6 | $T4^{131}I$ | 0.018 | 15.0 | | |
| | | 8.6 | " | " | 30.5 | | |
| | | 9.0 | " | " | 23.6 | | |
| Launay (1966) | cellulose acetate | EDTA-borate pH 9.0 | $T4^{131}I$ | 0.02 | 14.4-44.5 | 15.8-26.9 | 35.5-53.7 |
| | | | | 1.00 | 19.2-61.2 | 20.2-47.0 | 15.7-25.8 |
| | | | | | Also in alpha$_2$-gamma globulin region. | | |
| Deiss et al. (1953) | paper | veronal pH 8.6 | $T4^{131}I$ | 0.1 | some | more | most |
| | | | $T3^{131}I$ | " | none | some | most |
| | | | | | (no percentage given) | | |
| Tanabe et al. (1969) | cellulose acetate | barbital pH 8.6 | $T4^{131}I$ | 0.05 | 10.6 | 25.0 | 47.8 |
| Blumberg & Robbins (1960) | paper | ammonium carbonate pH 8.6 | $T4^{131}I$ | 0.075 | 15.0-40.0 | 10.0-27.0 | 42.0-75.0 |

Abbreviations used: PA . . . . . prealbumin
ALB . . . . . albumin
TBG . . . . . thyroxine-binding globulin.

lesser amounts. Smaller proportions of hormone are located in the prealbumin region at pH 7.4 than at pH 8.8.

b. Thyronine-Binding to Fish Plasma Proteins

1) T3* Binding. The author knows of no previous electrophoretic studies for plasma protein-T3* mixtures from fishes.

Electrophoresis of fish plasma-T3* samples on cellulose polyacetate was of no value in identifying hormone-binding plasma protein fractions. The affinity of T3* for cellulose polyacetate medium is greater than for fish plasma proteins; a fact which permits only minimal migration of the hormone from the point of application.

Although paper electrophoresis of fish plasma-T3* mixture made it impossible to assign binding properties to specific plasma protein fractions, radioactive peaks did occur in albumin- and postalbumin-like positions (Figures 9b, 10b). Salvatore et al. (1959), Leloup (1961a,b), Farer et al. (1969) and Tanabe et al. (1969) describe similar postalbumin-like fractions as binders of T4* (Figures 2 and 3; Table IV). This could mean that plasma protein fractions described as T4* binders also act as T3* binders; however, it is impossible to make direct comparisons. Furthermore, it is difficult to ascertain whether the hormone is in fact associated with these fractions or simply trailed behind a leading binding fraction due to an affinity for the electrophoretic medium.

Acrylamide gel electrophoresis at pH 8.8, although suggesting that a prealbumin-like fraction acts as a binder, presents a rather confusing picture with the occurrence of free and bound T3* in almost identical migratory positions. Also, the hormone-binding protein fraction

appears as a rather diffuse band and does not readily lend itself to fraction identification.

The acrylamide gel system at pH 8.1 proved most valuable for identification of T3*-binding proteins. Here the radioactive peaks and plasma protein fractions were sharp and clearly defined. Regardless of the added hormone concentration there was little variation in binding with the major radioactive peak located in the prealbumin-like to albumin-like area. The occurrence of a small radioactive peak in the beta-globulin-like region suggests a low capacity binding fraction in that area.

2) T4* Binding. It was impossible to identify hormone-binding plasma protein fractions following electrophoresis of T4*-plasma protein mixtures on cellulose polyacetate medium. Binding is indicated by the displacement of radioactivity in the direction of protein migration. This displacement exceeds that observed with T3* and suggests that T4* has the greater affinity for protein. No mention of this hormone-medium interaction is made by Tanabe et al. (1969) in their description of T4*-binding plasma protein fractions of Anguilla japonica, Carrassius giblio longsdorfi and Fugu niphobles (Table IV) following reverse-flow electrophoresis on cellulose polyacetate medium. Such an interaction would influence the interpretation of their results.

Although paper electrophoresis of fish plasma-T4* mixture made it impossible to assign binding properties to specific plasma protein fractions, radioactive peaks were apparent (Figures 14b and 15b). The occurrence of a major peak of radioactivity in the albumin-like to postalbumin-like (thyroxine-binding globulin-like) position is similar to results obtained by Leloup (1961a) on Salmo salar, Alosa alosa, Lampetra planeri and

Anguilla anguilla (Figure 2). However, the danger in making direct comparisons between this and other studies must again be emphasized. The trailing peak of radioactivity found in the beta-globulin-like to gamma globulin-like area corresponds to free hormone in migratory position and probably represents absorption of T4* to the electrophoretic medium.

The location of most radioactivity in a prealbumin-like position following acrylamide gel electrophoresis of fish plasma-T4* mixtures at pH 8.8 not only corresponds to free and apparently bound T3*, but also to free T4*. Although the prealbumin-like fraction may bind T4* the interpretation of results is complicated by the similar migration of free and bound hormones and by the poorly defined boundaries of the leading prealbumin-like protein fraction.

After acrylamide gel electrophoresis of fish plasma-T4* mixture at pH 8.1 the major radioactive peak was centered over the leading prealbumin-like band. The center of this peak was ahead of the center of the peak obtained following electrophoresis of fish plasma-T3* samples. The small radioactive peak in the beta-globulin-like region is similarly located for T3* and T4*, suggesting non-specificity for T3* and T4*. Similar binding properties for T4* have been assigned to the beta-globulin-like fractions of Scyllium stellare, Salmo salar, Protopterus annectens, the conger eel (Figure 2; Leloup, 1961a,b), the prealbumin-like fractions of the mullet (Figure 2; Salvatore et al., 1962), and of Anguilla japonica, Carrassius giblio longsdorfi and Fugu niphobles (Table IV; Tanabe et al., 1969). The lack of alteration in binding at increased added-hormone concentrations implies the prealbumin-like-binding fraction is of high hormone capacity and that of the beta globulin region

of low capacity. Salmo salar, Alosa alosa and Anguilla anguilla similarly exhibit no change in protein fractions binding T4* over added hormone concentrations of 1.5 to 10.0 µg/ml plasma (Figure 2; Leloup, 1961a).

c. Binding of Injected Radiothyronines

1) T3* Injected. Radioiodide present as an impurity in the injected T3* sample represented 11.2% of the total radioactivity. Thin-layer chromatography and trichloroacetic acid precipitation of plasma from fish injected with T3* (Tables XVII and XVIII) showed an increase in the percentage of radioiodide. This may represent in vivo deiodination of T3* or clearance of T3* from the circulatory system at a rate exceeding that of radioiodide. The large portion of radioactivity associated with the beta-globulin-like component following electrophoresis illustrates the importance of this fraction as a binder of thyroid hormones. The second radioactive peak, situated in a region identified as a binding site for T3*, trailed in the direction of the beta-globulin-like fraction to an extent greater than that observed after electrophoresis of plasma incubated with T3*. This latter situation was probably due to: (1) a loss of definition resulting from the size of plasma sample applied; (2) the greater proportion of radioactivity located in the beta-globulin-like area; and (3) the presence of T3* derivatives binding to other proteins. The position of radioactive peaks relative to plasma protein fractions conforms to that obtained after electrophoresis of T3*-plasma incubated mixtures.

2) T4* Injected. Radioiodide present as an impurity in the injected T4* sample was less than 1%. Thin-layer chromatography and trichloroacetic acid precipitation of plasma from fish injected with T4* (Tables XVII

and XVIII) showed an increase in the percentage of radioiodide. This increase in radioiodide was accompanied by the appearance of T3* suggesting that in vivo deiodination of T4* and T3* had occurred. The radioactive peaks identified after electrophoresis occurred in the prealbumin-like position, thought specific for T4*, the prealbumin- to albumin-like position, thought specific for T3*, and the beta-globulin-like position, thought specific for T3* or T4*. This agrees with chromatographic identification of both T3* and T4* in the pooled plasma sample (Table XVIII).

d. Binding of Endogenous Radioiodocompounds

The three peaks of radioactivity that occurred after electrophoresis of plasma from fish injected with $Na^{125}I$ are located in positions similar to T3* and T4* electrophoresed in incubated plasma-hormone mixtures. The location of these radioactive peaks shows little similarity to that found after reverse flow paper electrophoresis of plasma from $Na^{131}I$ injected ammocoete larva of Lampetra planeri (Figure 2; Leloup, 1961b). In this latter case a thyroxine-binding-globulin-like protein is the binder.

In summary, the beta-globulin-like fraction may be non-specific (bind T3* or T4*), of low capacity (relatively few binding sites) and of high affinity (types of binding sites) insofar as T3* and T4* are concerned. Furthermore, it may possess a greater affinity for T3* than T4*. This latter difference is suggested by alteration in the size of beta-globulin-like radioactive peaks following electrophoresis of pooled plasma samples from T3*- and T4*-injected fishes (Figures 17 and 18). The prealbumin-like and albumin-like protein fractions, while specific for T3*

or T4*, appear to have high hormone-carrying capacity. The low total thyronine values (as determined by Bio-Science Laboratories) found in this species (0.5-0.9 μg/100 ml) indicate that the added-hormone concentration used in the incubation studies probably exceeded the carrying capacity of the beta-globulin-like proteins with the excess hormone being taken-up by prealbumin-like and albumin-like proteins. This parallels the situation in mammals when thyroxine-binding globulin becomes saturated and excess hormone is taken up by albumin and prealbumin plasma proteins.

Following injection of T3* or T4*, far lower levels of radiothyronines would be found in the plasma. At these more physiological concentrations a higher proportion of the radioactivity was associated with the beta-globulin-like fraction. The endogenous radioiodocompounds which would be produced at physiological levels also showed prominent binding in the beta-globulin region.

Under normal circumstances, one might envisage prealbumin-like, albumin-like and beta-globulin-like fish-plasma-protein fractions transporting thyroid hormones. The immediate effect of excess free hormone could conceivably be buffered through binding to prealbumin-like and albumin-like proteins.

## SUMMARY AND CONCLUSIONS

1. The plasma proteins of brook trout (Salvelinus fontinalis) were found to bind T3* and T4*. This was verified by trichloroacetic acid protein-precipitation and by equilibrium dialysis. For both T3* and T4* the percentage of added hormone in the free or unbound state was less than 1% when determined by equilibrium dialysis and approximately 5% when determined by trichloroacetic acid protein precipitation.

2. Electrophoresis of human plasma-T3* and plasma-T4* mixtures on cellulose polyacetate, paper and acrylamide gel confirmed, at an added hormone concentration of 0.05 µg/ml, that thyroxine-binding globulin is the major thyronine binder while albumin and prealbumin proteins carry less. A smaller proportion of T4* is located in the prealbumin region at pH 7.4 than at 8.8. T3* showed binding to prealbumin fractions only on acrylamide gel medium, in which case a smaller proportion was located in the prealbumin region at pH 8.1 than at pH 8.8.

3. Due to thyronine adsorption to the supporting medium, electrophoresis on cellulose polyacetate and paper was of little value for identifying T3* and T4* binding proteins of fish plasma.

4. Incubation of fish plasma with T3* or T4* (0.05-5.0 µg/ml) followed by electrophoresis on acrylamide gel implicated prealbumin-like and albumin-like proteins as major hormone binders, and beta-globulin-like proteins as minor binders. The beta-globulin-like proteins appear

to be non-specific for T3* and T4*. The leading prealbumin-like protein fraction mainly binds T4* and the slowest prealbumin-like fraction mainly binds T3*.

5. The prealbumin-like to albumin-like proteins have a high hormone-binding capacity. Trichloroacetic acid protein precipitation, equilibrium dialysis and electrophoretic techniques all failed to establish any effect of increased hormone concentration (0.05-5.0 μg/ml) on the binding phenomenon.

6. As determined by ion-exchange-column, normal thyronine concentration in the plasma of brook trout (0.5-0.9 μg/100 ml) is approximately one-tenth that of humans. This suggests that added hormone concentrations used for incubation with fish plasma may have exceeded the binding capacity of proteins normally complexing with T3 and T4.

7. Electrophoresis of pooled plasma sample from fishes injected with T3* or T4* resulted in radioactive peaks at positions identical to those obtained after electrophoresis of incubated plasma-hormone mixtures. However, the proportion of radioactivity in these positions differed with that found after electrophoresis of incubated plasma-hormone mixtures. The plasma from injected animals had a much greater percentage of the total radioactivity in the beta-globulin-like region. This difference probably reflects differences in plasma-thyronine concentration. Radiothyronine concentration in the plasma of injected animals was less than that in incubated plasma-hormone mixtures.

8. Electrophoresis of pooled plasma samples from fish injected with $Na^{125}I$ resulted in radioactive peaks in regions corresponding to those described for T3* and T4*. Most of the radioactivity was equally divided between the slowest migrating prealbumin-like protein fraction and the beta-globulin-like proteins. A small amount of radioactivity paralleled the leading prealbumin-like protein.

9. T3* and T4* at high concentrations are mainly bound by the prealbumin-like and albumin-like proteins of trout plasma. Smaller amounts are associated with beta-globulin-like fractions. At more physiological thyronine concentrations the beta-globulin-like fractions appear to bind a greater proportion of the total hormone. All these plasma protein fractions may normally bind T3* and T4*. At higher thyronine concentrations prealbumin- and albumin-like fractions may serve to take up excess hormone with little resulting change in the circulating free thyronine levels.

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