THE PREPARATION, DISTRIBUTION, AND METABOLISM OF IODO-PROLACTIN LABELLED WITH I^{131}

by

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INTRODUCTION

The protein hormone, prolactin, has been of great interest to endocrinologists ever since the demonstration of its presence in the anterior pituitary by Stricker and Grueter in 1929. The action of this hormone was shown to be exerted on the fully developed mammary gland, causing the induction and maintenance of lactation (Nelson, 1936). This role of the hormone has even been of economic interest since prolactin for a time was used in Britian to prevent the decline in the spring in milk production of cows. More recently, there have been reports that prolactin may have other effects in the body besides its function in lactation. It is thought to induce the secretion of hormones from the corpus luteum; but here again, only when the gland is fully developed and ready to function.

In 1947 when the present problem was undertaken, almost nothing was known about the behavior of the hormone in the animal body. Questions such as "where does it go, and what does it do?" could not have been answered. The search for answers to questions like this are a proper responsibility of modern endocrinology.

As tools for investigations of this nature, radioactive isotopes are proving indispensible, because use of them allows study of the behavior of the hormones in minute doses, and these potent biological entities exert widespread effects when present in almost infinitisimal amounts. By use of radioactive isotopes,

not only can the distribution to, and the metabolic transformations of the hormones within the various end organs be analyzed, but exact cellular, and even exact cellular organoid localizations be obtained.

The basic techniques for such investigations are biochemical analyses carried out by means of the Geiger Muller tube and radio-autography, both of which have been employed in this investigation.

However, prolactin is a protein, in fact a simple protein, the intimate chemistry of which is unknown. Of itself, it is indistinguishable from other proteins. To study its behavior, a label was needed. C^{14} was economically impossible; N^{15} was undesirable in that it precluded autography which was deemed essential for ultimate proof of cellular localization; radioactive conversion of contained sulfur in an atomic pile would have led to destruction of the protein by charring. Therefore, an exogeneous label was sought; I^{131} was available, and would react with the protein--prolactin contains tyrosyl radicals, and Dr. Gross, formerly of this laboratory, had been successful in synthesizing I^{131} thyroxine by Horeau and Sue's (1945) method which involves iodination of the tyrosyl radicals of di-iodo-thyronine. But Li et al (1941 a) had shown that complete iodination of prolactin led to complete loss of biological activity. However, minimal iodination with I^{131} labelled iodine might result in a radioactive and biologically active protein hormone which then could be traced.

Thus the problem was undertaken; the evolution of the techniques used and the results obtained are the subject of this thesis.

The aim was to study the distribution of prolactin in the animal body in physiological doses. To do this, iodo-prolactin had to be used because prolactin had no distinguishing chemical or physical characteristics. To follow iodo-prolactin in physiological doses, it was labelled with radioactive iodine--I^{131}--single molecules of which can be detected due to emission of beta rays of the disintegrating nuclei of the isotope.

To iodinate prolactin, molecular iodine was required, which was obtained by the interaction of potassium iodide with potassium iodate in the presence of excess acid. The molecular iodine so formed was allowed to react with prolactin in a phosphate buffer at pH 7.6. Proof of iodination was obtained by dialysis while, routinely, radio-iodo-prolactin was freed of unincorporated inorganic iodide, stable and radioactive, by isoelectric precipitation and reprecipitation, a procedure which presumably further purified the original protein sample of the hormone used. The protein loss of such purification was determined by microkjeldahl; the degree of iodination obtained was determined by a study of yields, which incidentally elucidated the factors affecting such iodination.

To study the distribution, and more especially the

catabolism, of small doses of radio-iodo-prolactin given to mice, a means of separating organically-bound iodine from inorganic iodide was evolved in vitro - the Somogyi zinc sulfate method for fractionation of protein-bound iodine was thus adapted for use with this material.

Radio-iodo-prolactin having been made, purified, and recovered from tissues, could then be used to study its distribution in the animal body. Mice were chosen as the test animal, and intravenous injection of small amounts to a number of animals under a variety of conditions, with subsequent fractionation of their tissues by the Somogyi zinc sulfate method, indicated that technical details of experimentation, and the biological state of the animal, caused wide variations in the values obtained for any given organ. Distribution studies showed a progressive change with time after injection and physiological state. Fractionation studies showed a very rapid breakdown of radio-iodo-prolactin to some unknown metabolites and inorganic iodide. More detailed studies of the metabolism of radio-iodo-prolactin in the lactating mammary gland showed rapid breakdown and involvement of fat.

Autographic study at 7 minutes indicated that the distribution was diffuse, with concentration, perivascularly, in all tissues studied. Heavy concentrations were found in liver, thyroid, kidney, corpora lutea of the ovary, adrenal cortex, and over the alveolar cells of the lactating mammary gland. Precise intracellular localizations

were discovered.

A search of the literature revealed the following facts:

History of Prolactin

Bouin, in 1928, suggested to Stricker and Grueter that the anterior pituitary might contain a lactogenic factor. They injected an aqueous extract of anterior pituitary material to some ovariectomized pseudo-pregnant rabbits. Lactation was initiated. These workers followed up this lead and were able to show that such an extract would maintain lactation in hypophysectomized rabbits (1928), would increase milk production in cows, especially in declining lactation (Grueter and Stricker, 1929) and in goats (Grueter, 1930). Corner extended this finding to virgin rabbits in 1930 and so initiated the search for the active principle in these crude extracts of anterior pituitary. Various laboratories took up the problem: Turner's in Missouri, Evans' in California, Riddle's at Cold Spring Harbor, Allen's at Yale, and later the group at the Reading Dairy Institute in Britian. In the following 10 years, the actuality of this role in lactation was confirmed for many species; assays were proposed, evolved, and accepted (the best was the pigeon crop gland assay, for it was the simplest and probably the most reliable (Riddle et al, 1933)). With development of a suitable assay, the study of the distribution of prolactin throughout the animal kingdom was begun. Wherever it was sought,

prolactin was found. The content of the pituitary was assessed for many species and in many physiological states, natural and experimental (for details see Riddle and Bates, 1939), as well as that of urine (Lyons and Page, 1935; Tesauro, 1936; Langecker and Schenk, 1936; Hoffmann; 1937; Leblond, 1937; and Lyons, 1937 b), blood (Tesauro, 1936; Leblond, 1937; Cunningham, Bickell and Tanner, 1940-1941; and Meites and Turner, 1942), liver (Leblond and Noble, 1937; Riddle and Bates, 1939; and Rabald and Voss, 1939), placenta (Ehrhardt, 1936), brain (Leblond and Noble, 1937), and mammary glands (Geschickter and Lewis, 1936).

Meanwhile the work on the isolation and purification of prolactin had been progressing. Riddle, Bates, and Dykshorn (1932 a) had published a method--which they subsequently modified (1932 b, 1933, Bates and Riddle, 1935). Gardner and Turner (1933), Lyons and Catchpole (1933), and Fevold, Hisaw and Leonard (1931), used slightly different methods. Bergman and Turner (1937) made a comparison of the four methods then commonly in use. White, starting with the acid-acetone technique of Lyons (1936 - 37 a) obtained a crystalline preparation (White et al, 1937). Lyons (1937) and Schwenk et al (1943) also developed methods for the isolation and purification of prolactin which White (1943) showed to have about the same potencies on careful assay. C. H. Li, working in the same group as Lyons, began a careful study of the chemical and physical properties of this purified prolactin. In ten years a considerable

mass of information was thus collected; (Li et al, 1940 a) electrophoretic behavior; (Li et al, 1940 b) comparison of electrophoretic behavior of beef and sheep prolactin--no difference found; (Li et al, 1940 c) tyrosine and tryptophane content; (Li et al, 1941 c) solubility of sheep and beef hormone; (Li et al, 1941 b) reactions with iodine, (Li et al, 1941 a) molecular weight; (Li et al, 1942 c) a method of isolation; (Li, 1942 b) diffusion and viscosity measurements; (Li, 1943) content of sulfur amino acids; (Li, 1944) effect of a detergent; (Li and Kalman, 1946) reactions with ketene; (Li and Fraenkel-Conrat, 1947) effect of esterification with methyl alcohol; (Li, 1949) the amino acid composition of sheep lactogenic hormone; and finally, (Li et al, 1949 b) lack of effect of the lactogenic hormone upon organ weights, nitrogen and phosphorus balance, and the fat and protein content of liver and carcass in male rats. White and Lavin (1940) studied the ultra-violet absorption spectrum, and White et al (1942) and White (1943) gathered up in a review what was then known of the chemistry.

By 1937 or 1938, so many things had been claimed for prolactin that Riddle in writing a review the following year (Riddle and Bates, 1939), listed 8 separate functions which he pointed out included primary, as well as secondary and even tertiary effects. His list included the following biological functions: lactogenic, crop gland stimulation, maternal behavior induction, antigonadal action, elevation of basal metabolic rate, stimulation of body growth, splanchnomegaly, and increased carbohydrate metabolism.

Later, about 1940, a luteotrophic function was added also by Astwood (1941) and Evans et al (1941). However, as the available preparations of prolactin became purer, many of these initial claims had to be modified. Li et al (1949) found that pure prolactin did not induce splanchnomegaly, and the luteotrophic action of prolactin is currently under investigation in France (Desclin, 1948-1949; Mayer and Klein, 1949 a; Mayer and Klein, 1949 b; Mayer and Klein, 1949 c; Chambon, 1949). Probably, as Li et al (1949) point out, the whole biology of prolactin will have to be reassessed, using the purest preparations available if exact knowledge of what prolatin does and does not do is to be on a sound footing.

However, for the problem at hand, this matter of biology is beside the point, for the work to be reported here deals with the immediate behavior in the mouse body of an iodinated radioactive material which is presumably pure, and since the time elements involved are never more than 2 hours, and mostly less than 30 minutes, the functional effects of the hormone have not been considered. This approach also has been used by Sonenberg et al (1951 b) who reported their findings late in 1951 and at a time when this work was nearing completion.

The approach used here has been to study the immediate distribution of a small amount of radio-iodo-prolactin injected intravenously into the mouse, by following the radioactive label,

I^{131}. The results obtained would appear to indicate that such a material is rapidly catabolized, for by 30 minutes, as will be shown, 50% has been destroyed. This finding alone indicates that in this species at least prolactin must exert its influence very rapidly and is destroyed in the process.

EXPERIMENTAL METHODS AND RESULTS

INTRODUCTION

The aim was to prepare a radio-iodo-prolactin which would behave like natural prolactin.

To follow physiological amounts of prolactin in an animal body, a radioactive prolactin was required since prolactin itself has no distinguishing characteristics. Prolactin contains tyrosyl radicals (Li et al, 1940[c]) which could be readily iodinated (Olcott and Fraenkel-Conrat, 1947) with iodine--stable I^{127}, or radioactive I^{131}. Since I^{131} prolactin would be traceable by detection of the emission of beta rays by the I^{131} (detected either physically by Geiger Muller tube, or autographically by action of beta rays on photographic emulsion), the behavior of I^{131} prolactin could be used to follow iodinated prolactin but not prolactin itself necessarily. If, however, an iodinated prolactin could be prepared which resembled physiological prolactin in behavior, then trace-labelled I^{131} prolactin mixed with I^{127} iodinated prolactin could be said to behave like prolactin which could then be followed by I^{131} beta rays. But fully iodinated prolactin was almost biologically inert (Li et al,1941[a]). It was thought that minimal iodination would cause minimal reduction in biological behavior. Were it possible to prepare such a minimally iodinated I^{127} prolactin incorporating trace-labelled I^{131} prolactin, very small amounts, i.e. within presumed physiological range, could be injected

and the behavior of prolactin studied by tracing I^{131}. Such a preparation should contain iodine insufficient to affect the iodine balances of the body, and radioactivity insufficient to cause significant radiation damage. Therefore such an $I^{127 + 131}$ prolactin, hereafter designated I*P, was prepared.

PREPARATION OF I*P

The following materials were available and were prepared for use.

PREPARATION OF REACTANTS:

Prolactin:

Pellets, 10 mgm each, of International Standard Prolactin, Sample No. AP3004, with a biological potency of 10 International Units per mgm, were available and were used in preliminary work. This material was not a pure preparation, having a contaminant(s) whose properties were unknown but presumably those of biologically inert protein. A pH of 12, and at least 1½ hours were required to dissolve one pellet in 0.2 cc 1N NaOH. Subsequently, 0.8 cc of water could be added; but such a solution was never too satisfactory: always there were undissolved particles.

Doctor Schwenk of the Shering Company kindly gave the laboratory a gram of lyophilized purified prolactin, hereafter simply designated prolactin, extracted from sheep pituitaries. This preparation

had a potency of 24 International Prolactin Units per mgm. Its specifications as to its biological potency were not checked but accepted as stated. The material was kept at room temperature in the vial in which it was received, removed, and weighed with the precision prescribed in a quantitative chemistry laboratory. Care was required, for this fluffy white powder was lost in slight air disturbances. Weighing was therefore done within the housing of the Gram-atic balance and on filter paper from which the prolactin was transferred directly into, and packed down in, the test tube in which the I*P was to be prepared. Since many calculations were based on the weight of prolactin, a microkjeldahl check was run by Dr. Neiman at the Medical Laboratories of the Royal Victoria Hospital. Of two 1 mgm samples so weighed, 1.031 and 0.996 mgm of protein were found, an accuracy of better than $\pm$ 0.05 mgm.

In order to use prolactin, it had to be dissolved. It was not soluble in pure water nor in solvents of increasing pH. Not until strong alkali was tried, was solution obtained. A pH of at least 8 was necessary for initial solution, but once dissolved, prolactin could be kept in solution to a pH as low as a pH of 7.2; below this, it precipitated. This behavior suggested the formation of a sodium salt which was more soluble than the initial material. As a consequence, for each 5 mgm to be dissolved, 0.25 cc of 0.1N NaOH, + 0.25 cc of distilled water, and 0.5 cc of 0.5M Na_2HPO_4-KH_2PO_4, pH 7.6, buffer was

added to the weighed dry powder. (The 0.5M phosphate buffer was made by weighing and dissolving 6.81 grams of KH_2PO_4 in 100 cc of distilled water and 2 grams of NaOH pellets in 100 cc of water. To 50 cc of the former solution, 42.8 cc of the latter solution was added and the whole diluted to 200 cc.). 0.2 cc of such a solution contained 1 mgm of prolactin. Prolactin was found to be readily soluble also in strong acid, 1 mgm in 0.02 cc of 1N or 5 N HCl. This indicated that its hydrochloride was even more soluble than its sodium salt. This latter method of solution was used in more recent experiments as a means to reduce volume and to avoid transference of small volumes, for acid was a necessary reagent in release of I_2, and prolactin contained no cysteine radicals which would react with iodine at such a pH(Li, 1943).

The second necessary reagent was radio-active iodine.

Radio-active Iodine

Tellurium is bombarded with neutrons in the atomic pile (Ballantine and Cohn, 1947) at Chalk River. The metal is dissolved in acid and the iodine distilled off and collected in an alkaline solution which is neutralized to a physiological pH (Hevesy, 1948). The I^{130} (Siri, 1949) and the Te^{131} (B & C, 1947)* also present have such short half-lives, 12.6 and 30 hours respectively, that they will have disintegrated to Xe^{130} (Evans, 1947) and I^{131} (Ballantine and Cohn, 1947) which has a half-life of 8 days (Evans, 1947) before

* (B & C, 1947) = (Ballantine and Cohn, 1947)

use was made of these radioactive solutions. Thus the only isotope used in this work was I^{131}.

The material came to the laboratory regularly in an aqueous solution as NaI^{131}, sodium iodide. The actual weight of iodine present was usually less than could be detected by the usual chemical methods; in other words, it was usually "carrier free" and contained only radioactive isotopes of iodine. The volume containing 1 mc varied widely from shipment to shipment.

Since disintegrations of radioactive isotopes in aqueous solutions cause formation of hydrogen peroxide (Krenz, 1948), a reducing agent, bisulfite, was added as a counteractant. In the case of iodine, this was necessary for H_2O_2 (hydrogen peroxide) oxidized iodide ions to molecular iodine which then volatilized and was lost. For this work, iodide ions had to be oxidized to molecular iodine, and bisulfite ions were a contaminant which had to be removed. Therefore, the I^{131} solution as it came back from Chalk River was distilled by Chaney Distillation, as modified by Dr. Jack Gross of this Laboratory (1949) for use with the small amounts of iodide contained in such radio-active solutions. Of an initial quantity of I^{131}, 10 $\pm$ 5% was recovered in the distillate when 1 cc 0.01N NaOH was used in the trap, as compared to 80 $\pm$ 5% when 1 cc of 0.25N NaOH was so employed. This last proved a hindrance for the distillate (10-20cc) had to be evaporated to 0.1 cc or less (a time-consuming necessity), which also concentrated the alkali to strengths of greater than 2N.

Such manipulations meant loss of radioactivity as can be seen from the following typical example: Of 4.41×10^8 counts distilled, 3.58×10^8 were found in the fresh and 3.23×10^8 counts in the concentrated distillate, or a total loss of 28% of the initial radioactivity. Furthermore, to use this I^{131}, it had to be converted to molecular iodine, which required the presence of excess acid. In the volumes employed, to obtain such an excess acid, 5N strength had to be used to counteract the alkali present in the I^{131} distilled concentrated solution. This had two disadvantages: heat evolution, and the enhancement of the volatilization of the I_2 so formed. Also, it had been found at Oak Ridge that I^{131} was strongly adsorbed to glass in acid media, and since such small amounts were present in these samples, this factor undoubtedly played a large role in the otherwise unaccountable losses. Thus, in summary, to get rid of H_2O_2, bisulfite had to be added; to get rid of bisulfite, I^{131} had to be distilled; to retain distilled I^{131}, strong alkali had to be used; to counteract this strong alkali, strong acid had to be employed, which, in turn, enhanced the loss of $I_2{}^{131}$ both by volatilization and glass adsorption. To allow for these losses, at least twice as much I^{131} as was needed for a given experiment had to be available initially.

The third necessary reactant was molecular iodine (stable, $I_2{}^{127}$, and radioactive $I_2{}^{131}$, hereafter designated $I^*{}_2$).

Release of Molecular Iodine

To iodinated prolactin, the following two reactions were used routinely:

and

$$5\ KI^{127}(+\ NaI^{131}) + KIO_3 + 6HA \longrightarrow 6KA + 3H_2O + 3I^{*}_2$$

where  stands for the tyrosyl radical, and R for the rest of prolactin; I_2 stands for molecular iodine;  for radioactive di-iodo-tyrosyl radical; KI for potassium iodide; NaI^{131} for sodium radioactive iodine; KIO_3 for potassium iodate; HA for any acid; KA the potassium salt of the acid used; and H_2O for water. From the first equation: per molecule of prolactin, 2 molecules of molecular iodine are required for each tyrosyl radical to be iodinated. From the second formula: once the amount of iodine is known, the amount of KI and KIO_3 needed to produce this amount of iodine can be calculated.

Calculation

To calculate the amount of molecular iodine (the weight of I^{131} was never considered; too little was present (Leblond, 1951)) required for a determined amount of prolactin, the following facts were needed:

1. The number of tyrosyl radicals/molecule prolactin.
2. The number of prolactin molecules/gram prolactin preparation.
3. The number of iodine molecules needed to react with one tyrosyl radical.
4. The quantitativeness of the interaction of KI and KIO_3 in forming I_2.
5. The quantitativeness of the reaction between I_2 and the tyrosyl radical of prolactin.
6. The amount of KI and KIO_3 required.
7. The volume of the I_2 forming mixture.

In 1947, when the work was undertaken, the number of tyrosyl radicals per molecule of prolactin was unknown. For beef and sheep prolactin, the proportion by weight was known (Li et al, 1941[a]).

The molecular weight of prolactin was unknown; it had been set between the extremes of 16,000 and 35,000 (White, A, 1946).

The number of iodine atoms necessary to convert tyrosine to di-iodo-tyrosine was 4.

The completeness of the reaction between KI and KIO_3, in the presence of excess acid, was 100%. (This reaction is used to standardize sodium thiosulfate solutions for quantitative analyses. (Handbook of Chemistry and Physics 1945, 29th Edition, p. 1337 Nos. 27&28).)

The completeness of the reaction between tyrosyl radicals and available iodine was not known; it was assumed to be 100%, for Li, Lyons and Evans claimed in 1941 that "In about 45 minutes, iodine was completely used up by tyrosine, when equal moles of iodine and tyrosine were dissolved in 6.66 M urea solutions of pH 6.85 with 0.1M phosphate buffer at room temperature. Similarly, 1 per cent lactogenic hormone[7] ([7]Since the beef hormone contains 5.84 per cent

tyrosine, 1 per cent hormone solutions is equivalent to 3.2×10^{-3} mole of tyrosine. Therefore, 6.4×10^{-3} mole of iodine is required to iodinate completely the tyrosine groups in the hormone.) in the same solvent takes up 2.65×10^{-3} mole of iodine in about 40 minutes. The solution (after being dialysed) was analyzed for iodine and free tyrosine. The iodine was found to be 3.4 per cent; the calculated value based on the free tyrosine (3.0 per cent) was 3.6 per cent, which checks well with the value obtained from the iodine originally added to the reaction mixture. It thus appears that iodine acts only on the tyrosine group of the hormone and that the reaction kinetically resembles that involved in the formation of di-iodo-tyrosine." (quoted from page 49 of Studies on Pituitary Lactogenic Hormone, V. Reactions with iodine. J. Biol. Chem. 1941. 139, 43-55.)

The volume of solution to be used to release iodine, and hence the concentration of the KI and KIO_3 solutions, was subject to manipulations. During the course of this experimental work, they were changed several times.

On the basis of these assumptions and the facts stated above, calculations as shown in Table 1, were set up.

Conditions for Obtaining Molecular Iodine.

The gamma quantities of iodine (Column XIII) used to iodinate prolactin were obtained by employing minute volumes (Column XV) of accurate solutions of KI and KIO_3 (Columns XVI and XVII). The

For Table 1, see pocket at back.

preparation of these precise solutions depended on careful weighing and quantitative transfer of dissolved materials to accurate volumetric flasks, filling exactly to the mark, and mixing thoroughly by inversion twenty or more times.

To measure the volumes required (Column XV), graduated pipettes of 0.1 or 0.2 cc were employed. The pipette was filled by mouth suction (for solutions containing more than the minutest amounts of radioactivity, tuberculin syringes or pipettes fitted with an adapted greased 5 cc syringe were used) and stoppered by the ball of the thumb. Fluid was allowed to run out until the fluid miniscus just rested on the zero mark; the pipette was dried with kleenex -- all radioactive work was carried out on white enamel trays covered with kleenex to minimize radioactive contamination-- the wiped tip was carefully inserted into the small test tube where the reaction was to take place, and the volume required was allowed to leave the pipette slowly, by free dropping. When the exact amount had been delivered, the tip of the pipette was touched to the side of the test tube and the pipette carefully withdrawn. The pipette was read before, during, and after measurement, and as a rough check on accuracy the fluid level in the graduated test tube was observed after each such addition.

Once the amount of iodine had been calculated and the KI and KIO_3 solutions made, mixing of the distilled I^{131} and KI was

the next step-- the validity of labelling of carrier iodine depended on this for I^{131} and I^{127} have identical chemical behaviors.

The following reagents were placed in a graduated centrifuge tube in the following order: the acid, the KI, and the I^{131} solutions. The centrifuge tube was grasped lightly between thumb and index finger of the left hand and the tip of the tube struck sharply several times by the index finger of the right hand, thus agitating the small volume in the tapered tip of the centrifuge tube and so thoroughly mixing the ingredients. The required amount of KIO_3 was added, the tube agitated, and appearance of yellow I_2 colour was observed. If the various concentrations were correct, this yellow colour appeared rapidly and in proportion to the amount of iodine available for release. The real stumbling block to the release of iodine was eventually traced to the alkali in the distilled concentrated I^{131} solutions, a troublesome variant both to control and to assess. With the entire distillate, a small volume of concentrated acid, 0.07 cc of 5N HCl, was used. If less distillate was needed, the amount of acid was calculated accordingly.

After these three reactants had been so prepared, the iodination of prolactin could be undertaken.

IODINATION OF PROLACTIN

Theoretical -Li et al's Conditions for Iodination of Prolactin.

For full iodination of 100 mgm of the prolactin preparation (L283 B), Li et al (1941)[a] used the following conditions:

a. A 10% final concentration of the protein.
b. In an 0.25 M Na_2HPO_4-KH_2PO_4, pH 7, buffer,
c. A 2.5×10^{-2} N I_2 in a 0.125 N KI solution,
d. Room temperature,
e. Reaction time - one hour.

Experimental Conditions as used here:

Prolactin, as previously shown, was not soluble, even after initial solution in strong alkali, at a pH lower than 7.2. Therefore, 1 mgm was dissolved in 0.05 cc 0.1N NaOH + 0.05 cc H_2O + 0.1 cc 0.5M Na_2HPO_4-KH_2PO_4, pH 7.6, buffer, or a total volume of 0.2 cc and a concentration of 5%.

To this 0.2 cc solution containing 1 mgm of prolactin was added 0.2 cc containing I_2^*. The concentration of I_2 was reduced, for minimal rather than full iodination was desired; no excess iodide (KI) was present. In the 0.2 cc solution, the concentration was of the order of 2×10^{-5} N, an approximate figure for the actual weight of I^{131} was not included. Since excess acid was required to release I_2 from KI and KIO_3, and since I^{131} had been trapped in 1 cc of 0.25 N NaOH during distillation and subsequently concentrated to 0.1 cc or less, i.e., a concentration of 2.5N for 0.1 cc of distilled

concentrated I^{131}, 0.07 cc of 5N HCl was employed. The interaction of this HCl and NaOH should give a concentration of 1.25N NaCl in 0.2 cc, in which a negligible amount of KI was dissolved (approximately 2.5×10^{-5} N).

Since the tyrosyl radicals of prolactin will react best with I_2 at a mildly alkaline pH (the reaction of tyrosine with iodine has been shown by Li (1942)[a] to be most rapid at such a pH), and since the combination of the prolactin solution and the acid iodine-releasing mixture resulted in an acid solution, sufficient alkali had to be added to bring the pH back to that of the buffer (pH 7.6) in which prolactin was dissolved. For this purpose 0.04 cc of 0.5 N NaOH was added.

When this combination: 0.2 cc of prolactin solution, 0.2 cc of I_2-releasing mixture, and 0.04 of 0.5N NaOH, had been effected, the iodination of prolactin was allowed to proceed for at least 20 minutes at room temperature.

In order to study the yield of $I^{*}P$, and in fact to use it at all, a standard way of determining the emission of the beta rays of I^{131} had to be set up. (In the amounts to be so studied the emission of beta rays was the only measurable quantity.)

Beta rays can be detected, as has been said earlier, either by Geiger Muller tube or by photographic emulsion. The

latter was applicable to tissues of animals injected with I^{131}-containing materials; the former to chemical analyses as well. To use the G.M. tube to follow chemical reactions, "plates" had to be made and "counted".

Determination of I^{131} content of materials:

Plating:

All materials to be plated, unless already in solution, were digested in 2N NaOH with or without the aid of heat. 2N NaOH was chosen for the following reasons: 1. It was a known protein digestant if used in sufficient quantity for sufficient time and in the presence of heat if necessary; 2. NaOH was a chemical of known composition, and therefore a given volume of a given concentration would contain a given amount of solid material when dried; 3. Suitable drying converted NaOH to sodium carbonate (Na_2CO_3), which was non-hydroscopic, hence, the water content of the plate would not change upon exposure to air of different humidities, a troublesome variant in laboratories not air-conditioned.

Plates were made by evenly distributing on a 14 mm watch glass an aliquot of the solution to be counted. The concentration of the solution was brought to the equivalent of 1 cc 2N NaOH and left to dry at 37°C. until carbonatation had occurred. Plates of the standard (s) -- a dilution of the initial I^{131} material used to iodinate the prolactin and/or the $I^{*}P$ used for animal work--

were made at the same time in the same way, except that if the I^{131} standard was "carrier free", a 2N NaOH solution containing 10 gamma I^- as NaI was used to standardize the solid content of the resulting plate. Theoretically, the addition of the "carrier" iodide (NaI) reduced the loss by volitalization of the radioactive iodine (I^{131}). Duplicate and sometimes triplicates of each sample were plated. After carbonation, the plates were counted.

Counting:

Duplicate or triplicate plates were each counted twice for at least 1000 counts and/or for 100 seconds. Whenever possible, all of one experiment was counted in one day, in the same castle, and at the same distance from the Geiger Muller tube. These counts were indicative of relative rather than actual amounts of radioactivity present, only a small fraction of which, about 8%, was recordable.

The automatic control on the Berkley Decimal Scaler used was set to stop recording after a given number of counts. The time in seconds required for these counts (A count was equivalent to the emission of one beta ray by the disintegrating nucleus of one I^{131} atom) (See Appendix) as well as the number of counts counted were recorded. All counts were then adjusted by division or multiplication to the time required for 1000 counts, and recorded. By reciprocal, the count for 100 seconds was recorded, corrected for

coincidence, and for per cent error (see Appendix). From this corrected count, the background of the day for the castle used was subtracted, giving the "true", or here called "Plate", count for that aliquot of that sample. Since several, two at least, counts per aliquot were taken and several aliquots of the same plated, the plate count of these several samples were averaged and the average used for further calculations.

Below is a table showing the actual counting of one set of triplicate plates, each counted in duplicate: The background for that day was 21.

Table 2

Conversion of time for 2000 counts to corrected count per 100 seconds.

Sample	Total count	Seconds	Time 1000 counts	Counts 100 sec.	Corrected Count	Average
1	2000	161	80.5	1242.2	1221	
	"	161	"	"	"	
2	"	174	87.0	1149.4	1128	
	"	167	83.5	1197.6	1177	
3	"	178	89.0	1123.5	1103	
	"	178	89.0	1123.5	1103	
						1159

This table indicates the type of variation found with same duplicate plates of the sample, as wellas the variation found in counting the same plate successively.

Corrections

To correct for errors in the performance of the counting apparatus a uranium standard sample (Uranium has such a long half-life that any one sample can be counted time and again over long periods of time without appreciable change in the count obtained.) was counted routinely morning and night, and often at midday as well. When deviations from the known norm for that standard sample of uranium were found, such deviations could then be used to determine the degree of deviation of the counting apparatus and the proper corrective applied to the counts obtained during the period in question.

If different distances from the thin mica end-window of the G.M. tube had been used, the same plate counted under both conditions provided a corrective by which all counts could be reduced to those of a given distance from the tube.

If different castles had been used, two counts of the same plate, one in one, the other in the other castle, provided the necessary corrective..

The decay of I^{131} is exponential and its half-life is 8.0 days (Hevesy , p. 22, 1948). A table was set up showing the per cent activity remaining after daily intervals. Suitable application of these percentages were used to reduce all plate counts to that

of a given day when an experiment had been counted over a number of days. The following set of figures were obtained for successive days counting of two samples.

Table 3

Effect of Time on Plate Count/100 seconds of Two Samples

Day	Uranium Standard Count (Av.of 2)	Uranium Standard Average (of 4)	$I^{m}P$ Standard Found Count (Av.of 6)	$I^{m}P$ Standard Average (of 12)	$I^{m}P$ Standard Theoretical	$I^{m}P$ Standard Per Cent Decay	Background (Av. of 2)
2	AM 4026		237				
	PM 4045	4035	221	229	220	84.05	22
5	AM 3994		158				
	PM 4009	4002	157	157	157	65.0	23
6	AM 4010		149				
	PM 4043	4026	145	147	143	59.5	22
7	AM 4061		134				
	PM 4061	4061	130	132	131	54.5	21
8	AM 3992		120				
	PM 4142	4067	120	120	120	50.0	24
9	AM 4043		111				
	PM 4035	4039	108	109.5	110	45.8	21
		Av.4038					Av.22

From the above table it will be seen that the Uranium standard was found to be 4038 $\pm$ 36, or an overall error of 0.893%, with a daily spread of from 0 to 50, and an average of 21, or a daily error of 0.52%. The background, likewise, shows a spread of 3 counts / 100 seconds, an error of 6.8%. Furthermore, it will

be seen that the I*P standard varied from its theoretical by a range of + 9 to -3, and that it varied only half of the time. The corrected count, and these represent averages for duplicate readings of triplicate plates taken morning and evening, shows a variation of not more than + 8 to + 0.5, or an average of 2.3 counts in a day, a % of about 1 in the plate counts. This is to be expected for I^{131} decays exponentially to 50% in 8 days, and it will be noted that each AM count is higher than the corresponding PM count. The average, therefore, theoretically represented the count at the middle of the counting period.

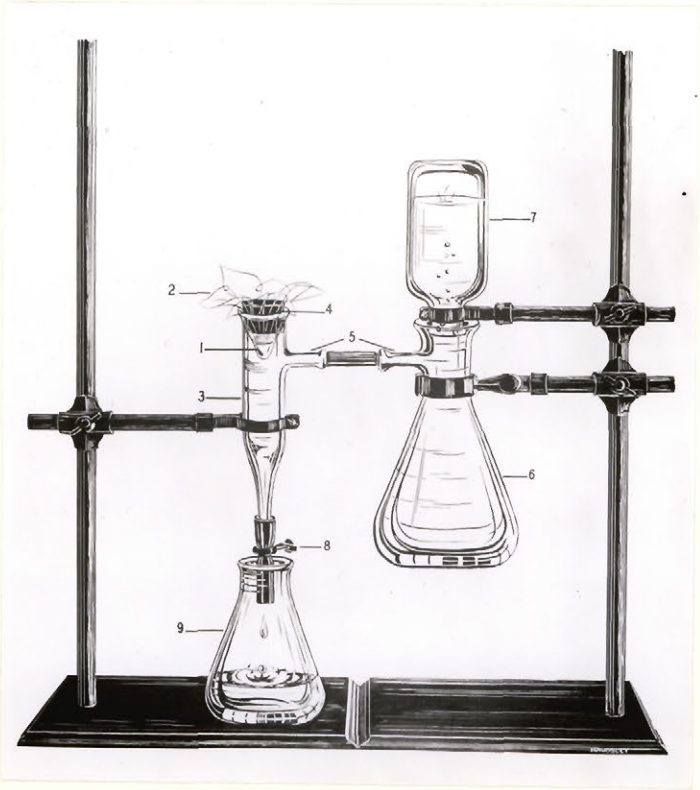
Of the I*P so prepared and measured, certain facts were desired. In the first place, it was necessary to establish that prolactin so iodinated was in truth iodinated. For this, dialysis was used.

Dialysis- To prove I*P truly iodinated

Small amounts of I*P and prolactin containing I* were dialysed in the apparatus illustrated in Fig. 1 . 1 cc or less, containing 0.5 to 1 mgm of protein, was put into a sac made by depressing a suitable square of cellophane into the top of the dialysing chamber and held in place with a rubber stopper. The dialysing chamber-- a modified, large centrifuge tube---, the side arm, and the suction flask were filled with the dialysing fluid-- 0.5 M Na_2HPO_4-KH_2PO_4, pH 7.6, buffer-- hereafter called pH 7.6

Fig. 1. Drawing of the apparatus used in the dialysis of small amounts of $I^{\pi}P$.

1) dialysis sac
2) cellophane square
3) dialysing chamber
4) rubber stopper
5) side arms
6) suction flask
7) 250 cc bottle reservoir
8) pinch clamp
9) Erlenmeyer flask


1
2
3
4
5
6
7
8
9

/

phosphate buffer, in such a way that the fluid surrounded the sac as high as the level of the bottom of the rubber stopper. Into the neck of the suction flask, a 250 cc reservoir, also filled with the same fluid, was inverted and so held that the bottom of its neck was on the same level as the bottom of the rubber stopper. By adjustment of the pinch-clamp on the rubber outlet tip of the dialysing chamber, a constant flow of fluid around the sac was maintained. The dialysate was collected in an Erlenmeyer flask which was changed at appropriate time intervals.

Experiment 1.

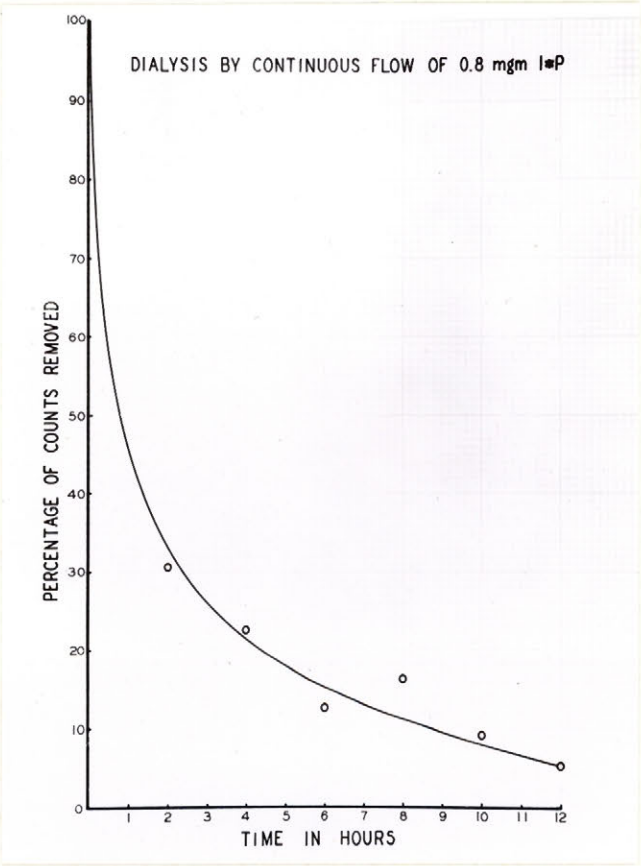
0.8 mgm of $I^{\alpha}P$ was thus dialysed for twelve hours against a continuous stream of pH 7.6 phosphate buffer. No record was kept of the flow rate. The dialysates were collected at 2-hour intervals, alkalinized, evaporated to dryness, made up to 10 cc, and 1 cc aliquots were plated and counted. From Fig. 2 a progressive fall in the activity of the dialysates was found. At the end of 12 hours, all but 8% of the initial activity used to iodinate the protein had been removed.

A question as to whether this was an adequate time for dialysis led to the following experiment.

Experiment 2.

Another sample of 0.8 mgm of $I^{\alpha}P$ was similarly dialyzed

Fig. 2. Curve showing percentage of counts removed from 0.8 mgm of I^{n} at 0-12 hours by continuous flow dialysis.


DIALYSIS BY CONTINUOUS FLOW OF 0.8 mgm I•P
PERCENTAGE OF COUNTS REMOVED
TIME IN HOURS
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12

2

for seventy-one hours. At the end of this time, 12.2% of the initial activity was found in the dialyzed protein. At the end of the first 24 hours, 42.3% of the initial activity was in the protein, at 48 hours, 29% was still retained. The flow rate for the first 24 hours had been 15 cc per hour, for the second 24 hours, 5 cc per hour. The latter flow rate was continued until the 70th hour, at which time the % activity remaining with the protein was 25%. In the last hour, the flow rate was stepped up to 200 cc an hour, and 50% of the remaining activity was removed. The dialysis had been carried out at room temperature, thus allowing the possibility of breakdown of the protein; this could explain the rapid drop in the last hour.

As a control, the third experiment was run.

Experiment 3.

0.8 mgm of prolactin, to which had been added an amount of I^{α} equivalent to that used to iodinate the sample dialyzed in experiment 2, was similarly dialyzed for 24 hours. A flow rate of 5 cc per hour was maintained for the first 18 hours, at which time 10% of the initial activity was retained in the protein. The flow rate was then increased to 30 cc an hour, for 3 hours, at which time the retained activity was 7.5%. The flow rate was again increased to 60 cc per hour for 3 hours, and the retained activity at the end of this period- i.e. 24 hours after the initiation of dialysis,

was 3.2%.

Discussion:

From the first experiment it can be seen that the removal of iodide from $I^{*}P$ was steady and exponential in character for the first 12 hours. From the second experiment, in which the iodination had resulted in a greater incorporation of the available iodine than in Experiment 1, only a little more than 50% of the I^{*} had been removed, while, at the same time, with 10 times less fluid used to dialyze, all but 3.2% of the non-incorporated I^{*} had been removed (Experiment 3). From these findings it was concluded that the iodination procedure was causing a true incorporation of I^{*} onto prolactin and not a mere adsorption.

Such dialysis was useful to demonstrate the incorporation of iodine onto prolactin but was too time consuming for routine separation of iodo-prolactin. Precipitation by acetate buffer at the isoelectric point of iodo-prolactin was therefore used.

PURIFICATION OF $I^{*}P$ ISOELECTRICALLY.

A protein is known to be most insoluble at a pH where the

ionization of its acidic and basic groups are equal in number. This pH is known as the isoelectric point, and this point is a characteristic of any protein. Usually the isoelectric point is determined by electrophoresis, as that point at which no movement of the protein molecules takes place in an electrical field.

Li et al (1941)[a] has shown that the isoelectric point of prolactin was 5.8 and of fully iodinated prolactin 4.8. (Iodination of phenolic radicals renders them more acidic (Neuberger, 1934). For minimal iodination, a pH of 5.4, and later of 5.7, obtained with an 0.2 N NaAc-HAc buffer was used for precipitation of I^{0}P made with Schwenk's purified prolactin. (For I^{0}AP3004, 10% acetic acid (dissociation constant, 1.75 x 10-5) was used in preliminary work and proved satisfactory. However, I^{0}P made with Schwenk's purified prolactin dissolved in 1% acetic acid used as a wash). The 0.2N NaAc-HAc, pH 5.4 or 5.7, buffer proved a satisfactory reagent, giving a clean precipitate easily handled.

Prolactin and its iodinated form were handled in volumes of 1 cc or less by alternate solution and precipitation. To remove reagents no longer desired, the precipitated protein was spun down by ordinary laboratory centrifuge and the resulting supernatant carefully pipetted off with a Pasteur pipette-- a length of glass tubing drawn into a fine capillary tip, fitted with a rubber eyedropper bulb, the latter allowing precise control of fluid removal

from 1 cc centrifuge tubes made by sealing off the end of a short length of pyrex glass tubing. Convenient lengths of glass rod drawn into a fairly fine point served as a mixing tool. Since small volumes were being employed, one stirring rod, centrifuge tube, and Pastuer pipette only were used for each preparation of I*P. By thus reducing the glass surface necessarily exposed to these small volumes, the loss of fluid and of I^{131} was kept to a minimum. Furthermore, aliquots rather than total samples were used to determine the amount of radioactivity per sample, since the quantitative transfer of material was more subject to error than that inherent in taking 1 cc aliquots of dilutions.

In the investigation of purification of I*P by isoelectric precipitation, the first point was how many washes were required?

Experiment 1.

1 mgm of prolactin was iodinated using 33.4 of I_2^* containing 24,00 counts/min. I*P was precipitated from 0.54 cc by 0.5 cc of 0.2N NaAc-HAc, pH 5.4, buffer, hereafter referred to as 5.4 acetate buffer. The mixture was centrifuged, the supernatant pipetted off, plated and counted. The precipitate was washed ten times with 0.1 cc of the 5.4 acetate buffer, centrifuged, the wash pipetted off, plated and counted. The remaining precipitate was dissolved in 2N NaOH, plated and counted. The % of the initial activity present in the various fractions are recorded in Table 4.

Table 4

EFFECT OF 10 WASHINGS AT ISO-ELECTRIC POINT

ON PURIFICATION OF RADIO-IODO PROLACTIN

	Counts/min.	% loss	
Total activity	24,000		
Supernatant	16,000		
Precipitation	8,000	33%	
Wash I	700	8.8	
	7,300		
II	251	3.4	
	7,049		
III	115	1.6	
	6,934		
IV	104	1.5	
	6,830		
V to VII	185(93)	2.7	0.9
	6,645		
VIII	70	1.1	
	6,574		
IX	47	0.7	
	6,528		
X	72	1.1	
Precipitation	6,456	12.1%	

In this particular experiment, 66.67% of the original activity was removed by the first supernatant, 8.8, 3.4, and 1.6% by the first three washes respectively. Thereafter, about 1% was removed per wash. The final precipitate contained 12.1% of the initial activity. From this experiment it was decided that three washes were sufficient.

From other work carried out in the laboratory: considerable radioactivity adsorbed onto precipitated material could be

removed by reprecipitation. This idea was tested in Experiment 2.

Experiment 2.

3 mgm prolactin were iodinated by 378 gamma I_2" containing 53,125 counts/min. I"P was precipitated with an equivalent volume of 5.4 acetate buffer, centrifuged, washed twice, and dissolved in 1.6 cc of 0.5N NaOH. An 0.2 cc aliquot was taken for counting, the remainder was reprecipitated with an equal volume of 5.4 acetate buffer, washed a third time, and dissolved in 2N NaOH. Aliquots of the various fractions were counted. The counts/min. and the % radioactivity of that originally present are recorded in Table 5.

Table 5

EFFECT OF ISO-ELECTRIC PRECIPITATION AND REPRECIPITATION

	Count/min.	%radio-activity
Iodine Mixture	53,125	100%
1st supernatant	43,475	83.5
1st wash	2,095	4.0
2nd wash	580	1.1
2nd supernatant	3,482	6.7
3rd wash	0	0.0
Dissolved radio-iodo-prolactin	2,298	4.4
		99.7

83.5% of the activity in this case was removed in the first supernatant. 5.1% in the two succeeding washes, 6.7% in

the second supernatant, and none in the third wash, leaving 4.4% in the final $I^{\beta}P$. 99.7% of the activity was recovered.

Experiment 3.

As a check on the effectiveness of the above purification procedure, quadruplicate samples of 1 mgm of prolactin each, to which I^{β} had been added, were subjected to a similar purification. The counts obtained and the % activity retained in the protein fractions are recorded in Table 6.

Table 6

RETENTION OF RADIO-IODIDE IN ISOELECTRIC PRECIPITATION METHOD FOR PURIFICATION OF RADIO-IODO-PROLACTIN WHEN ADDED TO PROLACTIN AT 20°C

Sample	Counts	% Activity retained in protein fraction
A1-combined supernatants and washes	17,800	
prolactin	60	0.33
B1-combined supernatants and washes	16,100	
prolactin	80	0.49
C1-combined supernatants and washes	17,280	
prolactin	40	0.23
D1-combined supernatants and washes	14,100	
prolactin	00	0.00
		Av. - 0.26%

99.74% of the activity had been removed.

On the basis of these findings, this method of purification was adopted and became routine in all subsequent preparations of I*P.

Removal of Histydyl-bound iodine

The iodination of prolactin as here used should result in preferential iodination of tyrosyl radicals (Olcott and Fraenkel-Conrat, 1947). However, the possibility remained that histydyl radicals might also react under these conditions. In order to rule out such iodination, at the suggestion of C.H. Li, after a suitable time elapsed for the iodination of the tyrosyl radicals, the reaction was stopped by the reacidification of the reaction mixture by addition of 1N HCl. Subsequent purification of four samples of prolactin, two so treated and the others not acidified, resulted in the % Yields recorded in Table 7.

Table 7

EFFECT OF ADDING STRONG ACID BEFORE PRECIPITATION OF R.I.P.

(TO REMOVE I^- ATTACHED TO HISTIDINE)

	With acid		Without acid
A	19.1% yield	C	22.42% yield
B	22.42% yield	D	18.77% yield
Average	20.76	Average	20.59

Table 7 indicates that there is no detectable difference between the samples of prolactin that were acidified after iodination and those that were not. From this it seemed safe to say that any iodination of histydyl radicals which had occurred was negligible. However, such acidification was routinely incorporated in the preparation of $I^{n}P$.

Discussion:

Since the G. M. tube cannot distinguish between I^{131} attached to protein and I^{131} adsorbed or free as a contaminating ion, and since this study was to concern itself with the behavior of $I^{n}P$, removal of non-incorporated I^{n} was essential. As a basis for calculation, and towards furthering the understanding of what we were dealing with, iodination of a single radical, tyrosine, of prolactin, was desired. These two conditions have been fulfilled, for all but 0.25% of non-incorporated I^{n} was removed by the isoelectric precipitation and reprecipitation method, and no iodine was apparently incorporated onto histydyl radicals of prolactin. Prolactin does not contain free SH groups (Li, 1943), which will also react with iodine, though not under the conditions used here, nor will tryptophane (Olcott and Fraenkel-Conrat, 1947), which is also a constitutant amino acid of the hormone (Li, 1940 c).

One further fact was required. It had been noted that the amount pf precipitate diminished considerably during the above

purification procedure, especially when samples of 1 mgm of prolactin were initally iodinated. Therefore, microkjeldahl analyses of two such samples of 1 mgm of prolactin were kindly done by Dr. Nieman.

Protein Loss During Isoelectric Purification

5 mgm prolactin were weighed and dissolved in pH 7.6 phosphate buffer. Two 0.2 cc aliquots (1 mgm prolactin) were quantitatively transferred to microkjeldahl flasks and made up with buffer to 1 cc. Two similar aliquots were iodinated and purified as detailed above. The supernatants and washes were pooled and quantitatively transferred to microkjeldahl flasks. The precipitates were dissolved in 0.2 cc 0.5 N NaOH and also quantitatively transferred to microkjeldahl flasks. These samples were then subjected to analysis and the amount of protein found in each are recorded in Table 8.

Table 8

Mgm of protein as determined by microkjeldahl determinations in samples of prolactin, combined supernatants and washes, and final precipitates of I^{n}P.

Samples	MGM PROTEIN
A. Untreated prolactin	0.986
B. " "	1.013
C. Pooled supernatants and washes	0.505
D. " " " "	0.500
E. Residual purified I^{n}P	0.469
F. " " "	0.500

From these analyses it can be seen that 50% of the protein is lost during purification. However, since isoelectric purification was done at a pH initially midway between that of pure prolactin and fully iodinated prolactin, and since, as will be shown, biological assay of such minimally iodinated prolactin indicated no loss of biological activity and even at times a gain, it was thought that the purification procedure as used not only removed inorganic ions, especially I^-, but also protein contaminants of the original prolactin sample, thereby purifying it.

With all these knowledges in hand, a study could be made of the factors affecting the yield of I*P.

FACTORS AFFECTING YIELD OF I*P

Calculation:

An aliquot of the I_2^{131} used to iodinate a given sample of prolactin was suitably diluted, an aliquot of this dilution was plated and counted as previously described. An aliquot of the purified I*P was likewise diluted, and an aliquot plated and counted. From the figures so obtained, suitably multiplied by the dilution factors, the % of activity found in the I*P as compared to the I_2* used to iodinate the protein could be determined. This figure was called % Yield. The aliquot of the purified I*P dilution was also used as the "Standard" for whatever other fractions

were to result from the use of the purified $I^{*}P$, suitably computed as to volume and amount used.

Theoretical Yield:

As stated previously (p. 3), the immediate aim of this problem was to incorporate iodine onto the tyrosyl radicals of prolactin. To repeat, to accomplish this end, the following conditions had to be met:

1. A suitable concentration of prolactin and iodine had to be provided.
2. A pH such that the iodine would react with the tyrosyl radicals in preference to other radicals.
3. A buffer to counteract the acidity of hydrogen iodide (HI), a by-product of the iodinating reaction.
4. A suitable time and temperature for the reaction to take place.

From the chemical formula involved in iodination (see p. 16), theoretically only half (50%) of the iodine would be available to react with the protein. If a theoretical yield is to be obtained, all the iodine in the reaction releasing I_2 (see p. 16) must be so converted and must not be lost before it comes into contact and reacts with prolactin. There were at least three sources of loss: volitalization from acid solution, adsorption onto glass, and reconversion of molecular iodine to either iodide or iodate ions, neither of which would react with tyrosyl radicals. The first two of these three conditions obtain in acid solutions; the third occurs when the pH of the solution containing the released

I_2 rises above pH 10. Both acid solution and a pH greater than 10 were present in this work; a full 50% theoretical yield was unlikely. Since 50% of the protein is lost during purification, a yield of 25% would represent maximum iodination. Actually, yields of 10 -15% were the average, with an occasional high of 20 + %.

Experimental

Table 9 has been set up to indicate the actual steps carried out in the preparation of any one sample of $I^{*}P$. It has been subdivided into those experiments in which the factors causing a low yield, those causing no appreciable effect on yield, and those which resulted in a high yield have been collected. Reading across the table there are several subdivisions, namely: the sections dealing with

1. dissolution of prolactin - amount used, final volume, and pH
2. iodine-releasing solution - amount, volume of combined KI and KIO_3 solutions, type, concentration, and amount of acid used.
3. I^{131} solution - state, as to whether distilled (D) or not (ND); concentration,as to whether it was evaporated down (bd) or not (nbd); and volume used.
4. Neutralization - as to concentration; volume and time at which NaOH was added, A stands for after buffer was added, and B for before.
5. Conditions of iodination - volume, volume per mgm prolactin, pH, time, and temperature.
6. Acidification to remove iodine from histydyl radicals - as to type and amount of acid added.
7. Purification - as to pH, volume of precipitating agent; volume of wash; strength and amount of alkali; volume of wash; and dissolving alkali, as to strength and volume used.
8. Percent Yield.

For Table 9, see pocket at back.

All experiments, except 17 and 19, were run on single samples; 17 and 19 are the average for two runs. Experiment 20 involved the iodination of 2 mgm of prolactin, one of which was purified by dialysis and the other by isoelectric precipitation. In all experiments, except 18 and 19, the iodine-releasing mixture was used immediately after addition of KIO_3. In 18 and 19, the mixture was allowed to stand for a half hour before use.

In experiment 1, 2% acetic acid was used to release I_2, which it failed to do. The yield was 1.6%, which was actually high for the method of purification was inadequate.

In experiment 2, a concentrated solution of I^0 was used, with alkali in excess of the acid added to release I_2. I_2 was not satisfactorily released, and the yield was 7.72%.

In experiment 3, non-distilled I^{131} was used; contaminating bisulfite ion prevented either the release of I_2 or interferred with its reaction with prolactin. The iodinating pH was less than 7.2 (a faint opalescence was noted), all of which resulted in a yield of 1.19%.

In experiment 4, an excess of alkali was added to the I_2-prolactin solution complex so that an iodination pH of greater than 10 was obtained. This overshooting of the pH converted the I_2 to an unreacting form, and the yield was 8.5%.

In experiment 5, too much acid was added to the I_2-releasing mixture, which was not sufficiently neutralized by the added buffer (prolactin solution). An iodination pH of 4 resulted, and the yield was 4.7%.

Thus either too much or too little acid, presence of contaminating ions, and excess alkalinity all affect the iodination adversely, causing low yields.

Experiments 6 to 17 show that the pH of the precipitating buffer, the time and temperature of initial precipitation, the time allowed for iodination, the addition of the neutralizing alkali before or after the buffer, provided the prolactin was already present, have very little effect upon the ultimate yield. An experimental error of plus and minus 10% is to be expected, and these values are within that range.

In experiments 18 and 19, the iodine-releasing mixture was allowed to stand for half an hour before use, and the concentration of the protein was 1.6 instead of 2.5%. Greater amounts of alkali were used for the final dissolution, and the vials in which the reaction was carried out were quantatively rinsed. All this resulted in the unusually high yield of 32.35%.

In experiment 20, the iodinated protein was precipitated for 48 hours in the cold (4°C.) after a long iodination period of

5.5 hours in the presence of excess iodine. This resulted in the unusual yield of 43.9%.

The 21st experiment, as numbered, was actually the first experiment, and was carried out on AP3004, while all the others were done on Schwenk's purified prolactin. Apparently, acidification with glacial acetic acid was adequate; the iodinating pH was 5.5; the purification was inadequate. This is an example of a false high yield.

Summary:

It has been demonstrated that $I^{a}P$ can be prepared in 1 mgm lots such that it incorporates an amount of iodine necessary for the iodination of 1 tyrosyl radical per molecule. Such $I^{a}P$ can be freed of non-incorporated iodide, but in the process 50% of the protein is lost. It has been shown that under the conditions used, the tyrosyl radicals but not the histydyl radicals react. Various factors influencing the iodination have been studied and discussed.

BIOLOGICAL ASSAY

After $I^{\eta}P$ had been prepared and purified, a series of experiments were set up to test biological potency.

The "local" intradermal squab assay for prolactin was used (Lyons 1937[a]). This test is less reliable than intramuscular or subcutaneous methods, but more sensitive, and requires less material. As used here, the day prior to initiation of assay, the crop gland area of pigeons, obtained from a local market and kept in a wire cage in the animal room, was plucked with care lest the skin be injured. A double twist of the wings, clipped unevenly to hinder free flight and to act as identification, was useful in controlling the birds.

To inject intradermally, the bird was held by the assistant in such a way that the crop gland to be injected was uppermost. The injector, following the line of the feather ridges to a point midway between the median plane and the coricoclavicular joint, instilled 0.1 cc of a suitable dilution of material of known potency-- 1 mgm of this prolactin in 2.4 cc of pH 7.6 phosphate buffer, equalled 0.1 I.U./ 0.1 cc-- so as to raise a circular bleb. The second crop gland was injected with the material to be assayed. Each bird was therefore injected twice daily for four days. Four 0.1 cc injections over 1 crop gland equalled one assay, i.e. twice the time and number of doses specified by Lyons (1937[a]). Ninety-six

hours after the first injection, the birds were sacrificed by severing the carotids through the opened beak. The birds were held head down to facilitate bleeding, and the beak kept open by inserted scissors until convulsive movements stopped. The crop gland was split up the middle, the food therein washed out under a stream of running water, and the skin stripped free of the underlying crop gland wall. The wall was stretched between thumb and index finger. Against the frosted glass covering the flourescent lamp over the work table of the amimal room, observation of the crop gland was made. Size of area affected and degree of opacity were recorded. The same operator read all the assays to insure uniformity of results. A system of assessment was established; area was recorded as relative to the area occupied by a quarter (25¢), and a gradient to that of a half-dollar was used as a basis for increments and diminutions. Opacity was tabulated as +'s, a visable characteristic increase being one +.

Experimental:

The first experiment was set up as follows:

These solutions were prepared:

A 1,2, and 3. contained 1,0.1, and 0.01 I.U. of untreated prolactin.
B 1,2, and 3. contained 1,0.1, and 0.01 I.U. of dummy iodinated prolactin (prolactin subjected to the manipulations involved in iodination and purification of I^P.).
C 1,2, and 3. contained 1,0.1, and 0.01 I.U. of iodo-prolactin.

Results obtained were:

Table 10

Effect of 3 dilutions of prolactin, dummy iodinated, and iodinated prolactin on Crop Glands.

Pigeon	Right Crop Gland Solution Injected	Response	Left Crop Gland Solution Injected	Response
No. 1	B3	+	C3	0
No. 2	A3	+++	B3	+
No. 3	B2	+ +	C2	++
No. 4	A2	++++	B2	++++
No. 5	B1	++++	C1	++++
No. 6	A1	"Tremendous"	B1	++++

These results may be summarized as follows:

A	0.01	I.U.	+++	0.01	I.U.	++++	1 I.U.	"Tremendous"
B	"	"	+	"	"	++	" "	++++
C	"	"	0	"	"	++	" "	++++

From the summary, B and C, i.e. dummy iodinated and iodinated--and this iodinated prolactin had been exposed to 12.6 gamma of iodine mgm of prolatin--produced identical reactions. An apparent difference existed, however, between the reactions produced by corresponding amounts of treated and untreated prolactin (A as compared to B or C). As shown in the preceding section, 50% of the protein is lost during purification of iodinated prolactin, which probably accounts for the differences

observed.

A second experiment was carried out six months later, at a time when the amount of iodine being used per milligram of prolactin was 32 gamma. In this experiment the following strengths were used:

A-- 0.1, 0.05, and 0.01 I.U. per 0.1 cc qd x 4 --- Untreated prolactin
B-- 0.1, 0.05, I.U. per 0.1 cc qd x 4 --- Dummy iodinated prolactin
C-- " " " " " " " " " --- Iodinated prolactin.

The results, obtained with a number of birds comparable to those used in the previous experiment (6), were assessed for area and opacity response. The findings are summarized in Table 11.

Table 11

Biological Potency of untreated, dummy iodinated, and iodinated (32 γI_2) prolactin

Sample			Response Area	Response Opacity
A	0.1	I.U./0.1 cc	40%	++++
A	0.05	I.U./0.1 cc	45%	++++
A	0.01	I.U./0.1 cc	5%	+
B	0.1	I.U./0.1 cc	25%	+++
C	0.1	I.U./0.1 cc	33%	++++

Experimental error obscured any difference between 0.1 and 0.05 I.U./0.1 cc qd x 4 for untreated prolactin. Actually, from

these findings, the iodinated prolactin appears to be more active than the dummy iodinated material. Both were less active than either the 0.1 or 0.05 I.U./0.1 cc dose of untreated prolactin. It must be remembered that these findings should be compared with the 0.05 I.U/ 0.1 cc dose of untreated prolactin rather than with the 0.1 I.U. dose due to protein loss. In this instance, more than twice as much iodine has been used, leading to a greater degree of iodination and hence, presumably, a greater loss in biological potency. Li et al (1941)[a] have claimed that the tyrosyl radicals were essential for this biological activity.

A third experiment was carried out using 63 gamma of iodine to iodinate 1 mgm of prolactin. Again, untreated prolactin, dummy iodinated prolactin, and iodoprolactin were assayed. Six birds and the same dilutions as in the previous experiment were used. The results are summarized in Table 12.

Table 12

Effect of untreated, dummy iodinated, and iodinated (63 γ) prolactin on Crop Glands.

Sample	Dose	Response Area	Opacity
Untreated Prolactin	0.1 I.U./0.1 cc	43.5¢	++++
	0.05I.U./0.1 cc	25.0¢	++ ±
Dummy iodinated prolactin	0.1 I.U./0.1 cc	25.0¢	++ ±
Iodinated prolactin	0.1 I.U./0.1 cc	17.5¢	+ ±

The dummy iodinated prolactin corresponded almost exactly in response to that obtained with half as much untreated prolactin. The iodo-prolactin showed a smaller response in both area and opacity than did its uniodinated control. An excess of iodine was used, in fact five times as much as was theoretically necessary to iodinate, to the di-iodo form, one tyrosyl radical; the probability existed that not one but several radicals were iodinated which, along with the protein loss, would account for the diminished activity. The loss in area was of the order of 30%, and that of opacity, 40%.

A fourth, much more extensive experiment, was done later. The iodo-prolactin was made with 37.5 gamma per mgm. Only prolactin plus iodide, subjected to the chemical manipulations involved in iodination and purification of iodo-prolactin in three dose levels, namely, 0.2, 0.1, and 0.05 I.U./0.1 cc (on the basis of the original protein used and not on the final protein content, probably of the order of 50%) was assayed against the corresponding iodo-prolactin. 36 birds, or 72 crop glands, were used; 144 readings were taken. Following intradermal injections of 0.1 cc each day for four days, the birds were sacrificed 96 hours after the first injection, and area and opacity of the reaction assessed as before. Table 13 summarizes the results.

Table 13

Biological Assay - I.D. Pigeon Crop Gland Method

(Average of 6 readings per group.)

Presumed Potency	Prolactin Area	Prolactin Opacity	Presumed Potency	IP (37.5 γ /mgm) Area	IP (37.5 γ /mgm) Opacity
0.20 I.U./0.1 cc	75	3.17	0.20 I.U./0.1 cc	62.5	2.83
0.10 " "	54	3.0	0.20 " "	50.0	3.0
0.10 " "	36	2.67	0.10 " "	20.8	1.67
0.05 " "	35	1.83	0.20 " "	47.5	2.5
0.05 " "	40	2.33	0.10 " "	46.67	2.17
0.05 " "	49	2.20	0.05 " "	32.5	2.20

Average							Retained Activity		Retained Activity
0.20	"	"	75	3.17	0.20	" "	53	(70.0%)	2.78 (87.8%)
0.10	"	"	45	2.83	0.10	" "	33.5	(74.5%)	1.92 (68.0%)
0.05	"	"	41	2.12	0.05	" "	32.5	(79.3%)	2.20 (108.0%)
								74.58% Av.	87.92% Av.

At these three dose levels, the least loss in area (100%-79.25%) occurred with the greatest dilution (0.05 I.U./0.1 cc), and the greatest loss (100% - 70.00%) with the smallest dilution (0.20 I.U./0.1 cc). The opacity findings are inconsistent, showing the

greatest loss for the intermediate dose, and an actual increase for the smallest dose. The averages indicate that when prolactin is iodinated so that 1 - 3 tyrosyl radicals are involved, there is a loss of reaction of about 25% in area and 12% in opacity, as compared with that due to material which has simply been subjected to the manipulations involved.

Discussion:

The first experiment showed that when 12.6 gamma of iodine was used to iodinate a mgm of prolactin, there was no detectable difference between dummy iodinated and iodo-prolactin. This degree of iodination corresponds to a 1 in 10 tyrosyl radical iodination to the di-iodo-state. There was a detectable difference between the untreated prolactin and the chemically manipulated and iodinated prolactin. Probably this difference was due to the loss of protein material in the purification of the iodo-prolactin.

When 32 gamma of iodine were used to iodinate prolactin, and this assayed against chemically manipulated material and untreated prolactin, a preparation somewhat more biologically active was obtained (that is, more biologically active than the corresponding material subjected simply to the chemical manipulations). Here two and a half times as much iodine was employed for the same amount of prolactin. It is conceivable that some oxidative reactions also took place with iodine as the effective agent which liberated active

moieties of the original protein, and that this liberation, apparently not affecting the bonds essential for biological activity, enhanced biological potency. It is possible also that the contaminating material of the original preparation was removed, thus concentrating the reactive protein. This enhancement of activity was found in none of the other experiments and therefore may be due to experimental error.

When 63 gamma of iodine per mgm of prolactin were used to prepare iodo-prolactin, and this assayed against untreated and chemically manipulated prolactin, it was found that the untreated prolactin was twice as active as the chemically manipulated prolactin at a dose of 0.1 I.U./0.1 cc. This suggests that 50% of the protein had been lost in the chemical manipulations. Iodo-prolactin was less active when assessed by area and opacity of reaction. The diminution in area reaction was of the order of 30%, that of opacity reaction of the order of 40%. The amount of iodine used here is 5 times the amount needed to effect a theoretical one-in-ten tryosyl radical conversion to di-iodo-tyrosine. These results indicated that the decrease in activity was proportional to the number of tyrosyl groups iodinated.

In the fourth experiment, the amount of iodine used was 37.5 gamma, an amount 2.5 times that theoretically necessary to iodinate to the di-iodo-form one of the nine tyrosyl radicals known to be present in any one molecule of prolactin. Presumably, 2 - 3

radicals, i.e., as many as one-third of those present, had been iodinated. In this experiment on a large number of birds, this iodinated prolactin was assayed against dummy iodinated prolactin in three dose levels. A consistent loss in biological activity was found in the iodo-prolactin as compared to that of the chemically manipulated material. This loss was detectable as a diminished area of response as well as a decreased opacity of reaction. The overall area loss was of the order of 25%, while that of the opacity was of the order of 12%. If as many as one-third of the tyrosyl groups had been iodinated, then, from this experiment, it can be said that the area loss is rather more indicative of the number of tyrosyl groups iodinated and is a more sensitive index than the opacity. It must, however, be remembered that these are purely subjective assessments, and far more careful work must be done to establish any such interrelationship.

Furthermore, it should be stressed that even after the rather heroic treatment this protein material received, it was still capable of producing a biological reaction, and that, apparently, the material was completely non-toxic as no irritative reactions were observed at any time throughout the course of these experiments.

Conclusions:

1. When the biological assay results on 6 birds of 0.05 I.U. of prolactin subjected to the chemical manipulations involved in

iodination and purification of I^P in the absence of iodine--here called dummy iodinated prolactin--were compared with those obtained with a similar amount of untreated prolactin no detectable difference in biological response was obtained. (Since 50% of the protein nitrogen was lost in the process of iodination and purification, this comparison had to be made between an original 0.1 I.U. of dummy iodinated prolactin and 0.05 I.U. of untreated prolactin.)

2. When 0.05 I.U. of prolactin containing 1 di-iodo-tryosine radical per molecule, was assayed against 0.05 I.U. of dummy iodinated prolactin, no difference in biological response was observed.

3. When 0.05 I.U. prolactin containing 4.5 radicals of di-iodo-tyrosine per molecule was assayed against an equivalent amount of dummy iodinated prolactin a definite loss in biological potency was found (30% in area of crop gland response and 50% in opacity response).

4. When 0.05, 0.1, and 0.2 I.U. of prolactin containing 2.5 di-iodo-tyrosyl radicals per molecule of prolactin were assayed against an equivalent amount of dummy iodinated prolactin on a much larger number of birds, an average of 25% loss in area of crop gland response and of 12% in opacity response was obtained.

From these findings it was tentatively concluded that the loss in biological activity was roughly proportional to the number of tyrosine radicals iodinated. The methods of iodination

and purification as here used seemed to have no effect on the biological activity of the protein per se other than that attributable to the loss of protein nitrogen in the process.

RECOVERY OF I*P FROM TISSUES:

INTRODUCTION:

Once I*P had been prepared, purified, proved biologically active and non-toxic, it was ready for use. To repeat: the aim of this investigation was the study of the distribution and metabolism of I*P within the animal body; the means were the detection of the presence or absence of radio-activity within a given biological sample. The tools available were the Geiger Muller Counter and photographic emulsion; both could indicate only how much radio-activity, due to emission of the beta rays of I^{131}, was present.

Tissues of an animal could be prepared for estimation of all the radio-activity present. Aliquots of tissue digested in 2N NaOH were plated and counted by the G.M. tube. The result of such determinations in no way indicated in what form I^{131}--and hence, presumably, carrier iodine (due to the careful mixing of these two isotopes)-existed. The aim, therefore, was to find a way to estimate the amount of iodine which was still bound to the protein, prolactin. For this, organic and inorganic iodine of the tissue

samples were separated. The work done on this problem is the subject of this section.

Preparation of Tissues for Biochemical Analyses:

Tissues from uninjected or injected animals were removed from freshly sacrificed animals, weighed, either digested in 2N NaOH, (such that for every 100 mgm of tissue 1 cc was used), or cut fine, put into either a Potter-Elvejehlm glass homogenizer or a Waring Blendor, with 10% TCA, 0.9% NaCl, buffered 10% TCA(5 g TCA in 25 cc H_2O, 4N NaOH added drop-wise until pH 5.4 by brom-cresol green paper, and 25 cc 0.2 NaAc-HAc, pH 5.4, buffer), Somogyi Zinc sulfate reagent No. 1. *, or diethyl ether, and homogenized. If the tissues could not be immediately homogenized, they were chilled to, and held at, 4°C., or rapidly frozen on solid CO_2.

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* Somogyi Zinc Sulfate Reagents-- 1.25 g $ZnSO_4 \cdot 7H_2O$ to 12.5 cc 0.25N H_2SO_4 in 100 cc water-- Reagent No. 1 0.75N NaOH was Reagent No. II.

Homogenization

Homogenization was used to so break up tissues that a virtual protein solution, i.e., a smaller than cell-size homogenate, would be obtained, and was carried out as follows: After the tissue had been weighed and cut into small pieces, the fragments were eased to the bottom of a Potter-Elvejehlm glass

homogenizer by its glass piston, the requisite amount of homogenizing fluid was added, the stem of the piston attached to the drive of a one-twelfth horsepower motor, and the rheostat slowly turned up until the piston was rotating at a fair speed. The outside jacket of the homogenizer was gently but firmly eased up and over the piston, by hand, until the fragments disintegrated. When no fragments of tissue could be detected by visual inspection, homogenization was considered complete. A creamy suspension resulted. Paranchymatous tissues were easy to homogenize, but if the tissue were fibrous, homogenization was broken into steps to allow for cooling; otherwise the protein could have coagulated. Fibrous tissues required time to homogenize to a suspension which could be handled in a graduated 5 cc pipette. The reagents used, except for 0.9% NaCl, undoubtedly caused chemical alterations of the tissue proteins.

Gelation

To facilitate homogenization, especially of fatty tissues, on the suggestion of Dr. O. Denstedt, three gelations on dry ice and thawings at room temperature were carried out along with diethyl ether homogenization.

Lyophilization

Tissues, chiefly lactating mammary glands, were cut,

placed in Potter-Elvejelhm glass homogenizers which were attached by rubber suction caps to a glass caterpillar, this to a trap, and that to a Duo-Seal Vacuum Pump. The caterpillar, with its attached homogenizers, was packed in solid CO_2 in a cardboard carton, insulated by newspapers, within a larger carton placed on a plate glass slab on the laboratory work bench. A temperature of less than -20°C. and a pressure of 4 mm Hg were maintained for 4 days. The adequacy of lyophilization was judged by the behavior of the material upon subsequent homogenization in anaesthetic di-ethyl ether. If the homogenization was easy, lyophilization was complete. Incidentally, the change in color, from old rose to chalk pink, of the tissues fragments was also used as a rough guide for the complete removal of water. Once such tissues had been thus lyophilized, care had to be taken to rapidly submerge them in di-ethyl ether when they were taken off the caterpillar, after the suction had been turned off - otherwise they rapidly took up water from the atmosphere.

Preliminary Experimental Results:

To effect a separation of organic iodine from inorganic iodide, precipitation of protein was selected. Various protein precipitatants were tried; trichloroacetic acid (TCA), isoelectric (at the isoelectric point of $I^{*}P$) precipitation, 95% ethyl alcohol, addition of carrier iodo-prolactin to facilitate precipitation of minute amounts of protein material (usually of the order of 0.1 mgm

of less, containing only a few gamma of iodine), and colloidal hydroxides of heavy metals. The most suitable precipitant of organic iodine was found to be zinc hydroxide. (Somogyi (1930) used acid zinc sulfate, neutralized with sodium hydroxide to free blood of protein material prior to blood sugar estimations).

Parallel studies on iodination, purification, in vitro recovery from tissues, and biological use of $I^{\eta}P$ were carried on simultaneously, and new factors were incorporated as they came to light. This progressive development made results less comparable. Therefore, illustrative experiments of preliminary experiments have been selected and are tabulated in Table 14.

For Table 14, see pocket at back.

From Table 14 the following significant facts were culled and progressively incorporated into further experimental work:

1. 10 and 5% TCA have pH's of less than, and about, 1. $I^{n}P$ is soluble in this pH range.

2. Addition to tissue homogenates did not sufficiently neutralize 10% TCA to cause satisfactory precipitation of $I^{n}P$.

3. Neutralizing 10% TCA to pH 5.4 caused complete precipitation of $I^{n}P$, but addition of tissue homogenates, under these conditions, resulted in only 75% precipitation.

4. 5% acetic acid, in place of 10% TCA neutralized to the same pH, is an unsatisfactory protein precipitant.

5. 10% TCA buffered to pH 5.4 is less satisfactory than neutralized 10% TCA as an $I^{n}P$ precipitant. 95% ethyl alcohol dissolves 10% TCA (pH 5.4) precipitated $I^{n}P$. Under these conditions, ether does not dissolve $I^{n}P$.

6. Addition of carrier IP under the conditions of the preceding experiment does not sufficiently increase the recovery of $I^{n}P$ to warrant its use.

7. Use of 0.2N NaAc-HAc, pH 5.4, buffer as a tissue homogenate precipitant is highly unsatisfactory.

8. The fat of lactating mammary gland interferred with attempts to precipitate protein containing $I^{n}P$.

9. Colloidal copper hydroxide gave a satisfactory precipitate with liver homogenate and water, but not with plasma, to each of which had been added $I^{n}P$.

From these experiments, it was concluded that isoelectric precipitation of tissue homogenates to which $I^{n}P$ had been added was futile, that the usual protein precipitants i.e., TCA and alcohol, dissolved rather than precipitated $I^{n}P$, and that a colloidal hydroxide of a heavy metal would prove satisfactory.

However, due to the colour of copper solutions, paper pH and liquid pH indicators were useless in determination of the exact neutrality needed. Use of the Beckman pH meter was both time-consuming and impractical due to potential cross contamination. For these reasons, copper hydroxide was abandoned in favor of zinc hydroxide, and the Somogyi zinc sulfate method for the determination of protein-bound iodine was adapted for use as indicated in the following series of experiments.

SOMOGYI ZINC SULFATE FRACTIONATION OF ORGANICALLY-BOUND IODINE FROM INORGANIC IODIDE

In 1930 Somogyi described a method using acid zinc sulfate neutralized to pH 7 to obtain protein-free blood filtrates. His purpose was to determine blood sugar levels. However, his reagent (12.5 cc of 0.25N H_2SO_4 plus 1.25 g $ZnSO_4 \cdot 7H_2O$ in 100 cc water and 0.75N NaOH) and the proportions used in his "second procedure"(to 1 part plasma protein add 8 parts of acid zinc sulfate and 1 part of alkali) have been adopted for many uses, and were adaptable here.

Not only the proteins of plasma, but of other tissues as well, were to be precipitated.

The biological material-- to which small amounts of iodine, as inorganic iodide (I^n), or organically-bound iodine

($I^{\eta}P$) was added-- was obtained from freshly sacrificed rats. Heparinized blood, drawn from the inferior vena cava, was freed of red blood cells by centrifugation and the resulting plasma used. Organs were removed, weighed, and homogenized in Somogyi zinc sulfate reagent such that for 500 mgm wet weight, 8 cc was used. Of such an homogenate, suitable aliquots (4 cc usually) were put into graduated centrifuge tubes, and the amount of $I^{\eta}P$ (0.1 mgm in 0.2 cc) or I^{η} (0.2 cc), one drop of 1% phenolphthalein solution, and 0.5 cc or more of 0.75N NaOH were added. The resulting mixtures were centrifuged, the supernatants pipetted off, the precipitates washed with 3 cc of triply distilled water, and the supernatants and washes were pooled, 1 cc aliquots were plated with 1 cc 2N NaOH and counted. Precipitates were digested in 3 cc 2N NaOH, and 1 cc aliquots plated and counted. All plates of a given experiment were counted on the same day, in the same castle, and at the same time of day. The precipitate fractions were calculated as the percent of initial activity, added, and recorded in Table 15 . The extremes of these percentages for quadruplicate or duplicate samples processed at one time, as well as the averages, are recorded in the last columns of the same table.

Effect of tissue type, pH, tissue concentration, time, precipitation in the cold, replacement of H_2O by isotonic sodium sulfate solution as a wash, and effect of $I^{\eta}P$ concentration were

For Table 15, see pocket at back.

systematically studied. The results of the various experiments are summarized in Table 15.

RESULTS

EVOLUTION OF METHOD

Effect of Tissue Types.

Recoveries of $I^{\Omega}P$ added to plasma were most consistent, as indicated by the small spread of percentages for the four samples tested. From it, most inorganic iodide was eliminated. Recoveries of $I^{\Omega}P$ from liver showed the greatest spread; most iodide was retained. Recoveries of $I^{\Omega}P$ from kidney were best, removal of iodide was intermediate in amount and spread.

Sufficient alkali to just turn phenolphthalein red-- an excess of the amount required for the proportions established by Somogyi-- was used. The unsatisfactory results obtained with liver and plasma were attributed to this excess.

Effect of pH.

Addition of the exact proportions of 8 parts of Somogyi zinc sulfate reagent and 1 part of alkali to 0.3 cc of plasma resulted in a pH of 7.0. Recovery of organic iodine added to plasma increased from 87 of the control experiment to 91%; the

spread in the four samples was somewhat less. Removal of iodide was, however, quite unsatisfactory in both instances. Recovery of $I^{\uparrow}P$ added to liver at a pH of 7 was considerably better than that of the previous experiment, as well as that of its own control. The spread here was reduced. Iodide was as unsatisfactorily removed with liver as with plasma, probably due to poor mixing during washing of precipitates.

Effect of Tissue Concentration:

What the protein concentration of the 1:8:1 ratio should be, was studied in five graded concentrations of rat liver obtained by suitably diluting a 500 mgm to 8 cc homogenate with homogenizing reagent. A small but steady improvement in the amount of organically-bound iodine recovered in the precipitates as the amount of protein material was increased, was found. Spread of percentages was small. The iodide adsorbed onto the precipitated protein in greater amounts than was desired. From this, the highest concentration here investigated, namely, 500 mgm of wet tissue per 8 cc of Somogyi zinc sulfate reagent, was adopted as routine. (This represented a 1.875% protein concentration, if wet tissue weight is 70% water; the 30% solids would then be largely protein in nature).

Effect of Cold and Time:

Though initial precipitation gave water-clear supernatants, washes were milky, indicating loss of precipitate, which led to an investigation of the effect of cold and time on completeness of precipitation. Two groups, one treated as described above, the other, without centrifugation and kept in the icebox over night, were set up. After washing, on the second day, combined supernatants and washes were again kept in the icebox overnight. More care in mixing the resulting precipitates with the washes reduced the amount of iodide in the final precipitates. (The final precipitates of the table are those recovered from this second icebox treatment.) In both instances, 10 to 15% more organically-bound activity was recovered in the secondary precipitates, while the amount of extra inorganic iodide here precipitated, was less than 1%, i.e., 0.85% in the first and 0.39% in the second instance. This latter finding led to the routine use of the icebox treatment prior to centrifugation.

Effect of isotonic sodium sulfate in place of water as wash.

The washes were still milky, and since the supernatants were clear, a Na_2SO_4 wash solution, isotonic with the original supernatant, was tried. Conditions similar to those in the first run of the previous experiment, i.e., centrifugation prior to initial icebox treatment, were used. A slight improvement in the

average recovery of organically-bound iodine, and a spread of 0.2% was obtained. Precipitated inorganic iodide was negligible. Isotonic Na_2SO_4 made manipulation easy; washes were clean and the precipitate compact.

Effect of $I^{\wedge}P$ concentration

When the method had been worked out as detailed above, the effect of $I^{\wedge}P$ concentration was studied. Rat plasma, to which half as much $I^{\wedge}P$ as used previously, was processed. In two samples, an average of 65% $I^{\wedge}P$ was recovered in the precipitate fractions-- a recovery 25% poorer than that obtained in the effect of pH investigation. Adsorption of $I^{\wedge}$ resulted in an average of 11.85% in two similar samples-- a figure closely resembling that obtained before. Here, however, the spread was much less.

From the above series of experiments, these modifications were deemed satisfactory for most tissues. Plasma, a more concentrated protein solution (6%), still caused poor recoveries, and tissues with a high fat content, especially lactating mammary gland, proved difficult both to manipulate and from which to obtain good recoveries. Further study on the latter tissue was therefore undertaken.

Mammary Glands: Effect of Tissue Concentration:

Effect of tissue concentration studies, (See Table 15),

identical to that carried out on liver, proved disappointing. Poor recoveries, with poor duplication of results in presumably duplicate samples, were found. Extraction of inorganic iodide was unsatisfactory. Evidently the interferring fat had to be removed.

Effect of Ether Extraction:

Since alcohol dissolved $I^{\eta}P$ (See Preliminary Studies, p. 69 , and Fleischer, 1943) and ether did not, extraction with ether before and after precipitation of Somogyi zinc sulfate homogenates of mammary glands (homogenization was difficult, due to presence of fat and fibrous tissue) showed that recovery of $I^{\eta}P$ in the precipitate after ether extraction was unsatisfactory, whereas that done before precipitation was better. Good removal of iodide was obtained by both methods. Effect of removing fat with ether prior to homogenization-- to facillitate homogenization itself, the tissue was frozen and thawed three times to break down the tissues cells without disrupting the tissue protein-- was tried. (A smaller than cell-size homogenate was desired because injected inorganic iodide might find its way into the tissue cells. Were a larger than cell-size homogenate to be prepared and subsequently treated as described above, material found in the precipitate would be called organically-bound iodine, whereas, in actuality, it would be intracellular inorganic iodide). With these two modifications in mind; to extract the tissue prior to Somogyi

zinc sulfate homogenization, and to attempt a smaller than cell-size homogenate, the following experiment was set up.

Experimental:

The mammary gland of a lactating rat was removed, weighed, and cut into fine pieces. 10 cc of diethyl ether per gram of mammary gland tissue was added, the mixture rapidly frozen on dry ice, allowed to thaw, homogenized, and these processes repeated twice more. The ether supernatant was pipetted off and set aside. The gummy homogenate mass, containing some sand (homogenization had been carried out in a Waring Blendor) was rehomogenized in a Potter-Elvejehlm glass homogenizer in the requisite amount of Somogyi zinc sulfate reagent. Four 2 cc aliquots were taken, and 0.1 cc containing 0.05 mgm of $I^{\eta}P$ containing 0.9 gamma of I^{η}, were added. To four similar 2 cc aliquots, 0.1 cc containing 0.98 gamma of I^{η}, were added. These 8 aliquots were then treated in the usual manner.

Results:

Under these conditions, 85% of the organically-bound iodine and 12.6% of the inorganic iodide was found in the respective precipitates (See Table 15)-- a distinct improvement. The spread had been cut down to about 3.5% for organically-bound idoine and to a little more than 2% for the inorganic iodide.

Discussion:

This experiment was carried out in vitro and, therefore, was not strictly comparable to conditions usual in tissue work from animals injected with either I^{β} or $I^{\beta}P$. In the case here described, mammary gland tissue presumably had had its fat extracted prior to addition of iodine-containing material, which under these conditions was certian to be extracellular. However, in tissues taken from injected animals neither fat removal nor extracellularity of material could be expected. Such fat removal facilitated handling, but mammary gland fractionation was still as much of a problem as ever.

This modification of the Somogyi zinc sulfate fractionation of organic iodine from inorganic iodide was based on Barker's (1948) study of the determination of protein-bound iodine of plasma. For that he described four distinct steps, only the first of which, precipitation and washing of proteins, has been used here. In his method, 2 ml of heparinized plasma was treated with an unspecified amount of Somogyi acid zinc sulfate reagent, centrifuged 10 minutes, the supernatant poured off, and the precipitate washed free of inorganic iodide by four successive washings of 25 ml each of triply-distilled water. Barker also found that 500 mgm of rat liver, kidney, heart, and skeletal muscle could be homogenized with 8 cc of acid zinc sulfate reagent, subsequently transferred to a 50 ml

centrifuge tube by four rinsings of the homogenizer with 4 ml each of the reagent -- making a total of 24 cc per each 500 mgm of tissue used. This was then precipitated with sufficient 0.75 N NaOH (about 3 cc) to cause phenol red to turn a permanent pink (pH 6.8 to 8.4--Handbook of Chemistry and Physics, 29th Edition, p. 1365, 1945.)

Under these conditions, 99.92% of 1000 gamma of inorganic iodide per 100 ml of plasma could be removed, whereas 5 gamma % of added thyroxine iodine was retained completely, and 5 gamma % of added di-iodo-tyrosine was "retained to 75% completion".

The purpose here was to determine recovery of the organically-bound iodine of $I^{*}P$ present in, or added to, tissue. Representative aliquots rather than total samples were to be used, and the iodine content was determined by measurement of the amount of I^{131} by the G.M. tube. The amounts of I^{131} so determined, in no way indicated the endogenous iodine content of the tissue studied but were assumed to represent the added iodine. Hence quantitative recovery of total iodine was not the aim as was the case with Barker. The modifications of the first step of his procedure described above resulted in a recovery of 96.5% of 0.1 mgm of known protein-bound iodine, $I^{*}P$, from 250 mgm of rat liver and the elimination of all but 1-2% of 1 - 2 gamma of inorganic iodide, I^{*}. From these findings, it was assumed that organic iodine moieties down to as small a molecular size as di-iodo-tyrosine (Barker, 1948), would be recovered in the precipitate. Therefore,

this precipitate fraction was called "organically-bound" rather than "protein-bound" iodine.

Summary:

When, for every 8 cc of Somogyi zinc sulfate reagent, 500 mgm of wet rat tissue was homogenized, neutralized by 1 cc of 0.75N NaOH, held in the cold for 24 - 48 hours twice, and the resulting precipitate washed with a volume of isotonic Na_2SO_4 equivalent to the homogenate aliquot investigated, 96.5% of 0.1 mgm of $I^{\eta}P$ containing about 2 gamma of I^{η} can be recovered, and all but 1.5% of a similar quantity of $I^{-\eta}$ can be removed from such a tissue preparation.

From 0.5 cc of plasma, 65% of 0.05 mgm and 90.6% from 0.3 cc of plasma of similarly iodinated prolactin can be recovered int the precipitate, while about 88% of a like amount of I^{η} can be removed.

By ether homogenization of thrice frozen mammary tissue, followed by Somogyi zinc sulfate fractionation, 85.2% of 0.05 mgm of $I^{\eta}P$ containing about 1 gamma of I^{η} can be recovered in the precipitate fraction, while about 87% of a similar amount of $I^{-\eta}$ can be removed.

Since the biological work of this investigation was to be done on mice rather than rats, the effectiveness of this modified method in recovering known organically-bound iodine, $I^{\eta}P$, and eliminating inorganic iodide, $I^{-\eta}$, was checked on mouse tissues:

In Vitro Recovery of $I^{\Omega}P$ and I^{Ω} From 3 Day Lactating Mouse Tissues:

Experimental:

Homogenates of liver, kidney, thymus, spleen, adrenal, and ovary were prepared, using Somogyi zinc sulfate reagent. To 2 cc or smaller aliquots of these homogenates, 0.1 cc containing 0.05 mgm of $I^{\Omega}P$, or 0.1 cc containing 3.9 gamma of I^{Ω}, were added. These mixtures were processed as previously described. The percentage of added radioactivity retained in the precipitate, i.e. organically-bound iodine, are recorded in Table

Four aliquots of plasma were divided into two groups. To one group a similar amount of $I^{\Omega}P$ was added; to the other, I^{Ω}. One aliquot of each group was treated with twice, the remaining two aliquots with four times the amounts of Somogyi zinc sulfate reagents previously used. The organically-bound fractions are likewise recorded in Table 16.

Table 16

Percentage Recovery in Precipitates of 0.05 mgm $I^{\Omega}P$ and 3.9 gamma of I^{Ω} Added to Tissues of a 3 Day Lactating Mouse Subjected to Somogyi Zinc Sulfate Fractionation Procedure.

Organs	$I^{\Omega}P$ % Organic Iodine	I^{Ω} % Organic Iodine
Plasma I	91.0	6.8
Liver	86.0	6.1
Kidney	81.5	3.3
Adrenal	81.0	6.8
Ovary	85.0	9.7
Thymus and Spleen	60.0	6.3

Plasma I treated with 2 X quantity acid $ZnSO_4$ usually used.

In this experiment half as much $I^{\eta}P$, 0.05 mgm as compared with 0.1 mgm, has been used. Recoveries with liver, kidney, adrenal, and ovary average 83.4%. Elimination of inorganic iodide has been about 94% complete for these same organs. Recovery from plasma has been as complete with twice as much Somogyi zinc sulfate reagents as it was with twice as much $I^{\eta}P$ and the usual amount of Somogyi zinc sulfate from rat plasma. These findings suggest that a corrective of 20% needs to be applied to the organically-bound iodine figures, and one of 6% to those of inorganic iodide, on the assumption, of course, that the various tissues or the method have not caused a breakdown of the $I^{\eta}P$, with a removal of the iodine label. The latter possibilities, though not rulled out, seem unlikely in the light of the findings of Barker.

The findings that only 80 - 85% of the organically-bound iodine has been recovered here suggests that in the case of plasma, or tissue fragments in tissue homogenates, such recovery depends upon adsorbability of $I^{\eta}P$ to other protein molecules, as well as to the colloidal $ZN(OH)_2$ formed, and that the smaller the amount of $I^{\eta}P$ to be precipitated, the greater that adsorptive surface needs to be. The protein content seems more important in this respect than the colloidal $Zn(OH)_2$, for twice as much $ZN(OH)_2$ with the same amount of plasma in the second plasma sample studied here, led to a marked reduction in recovery. That plasma -- a 6% protein solution -- provides a more effective precipitating agent when twice the usual

amount of Colloidal $Zn(OH)_2$ is also present, is indicated by the recovery obtained. This result suggested that the finer the homogenization -- hence the greater the number of protein particles available- - the better the recovery of such small amounts of organically-bound iodine ought to be. Use of twice as much Somogyi zinc sulfate reagents with plasma brings the % protein present in the solution to about that of the tissue homogenates, such a 3% solution of protein seems to be about the optimum concentration for recovery of small amounts of $I^{\alpha}P$.

In this experiment, twice as much inorganic iodide has been added, 4 gamma as compared to 2 gamma used previously. The more removal seems to indicate that the/iodide to be removed, the more carefully must the precipitates be washed.

Summary:

From this experiment it was concluded; that plasma should be treated with twice the usual amount of Somogyi zinc sulfate reagents; that as fine a homogenization as possible should be obtained; and that the greater the amount of iodide to be removed, the more stringent the washing of the precipitate should be.

So far the method had only been applied to tissues to which either $I^{\alpha}P$ or I^{α} had been added. In tissues from an animal injected with $I^{\alpha}P$ some time previously, both $I^{\alpha}P$ or its degredation products

and I^{α} would be present. The question arose as to what effect these conditions would have on the accuracy of the method.

Application of The Somogyi Zinc Sulfate Fractionation Method to Tissues From Injected Mice

Triplicate samples of liver taken from two C_3H male mice which had been given an intravenous injection of either 0.05 mgm of $I^{\alpha}P$ or 1.3 gamma of I^{α} twenty minutes prior to sacrifice were fractionated with Somogyi zinc sulfate reagents as described above. The percentages of organically-bound iodine recovered are recorded in Table 17.

Table 17

Somogyi Zinc Sulfate Fractionation Studies of Liver Aliquots from Two C_3H Mice Injected I.V. 20 Minutes Prior to Sacrifice with 0.05 mgm Of $I^{\alpha}P$ and 1.3 gamma of I^{α}

Sample	$I^{\alpha}P$ % Organic Iodine	I^{α} % Organic Iodine
A	66.9	22.9
B	63.8	23.0
C	63.7	22.5
Average	64.8 (81%)	22.8 (21.5%)
Coefficient of Variation	2.8%	1.1%

An average of 64.8% of the radioactivity present in the liver of the animal injected with $I^{\alpha}P$ was recovered in the precipitate. The coefficient of variation for the three samples was 2.8%. When corrected, as indicated in the previous experiment, this figure rose to 81%. An average of 22.8% of the activity present in the liver of the inorganic iodide-injected animal was found in the precipitate fraction. When corrected this came to 21.5%. The coefficient of variation here was 1.1%.

Of the 0.05 mgm $I^{\alpha}P$ activity injected into this C_3H male mouse, 30% was found in the liver at 20 minutes. Of this, 81% was precipitable and so organically-bound. Therefore 20% was inorganic iodide. When 1.3 gamma of inorganic iodide activity was injected, only 6.6% was found in the liver after the same time interval, and of this 21% was precipitable, and hence organically-bound. These figures represent what was so at the time the sample was taken - i.e., at the moment of sacrifice.

It is, however not likely that 0.017 gamma of inorganic iodide had been converted to organic iodine and entered the liver 20 minutes after intravenous injection. Possibly the radio iodide may be so intimately associated with tissue components as to precipitate with some of them. The finding of 30% of the injected radioactivity from $I^{\alpha}P$ in the liver 20 minutes after intravenous injection does not mean that 30% of 0.05 mgm of $I^{\alpha}P$ in the same state in which it was injected was now in the liver. It only means

that 30% of the I^{131} incorporated on that 0.05 mgm of I*P was in the liver at that instant, and that of that 30% activity, 80% was precipitable, and hence presumably organically-bound iodine. In spite of the fact that only approximate data were obtained by the method, it was found a useful tool in indicating the fate of injected I*P in the body.

Summary:

Triplicate aliquots of livers removed from C_3H male mice injected intravenously 20 minutes prior to sacrifice with 0.05 mgm of I*P containing 0.05 gamma I^*_2 and 1.3 gamma of I* were found to contain 65% and 22.8% respectively of the activity present in the precipitate fractions. The coefficient of variation for the two sets of triplicate samples was 2.8% in the case of I*P, and 1.1% in the case of inorganic iodide.

However, this Somogyi zinc sulfate fractionation method could not be satisfactorily applied to fatty tissues, especially lactating mammary gland. The fat had to be removed. In preliminary work on rat tissues, extraction by ether had appeared promising. This line was pursued in the following experiments:

Somogyi Zinc Sulfate Fractionation of Mammary Gland Tissue Previously Extracted with Ether

0.05 mgm of I*P and, in a control experiment, 3.9 gamma of

inorganic iodide (I^n) were added to equal weights of fresh mammary gland tissue removed froma 3 day lactating mouse. For each gram of tissue, 16 cc of ether was added, and the mixture homogenized in a Waring Blendor with a little sand. These homogenates were chilled on dry ice thrice and rehomogenized in Potter-Elvejehlm glass homogenizers. The ether supernatants were pipetted off. The residual gummy mass was subjected to Somogyi zinc sulfate fractionation in the usual manner. The ether extract was evaporated to dryness, re-extracted with a similar amount of (presumably water-free) ether, the ether extract removed, evaporated to dryness, and the residue digested in 2N NaOH, plated and counted separately. The residue from the first ether extraction, i.e., the water soluble material carried over by the ether, was added to the pooled supernatant and washes prior to their second icebox treatment, and subsequently fractionated.

The stomach of the three day old young to which 3.9 gamma of I^n had been added were similarly treated.

The results for the % organically-bound and the ether extractable iodine are recorded in Table 18.

Table 18

% Recovery of 0.05 mgm $I^{\eta}P$ and 3.9 gamma I^{η} in Mammary Gland Tissue And Stomach Contents of 3 Day Old Young, Extracted With Ether Prior To Somogyi Zinc Sulfate Fractionation

	$I^{\eta}P$		I^{η}	
	% Organic Iodine	% Ether Extractable Iodine	% Organic Iodine	% Ether Extractable Iodine
Mammary Gland	52.5	12.8	4.6	2.64
Stomach Contents			2.4	0.98

Since ether extraction was done at the beginning of the procedure, 12.8% of the activity was removed by the ether before the Somogyi zinc sulfate procedure. This left 87.2% of the activity to be fractionated. Of the total, 52.6% (or 60% of what remained there) was recovered in the organically-bound fraction. From the experiment on the other mouse tissues also containing 0.05 mgm of $I^{\eta}P$, a corrective of 15 - 20% was deemed necessary. If a 20% corrective is applied, the organically-bound iodine should be 69.75%, which leaves nearly 20% as inorganic iodide. Under the conditions of this experiment, the fresh mammary gland with its added $I^{\eta}P$ was homogenized at room temperature, with ether as a homogenizing agent. The homogenization was most difficult, resulting in a gummy mass. A question arises as to whether any proteolytic enzymes present in this tissue may not have broken up $I^{\eta}P$ and hence account for the inorganic iodide fraction - the result of further

dehalogenation - for mere splitting of the I^{n}P should first result in fragments not smaller than di-iodo-tyrosine, which should precipitate as organically-bound iodine, according to Barker (1948).

The 12.8% ether extractable activity may be due to solubility of I^{n}P in ether or in the water which is carried by ether. Ether will take up one-thirteenth of its weight in water. Or, I^{n}P may be soluble in mammary gland fat which, in turn, is soluble in ether. These possibilities required further investigation.

All but 4.6 and 2.4% of added inorganic iodide (3.9 gamma) were removed from the precipitates of mammary gland and stomach contents. Only 2.64 and 0.98% of such iodide was extractable by ether under the conditions used. These findings ruled against activity being present in ether extracts purely on the basis of the weight of water taken up, for, surely, inorganic iodide is more soluble than I^{n}P.

Out of this experiment the question arose as to what ether extraction would do to the recovery of I^{n}P added to other tissues. The following experiment was therefore set up:

Effect on Recovery of I^{n}P Added to 3 Day Lactating Mouse Tissues, Ether Extracted Prior to Somogyi Zinc Sulfate Fractionation;

To plasma, liver, kidney, adrenal, ovary, spleen, and thymus, and mammary gland of a 3 day lactating mouse 0.025 mgm of

$I^{\Lambda}P$ was added. 2 aliquots of a similar amount of $I^{\Lambda}P$ were also set up. These mixtures were rapidly frozen on dry ice. Amounts of dry ether--16 cc for every gram of tissue--were added. These mixtures were allowed to thaw at room temperature, were homogenized, refrozen, rehomogenized twice more and centrifuged. The ether extracts were removed and subsequently treated as described for mammary gland in the preceding experiment. To precipitated homogenates volumes of acid zinc sulfate reagent, equivalent to the ether used, were added, and the mixtures carefully rehomogenized. 2 cc or smaller aliquots of these mixtures were taken, precipitated, and subsequently treated as usual. The findings are recorded in Table 19.

Table 19

Somogyi Zinc Sulfate Fractionation After Ether Extraction of 0.025 mgm Of $I^{\alpha}P$ Added to 3 Day Lactating Mouse Tissues

Tissues	To Ether Extractable Iodine				% Organic Iodine		
	% Found	% H_2O Soluble		% Found -% H_2O Soluble	Expected	Found	% of Expected Activity Recovered
Plasma	14.05	- 10.7	=	3.35	86.	74.9	87.0
Liver	25.0	- "	=	14.3	75.	57.25	76.25
Kidney	33.7	- "	=	23.0	66.3	58.5	92.5
Lymphatic Tissue	28.0	- "	=	17.3	72.	57.3	79.5
Adrenal	22.9	- "	=	11.2	77.1	54.5	70.75
Ovary	18.8	- "	=	8.1	81.1	62.0	76.5
Mammary Gland	43.5	- "	=	32.8	56.5	37.9	67.0
$I^{\alpha}P$	9.1	- "	=	---	90.9	63.25	69.75
$I^{\alpha}P$	12.25	- "	=	2.6	87.75	57.0	65.1
Average							74.93

Considerable amounts of radioactivity were extracted by ether. Even $I^{\alpha}P$ alone showed an average extractability of 10.7%. Since this $I^{\alpha}P$ was in an aqueous solution to which the ether had been added, this extractability probably was due to its solubility in the water extracted by the ether. In the other tissues, the $I^{\alpha}P$ may have dissolved in the tissue water which was then ether extractable.

A corrective of the amount of $I^{\eta}P$, itself soluble in ether, was applied to all the other tissues, and the values obtained are recorded in the third column under % ether extractable iodine. Plasma behaved like $I^{\eta}P$ itself, while all the other tissues (average of 16.8%) and especially mammary gland (32.8%) showed a high % of extractable iodine activity.

Since the contained $I^{\eta}P$ was first subjected to ether extraction, only what was left after the extraction was fractionable by the Somogyi zinc sulfate method. The variation in the amount of organic iodine so recovered was considerable (65 - 92%).

In this experiment the tissues had been rapidly frozen prior to addition of ether, which should have eliminated proteolytic activity, and repeatedly homogenized, which should have increased the number of protein particles on which $I^{\eta}P$ could adsorb. Such homogenization should also have freed any tissue fat which was present, which would then have allowed its extractibility by ether. Of the tissues investigated, the fattiest was mammary gland, and from this, ether extracted at least twice as much more $I^{\eta}P$ activity as from any other tissue, except kidney, and lymphatic tissue. Why from the last two, which are particularly paranchymatous tissues, such a high proportion, of $I^{\eta}P$ activity should be ether extractable is not understood. Perhaps their very paranchymatousness meant they had more tissue water and hence dissolved more $I^{\eta}P$.

To rule out a tissue water factor, the following experiment was performed:

Effect of Lyophilization, Ether Extraction, and Somogyi Zinc Sulfate Fractionation of 17 Day Lactating Mouse Mammary Gland Tissue to Which 0.05 mgm $I^{\eta}P$ Had Been Added

Three aliquots of mammary gland from a 17 day lactating mouse were rapidly frozen on dry ice. 0.1 cc containing 0.05 mgm of $I^{\eta}P$ were added to two and 0.025 mgm to the third aliquot. The mixtures also were rapidly frozen, and then lyophilized for four days at less than -20°C. and 4 mm Hg. Dry ether, 16 cc per gram of tissue was added rapidly--as soon as the homogenizers were removed from the caterpillar to which they had been attached during lyophilization. The ether mixtures proved easy to homogenize. After homogenization, the ether extract was removed, and the residue was fractionated as usual by Somogyi zinc sulfate reagents. The results obtained are recorded in Table 20.

Table 20

Effect of Lyophilization, Ether Extraction and Somogyi Zinc Sulfate Extraction of Mammary Gland Tissue From a 17 Day Lactating Albino Mouse to Which $I^{\eta}P$ Had Been Added

Amount of $I^{\eta}P$ added	% Ether Extractable Iodine	% Organic Iodine		
		Expected	Found	% of Expected Activity Recovered
0.05 mgm with 0.03 µg I^{η}	26.7	73.3	69.0	93.4
" " " " " "	22.1	78.8	74.3	94.1
0.025 mgm with 0.15 µg I^{η}	30.8	69.2	65.0	94.0

In this experiment the % organic iodine present after ether extraction, was about 74% of which 68.3% was precipitated by the Somogyi zinc sulfate method. This represents a 94% recovery of the organic iodine remaining after ether extraction. Since no water was present, and since homogenization was so much easier, the high percentages of ether extractable activity suggest that I^{n}P must be soluble in something that is extractable by ether, possibly fat. The 6% not accounted for as organic iodine under these conditions may represent breakdown of I^{n}P, or more likely, loss somewhere along the way.

From this series of experiments it was concluded; that one-eighth of the small amounts of I^{n}P used were soluble in the water that was extracted by ether, that from 25 - 30% of such amounts of I^{n}P were soluble in the fat of mammary gland; and lesser amounts, about 17%, in some ether-extractable materials of other tissues; that following complete lyophilization, about 94% of the remaining activity was recovered as organically-bound iodine; and that there was at least a 6% loss in the method.

USE OF $I^{n}P$ IN THE ANIMAL BODY

INTRODUCTION

After the methods for the preparation and purification of $I^{n}P$ and the fractionation of tissues containing the iodinated hormone had been evolved, the next step was to inject the prepared material into animals and to study their tissues to determine where and in what form the injected radioactivity was. For this purpose, C3H and albino mice were used. These animals were raised in the animal room of the laboratory and fed Purina Fox Chow and water ad lib.

Most experiments were acute, consisting of intravenous injection of 0.1 cc containing a small quantity of $I^{n}P$ or, for control purposes, I^{-n}. In mice, the jugular was found the most suitable vein for this type of injection. Routinely, animals were exsanguinated immediately antemortem to increase accuracy of biochemical and autographic studies. The organs for chemistry and/or autography were removed rapidly and weighed, aliquots were placed in fixative or treated as previously described for biochemical estimations. The carcass, any unused tissue, and any bits of adsorbent cotton containing traces of blood were digested in 2N NaOH (1 cc/ 200 mgm tissue) and subsequently counted; in this way the recovered radioactivity was held within 10% of that injected. All weights of animals, organs, and aliquots of organs used, as well as all volumes of homogenates and aliquots thereof, were recorded, as were such

pertinent data as time of injection and sacrifice, condition and reaction of the animal. From such experimental findings, biological data has been calculated.

Calculation of Results:

To study distribution within a given animal, the results were expressed as per cent recovered dose. To study distribution in the same organs of different animals, the results were expressed as concentration. To study what was taking place within a single organ, the percentages of organically-bound and ether-extractable iodine were estimated.

The per cent of the recovered dose was calculated from the corrected plate count/100 seconds. The total recovered dose was obtained by summation of the total organ counts for a given animal. (In the case of plasma, only the aliquot actually used was included, since the rest was either still in the organs or in the carcass; using the whole plasma meant counting it twice.) The per cent of the total recovered dose attributable to a single organ was then determined.

Concentration is a pure number, i.e., it has no units, for those of weights and counts/100 seconds cancel out in computation. It is interpreted as follows: A concentration of 1 means a random distribution; of greater than 1, specific pickup; of less than 1, interference with random distribution. Concentration is obtained by dividing -

$$\frac{\text{total organ count}}{\text{total organ weight (in mgm)}} \text{ by } \frac{\text{total recovered count}}{\text{body weight (in mgm)}} .$$

The reliability of these two indices of measurement depended upon the accuracy of the G.M. counter and the preparation of aliquots studied. The G.M. counter has an accuracy of 100% ± 3%; duplicate or triplicate aliquots were prepared wherever possible, thereby reducing experimental error to ± 10%.

Percentages of organically-bound and ether-extractable iodine has been explained in considerable detail in the preceding section. Their value has also been assessed critically.

In the presentation of data to follow, the methods used on the organs of an experimental animal will be indicated simply as; 1) used for autography; 2) digested in 2N NaOH; 3) fractionated by Somogyi zinc sulfate method; or 4) ether-extracted and fractionated as in 3).

The findings will be divided into two large groups--biochemical and autographic--and the former will be further subdivided into technical and biological factors affecting distribution.

BIOCHEMICAL FINDINGS:

The experiments to be presented were done on single animals in many cases and their validity as such only is claimed. However, the findings obtained suggest avenues for future investigation. In

these experiments the following routine and symbolization were used:

The intravenous route was chosen to insure uniformity of distribution and because endogenous prolactin is probably so distributed.

The amount of protein injected will be indicated as a decimal-- i.e., 0.05 mgm $I^{\alpha}P$. The amount of iodine so injected varied from 2 to 0.2/ug. The degree of iodination will be expressed as the number of iodine atoms/molecule of prolactin, in the following way---(7.1 I_2 atoms/molecule prolactin). The exact time of day of injection will be recorded; the experimental duration will be specified in minutes. Sex, species, and physiological states, other than "normal", will be stated.

Technical Factors Affecting Distribution of $I^{\alpha}P$

In C_3H male mice, the amount of protein injected, the degree of iodination, and the time of day of injection were found to affect distribution of $I^{\alpha}P$ at 20 minutes after intravenous administration.

Effect of Amount of Protein Injected:

This experiment consisted of a comparison of the distribution of $I^{\alpha}P$ in two C_3H male mice, one of which was given 0.1 mgm of $I^{\alpha}P$, and the other, half that dose.

Experimental:

A. One C3H male mouse was given 0.1 mgm $I^{\alpha}P$ (7.6 I_2 atoms/ molecule prolactin) at 10:30 A.M. The animal was sacrificed twenty minutes later and the organs fractionated by the Somogyi zinc sulfate method.

B. One C3H male mouse was given 0.05 mgm $I^{\alpha}P$ (7.1 I_2atoms/ molecule prolactin) at 9:30 A.M., and sacrificed twenty minutes later. The organs were fractionated by the Somogyi zinc sulfate method.

The findings are recorded in Table 21.

Table 21

Effect of 0.1 and 0.05 mgm $I^{\alpha}P$ (Containing 7.6 and 7.1 I_2 Atoms/Molecule Prolactin, Respectively) in C3H Male Mice At Twenty Minutes

Animals	% Recovered Dose		Concentration		% Organic Iodine	
	A	B	A	B	A	B
Organs	(0.1 mgm)	(0.05 mgm)	(0.1 mgm)	(0.05 mgm)	(0.1 mgm)	(0.05 mgm)
Plasma	25.5	11.27	7.64	4.24	98.5	87.5
Muscle	25.8	13.6	0.51	0.33	96.0	74.0
Adrenal	0.24	0.08	12.6	2.06	95.0	75.0
Thyroid	0.09	0.15	3.95	2.07		99.0

Findings:

From Table 21 , more activity was in the plasma of Animal A; was of that contained activity, most/organically-bound. This was also true of muscle. Even though these animals were exsanguinated, this high figure in the muscle suggested that part at least was due to contained blood. The smaller per cents of organically-bound iodine,

and especially the higher total in the thyroid of Animal B, suggested a larger breakdown of the smaller dose. The iodine in the thyroid of the latter animal had apparently been rapidly incorporated into thyroglobulin, a fact suggested by the high % organic iodine figure for that organ in that animal. These findings suggested that there was a threshold both for removal from the blood and for rate of breakdown of I$^{\eta}$P. The figures obtained for Animal B suggested that the muscle and possibly the adrenal were sites of catabolism.

Effect of the Degree of Iodination of Protein Injected:

This experiment consisted of a comparison of the distribution of 0.05 mgm of I$^{\eta}$P in two C$_3$H male mice, one of which was given a dose of I$^{\eta}$P containing 7.1 I_2 atoms per molecule, while the other was given the same amount of I$^{\eta}$P containing 2.1 I_2 atoms per molecule.

Experimental:

A. One C$_3$H male mouse was given 0.05 mgm of I$^{\eta}$P (7.1 I_2 atoms/ molecule of prolactin) at 9:30 A.M. and sacrificed twenty minutes later. Its organs were subjected to Somogyi zinc sulfate fractionation.

B. One C$_3$H male mouse was given 0.05 mgm of I$^{\eta}$P (2.1 I_2 atoms/ molecule prolactin) at 9:50 A.M; and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

The findings are recorded in Table 22.

Table 22

Effect of Degree of Iodination on Distribution of 0.05 mgm $I^{\eta}P$ in C_3H Male Mice at 20 Minutes

Animals Organs	% Recovered Dose A (7.1)	% Recovered Dose B (2.1)	Concentration A (7.1)	Concentration B (2.1)	% Organic Iodine A (7.1)	% Organic Iodine B (2.1)
Plasma	11.27	15.96	4.24	3.16	87.5	33.2
Liver	27.5	29.33	5.6	5.8	87.0	82.0
Adrenal	0.08	0.17	2.06	0.73	75.0	19.0
Thyroid	0.15	0.57	2.07	7.7	99.0	

Findings:

From Table 22 , though there was more total activity in the plasma of the animal given the less heavily iodinated $I^{\eta}P$, the concentration and the % organic iodine were less, indicating that more $I^{\eta}P$ had been removed from the blood and catabolized to the extent that the freed inorganic iodide had re-entered the circulation. This supposition was confirmed by the larger % recovered dose and higher concentration in the thyroid. The adrenal appeared to contain more activity on the basis of per cent recovered dose, but this proved not to be true on concentration--breakdown here seemed to be considerable. The liver appeared to collect protein regardless of its degree of iodination. No significant difference in % organic iodine was found for this organ in these two animals. From these findings it was concluded that less heavily iodinated prolactin left the blood stream more rapidly than more heavily iodinated material, and that breakdown or dehalogenation, without true catabolism of the prolactin itself,

was considerably greater with the less heavily iodinated material.

Effect of Time of Day of Injection on the Distribution of I$^{\eta}$P

This experiment consisted of the comparison of the distribution of I$^{\eta}$P in 2 C_3H male mice, one of which was injected at 9:30 A.M. a and the other at 4:00 P.M.

Experimental:

A. One C_3H male mouse was given 0.05 mgm I$^{\eta}$P (7.1 I_2 atoms/ molecule prolactin) at 9:30 A.M. and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

B. One C_3H male mouse was given 0.05 mgm I$^{\eta}$P (7.1 I_2 atoms/ molecule prolactin) at 4:00 P.M. and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

The findings are recorded in Table 23.

Table 23

Effect of Time of Day of Injection of 0.05 mgm I$^{\eta}$P in C_3H Male Mice

Twenty Minutes Before Sacrifice

Animals	% Recovered Dose		Concentration		% Organic Iodine	
	A	B	A	B	A	B
Organs	(9:30)	(4:00)	(9:30)	(4:00)	(9:30)	(4:00)
Plasma	11.27	7.4	4.24	1.42	87.5	90.0
Liver	27.5	17.5	5.6	3.42	87.0	84.5
Kidney	7.1	4.9	5.18	2.98	81.5	73.3
Muscle	13.6	9.0	0.33	0.18	74.0	77.5
Adrenal	0.08	0.04	2.06	1.24	75.0	69.5
Thyroid	0.15	0.18	2.07	3.30	99.0	90.0

Findings:

From Table 23 , the per cent recovered dose and the concentration figures were lower throughout for all the organs here studied in the animal injected in the afternoon except for the thyroid which had both a higher total content and concentration and a lower % organically-bound iodine figure, indicating that of the greater amount there, less had been converted to thyroglobulin. The kidney figures also indicated greater breakdown for the afternoon than the morning injected animal, though this may be merely a reflection of the smaller concentration in that organ. From these findings it was concluded, that though the material appeared to diffuse out of the blood faster, probably it was pooled somewhere else, and that distribution was slower in the afternoon than in the morning. The animals were observed to be more sluggish in the afternoon.

Summary:

From these three experiments, it was concluded that: the larger the amount of protein injected, the more slowly it was removed from the circulation and the slower the breakdown; the more heavily iodinated the injected protein, the more slowly it was removed from the blood and more slowly catabolized or dehalogenated. Distribution was probably slower in the afternoon, but that time of day of injection made very little difference in degree of catabolism.

The muscle, kidney, and adrenal seemed to be sites of breakdown, while the liver seemed to be a protein-concentrating organ.

Attention was then turned to biological factors which might affect the distribution of $I^{\Omega}P$. Sex, physiological state, and time after injection were investigated.

Biological Factors Affecting the Distribution of $I^{\Omega}P$

Effect of Sex on the Distribution of $I^{\Omega}P$

This experiment consists of the comparison of the distribution of $I^{\Omega}P$ in one male and one female C_3H mouse.

Experimental:

A. One C_3H male mouse was given 0.05 mgm $I^{\Omega}P$ (2.1 I_2 atoms/molecule prolactin) at 9:50 A.M. and was sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

B. One C_3H female mouse was given 0.05 mgm $I^{\Omega}P$ (2.3 I_2 atoms/molecule prolactin) at 12:00 noon and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

The findings are recorded in Table 24.

Table 24

Effect of Sex on the Distribution of 0.05 mgm $I^{\alpha}P$ in C_3H Mice

Twenty Minutes After Injection

Organs	% Recovered Dose		Concentration		% Organic Iodine	
	Male	Female	Male	Female	Male	Female
Plasma	15.96	9.26	3.16	2.17	33.2	73.1
Liver	29.33	45.26	5.8	8.35	82.0	90.5
Adrenal	0.17	0.98	0.73	7.18	19.0	11.3

Findings:

Though this female was injected two hours later in the day than the male, there was less activity in the blood and more in the liver and adrenal than in the male, and of what was there, more was organically-bound, indicating that less catabolism had taken place or that the freed iodide had been more rapidly removed. The concentration in the adrenal here appeared to be ten times that in the male, and though the figures for per cent organic iodine were not too different, that for the female indicated tremendous breakdown. Such a finding suggested that the adrenal may be a true target organ for prolactin.

Effect of Physiological State on Distribution of 0.05 mgm $I^{\alpha}P$ at Twenty Minutes

This experiment consisted of a comparison of the

distribution of I^{n}P in a C_3H normal, a 3 day post partum albino, and a three day lactating albino mouse.

Experimental:

A. One C_3H female mouse was given 0.05 mgm I^{n}P (2.3 I_2 atoms/ molecule prolactin) at 12:00 noon and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

B. One 3 day post partum albino mouse was given 0.05 mgm I^{n}P (1.0 I_2 atom/ molecule prolactin) at 5:00 P.M. and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

C. One 3 day lactating albino mouse was given 0.05 mgm I^{n}P (1.0 I_2 atoms/molecule prolactin) at 5:30 and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

The findings are tabulated in Table. 25.

Table 25

Effect of Physiological States on the Distribution of 0.05 mgm I^{n}P

In Mice at Twenty Minutes

Animals	% Recovered Dose			Concentration			% Organic Iodine		
State	A	B	C	A	B	C	A	B	C
Organs	Normal	3dpp	3dl	Normal	3dpp	3dl	Normal	3dpp	3dl
Plasma	9.26	5.3	1.59	2.17	1.6	0.47	73.1	71.3	81.5
Liver	45.26	19.75	8.6	8.35	2.9	1.51	90.5	70.2	82.5
Kidney	9.26	4.22	2.13	8.55	3.8	1.25	61.0	50.5	68.8
Adrenal	0.98	0.03	0.07	7.18	1.3	2.94	11.3	99.5	99.9
Ovary	0.25	0.2	0.07	1.03	1.6	0.40	77.0	40.9	95.0
Mammary Gland		4.3	19.3		0.69	3.5		38.8	22.9
Thyroid		0.4	0.97		6.67	22.4			
Sum	65.01	34.30	32.73						

Findings:

By summing the per cent recovered dose for these seven organs in these three animals, twice as much total activity was present in the normal as in the postpartum or lactating animal. The liver contained the largest amount in the normal and postpartum animals while most was found in the mammary gland in the lactating animal. A decreasing total activity was found in plasma, kidney, and ovary, Mammary gland and thyroid showed increasing totals in the lactating as compared to the postpartum animal. The total activity of the adrenal was lowest for the postpartum, highest for the normal, and intermediary for the lactating animal.

The concentration figures showed the same trends. All except that of the mammary gland in the postpartum and the ovary and plasma in the lactating animal were greater than 1, indicating specific pick-up of injected material. Concentrations for liver, kidney, and adrenal in the normal, of liver, kidney, and thyroid in the postpartum, and of liver, kidney, adrenal, mammary gland and thyroid in the lactating animal were greater than that of the respective plasmas. Such findings indicated that not only was there selective pick-up, but retention as well of material in these sites. The per cent organic iodine figures suggested that the kidney and adrenal of the normal, the kidney, ovary and mammary gland in the postpartum, and the kidney and mammary gland in particular in the lactating

animal were the presumed sites of breakdown.

Distribution of $I^{n}P$ and I^{131} in C_3H Male Mice at About 2 Hours After Injection

This experiment consisted of the comparison of the distribution of 0.05 mgm of prolactin-- one sample of which contained 2.0 I_2 atoms per molecule, and the other of which contained 1.0 I_2 atoms per molecule, injected into single C_3H male mice--with the distribution of an equivalent amount of carrier free I^{131} iodide ions also injected into a C_3H male mouse. All three animals were sacrificed about two hours after injection.

Experimental:

A. One C_3H male mouse was given 0.05 mgm $I^{n}P$ (2.0 I_2 atoms/molecule prolactin) at 10:40 A.M. and was sacrificed 155 minutes later. The organs were digested in 2N NaOH.

B. One C_3H male mouse was given 0.05 mgm $I^{n}P$ (1.0 I_2 atoms/molecule prolactin) at 10:30 A.M. and was sacrificed 120 minutes later. The organs were digested in 2N NaOH.

C. One C_3H male mouse was given 0.1 cc containing an equivalent amount of "carrier free" I^{131} as NaI. at 10:30 A.M. and sacrificed 135 minutes later. The organs were digested in 2N NaOH.

The findings for % recovered dose and concentration are to be found in Table 26.

Table 26

Distribution of $I^{*}P$ and I^{-131} in C_3H Male Mice at About 135 Minutes

Organs	% Recovered Dose $I^{*}P$ A	% Recovered Dose $I^{*}P$ B	% Recovered Dose I^{-131} C	Concentration $I^{*}P$ A	Concentration $I^{*}P$ B	Concentration I^{-131} C
Plasma	6.3	5.4	4.5	1.32	1.0	1.34
Liver	9.5	5.4	2.7	1.33	0.76	0.42
Kidney	2.3	3.6	3.5	1.68	1.51	1.50
Heart	0.14		0.17	0.43		0.30
Lung	1.08		0.24	1.61		0.58
Stomach	7.63		14.05	6.03		4.32
Small Intestine	22.7		3.83	3.87		0.35
Muscle		17.1	30.4		0.33	0.66
Skin		20.4	16.3		0.58	0.77
Submaxillary Gland		0.12	0.05		0.41	0.35
Spleen		7.4	3.78		3.61	2.77
Testis		0.23	0.50		0.21	0.56
Prostate		0.42	0.25		1.96	1.56
Seminal Vesicles		1.68	1.75		0.76	2.79
Brain	0.08	0.06	0.04	0.07	0.04	0.03
Pituitary	0.01		0.002	3.51		0.50
Adrenal	0.02	-	0.005	0.39		0.20
Thyroid	-	1.65	2.6		73.32	177.90
Carcass	62.5	33.2	16.7		0.71	0.28

Discussion:

Stomach, small intestine, muscle, and skin account for 67.83% and 64.58% of the recovered dose in the animals given the $I^{*}P$ and I^{-131} respectively. Plasma, liver, and kidney account for 18.1%, 14.4% and 10.7% more in the three animals respectively. The spleen, seminal vesicles, and thyroid account for 10.73 and 8.13% in the second and third animals here tabulated. These organs together,

therefore, account for 91.83% of the total recovered dose in the $I^{\Omega}P$-injected animals, and 82.58% in the I^{-131}-injected animal. Therefore, at this time interval, the distribution is similar. Note that the less iodinated protein material has a distribution closer to that of the I^{-131}-injected animal than the more heavily iodinated material.

Comparison of the concentration figures for these three animals shows that liver, lung, small intestine, and spleen have higher concentrations in the $I^{\Omega}P$-injected animals, while muscle, seminal vesicles, and thyroid have smaller concentrations. Liver, lung, and small intestine have extensive capillary beds, while small intestine and spleen have large accumulations of lymphatic nodules which probably have trapped and held the protein molecules. This is particularly true of the lung, the first capillary bed which the $I^{\Omega}P$ has to traverse after injection into the jugular vein. The smaller concentrations may be considered as interference with the free distribution of iodide ions by the circulation. Not only was the I^{-131}-injected animal given free iodide ions, but also it was given far fewer ions. Thus, this apparent interference with iodide distribution may be only a reflection of this difference in numbers of ions available. The higher concentration in such organs as the thyroid in particular, would give rise to such figures. The fact that there is as much concentration in the thyroid of the animal given $I^{\Omega}P$ as there is, is evidence of the extent to which the injected

protein-bound iodine has been freed.

Conclusion:

From these findings it can be concluded, that, by two hours, injected $I^{\eta}P$ (iodinated so that there are 1 or 2 iodine atoms per molecule) has been destroyed to such an extent that its distribution resembles that of iodide ions. The discrepancies found are explainable on the basis of stasis in capillary bed, or ensnarement by lymphoid accumulations of those iodinated protein molecules not yet destroyed, and the differences in the number of potential iodide ions here injected.

Since two hours proved too late--the distribution found approached that of inorganic iodide too closely for differentiation--a distribution study in 3 day lactating albino mice at twenty minutes was undertaken.

Distribution of $I^{\eta}P$ and I^{η} in 3 Day Lactating Albino Mice at 20 Minutes

This experiment consisted of the comparison of the distribution of $I^{\eta}P$ and I^{η} in 3 day lactating albino mice twenty minutes after injection.

Experimental

One 3 day lactating albino mouse was given 0.05 mgm of $I^{\eta}P$ (1.0 I_2 atoms/molecule prolactin) at 5:30 P.M. and sacrificed twenty minutes later. The organs were subjected

to Somogyi zinc sulfate fractionation.

Three 3 day lactating albino mice were given 0.1 cc containing 0.78 gamma of I^{β} at 3:30, 4:30, and 5:30 P.M. They were sacrificed twenty minutes later and their organs subjected to Somogyi zinc sulfate fractionation.

The findings for % recovered dose, concentration, and % organic iodine are recorded in Table 27.

Table 27

DISTRIBUTION OF $I^{\beta}P$ AND I^{β} IN 3 DAY LACTATING ALBINO MICE AT 20 MINUTES

Organs	% Recovered Dose $I^{\beta}P$	% Recovered Dose I^{β} Av. of Three	Concentration $I^{\beta}P$	Concentration I^{β}	% Organic Iodine $I^{\beta}P$	% Organic Iodine I^{β} Av. of Three
Plasma	1.59	4.28	0.47	1.31	81.5	2.3
Liver	8.60	2.99	1.51	0.51	82.5	8.9
Kidney	2.13	0.84	1.25	0.59	68.8	6.4
Adrenal	0.07	0.01	2.94	0.35	99.9	3.6
Ovary	0.07	0.04	0.4	0.33	95.0	1.2
Mammary Gland	19.30	6.98	3.5	1.34	22.9	3.4
Thyroid	0.97	0.76	22.4	40.0		
Carcas	67.27	86.17				

Discussion:

Here there are differences. There is more total activity in the liver, kidney, adrenal, ovary, and mammary gland of the $I^{\beta}P$-injected animal, and less in the plasma than in the same organs of the $I^{-\beta}$- injected animals. This low plasma % recovered dose and concentration was unusual. In most cases, plasma concentration was higher in the $I^{\beta}P$-injected animals than that of the corresponding

$I^{-\pi}$-injected control animals. On the basis of concentration, there is more throughout in the $I^{\pi}P$ than in the $I^{-\pi}$- injected animal, except for the plasma, as already mentioned, and except in the thyroid where there is twice as much in the $I^{-\pi}$- injected animals. That, in the thyroid, the concentrations do not show a greater difference, indicates how much of the injected $I^{\pi}P$ has been broken down at this time interval in this 3 day lactaing animal. From the per cent organic iodine figures, the major breakdown seems to be taking place in the mammary gland. From their per cent organic iodine findings, the $I^{-\pi}$- injected animals show practically no incorporation of I^{π} into any organic conpounds by this time.

The concentration in the liver of the $I^{\pi}P$-injected animal is three times that of the $I^{-\pi}$-injected animal, while that of the kidney is twice, of the adrenal nine times, and of the mammary gland about three times. The concentration in the ovary is the same in both cases. The high concentration in the mammary gland of the $I^{-\pi}$-injected animal probably indicates the extent of the blood flow to that organ in this lactating state, and also the avidity of the gland for everything. Iodide is known to be exereted in milk (Cushny, 1940). From these figures, liver and kidney seem to be protein concentrators, and kidney too seems to be causing some breakdown of $I^{\pi}P$, though the smaller recoverable per cent of organic iodine here may be a reflection of breakdown somewhere else, such as in the mammary gland, with excretion of iodide by the kidney.

The high concentration in the adrenal of the $I^{*}P$-injected animal in comparison to the I^{-*}-injected animal is suggestive of specific pickup of $I^{*}P$.

Summary:

From this study of distribution, it seems safe to say that in 3 day lactating mice a difference does exist between the distribution of $I^{*}P$ and I^{*} at twenty minutes after injection.

From these studies it was thought that an investigation of the effect of time after injection on the distribution of $I^{*}P$ in C_3H and 3 day lactating albino mice would be advisable. The following studies were carried out.

Effect of Time After Injection on The Distribution of 0.05 mgm of $I^{*}P$

This experiment consisted of the comparison of the distribution of $I^{*}P$ given to series of C_3H male and 3 day lactating albino mice which were sacrificed after increasing intervals following intravenous injection.

Experimental:

One C_3H male mouse was given 0.05 mgm $I^{*}P$ (0.4 I_2 atoms/molecule prolactin) at 12:00 noon and sacrificed 1-2 minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

One C_3H male mouse was given 0.05 mgm $I^{*}P$ (1.1 I_2 atoms/molecule prolactin) at 4:00 P.M. and sacrificed 10 minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

One C_3H male mouse was given 0.05 mgm $I^{\alpha}P$ (2.1 I_2 atoms/ molecule prolactin) at 9:50 A.M. and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

One C_3H female mouse was given 0.05 mgm $I^{\alpha}P$ (2.3 I_2 atoms/ molecule prolactin) at 12:00 noon and sacrificed twenty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

One C_3H male mouse was given 0.05 mgm $I^{\alpha}P$ (1.1 I_2 atoms/ molecule prolactin) at 12:00 noon and sacrificed thirty minutes later. The organs were subjected to Somogyi zinc sulfate fractionation.

Two C_3H male mice were given 0.05 mgm $I^{\alpha}P$ (2 I_2 atoms/ molecule prolactin) at 12:30 P.M. and 12:35 P.M. and sacrificed 130 and 160 minutes later respectively. The organs were digested in 2N NaOH. The findings were averaged.

The findings for these animals are recorded in Table 28.

Three 3 day lactating albino mice were given 0.05 mgm $I^{\alpha}P$ (0.77 I_2 atoms/molecule prolactin) at 3:30 P.M., 4:00 P.M and 3:10 P.M. and sacrificed 5, 20, and 60 minutes later respectively. Their organs were subjected to ether extraction and Somogyi zinc sulfate fractionation.

The findings for these animals are recorded in Table 29.

Discussion:

At 1-2 minutes after injection in C_3H mice, 97% of the activity was found in the blood, what was found in the other organs investigated was what plasma was contained thereinafter exsanguination. At 10 minutes, the activity which was in the blood had largely moved out of that vehicle into the extracellular spaces, chiefly perivascular in location, and so the plasma total activity had fallen, and that of

Table 28

Effect of Time after Injection of 0.05 mgm $I^{\eta}P$ in C_3H Male Mice

Organs	% Recovered Dose 1-2 min.	10 min.	20 min.	30 min.	145 min.	Concentration 1-2 min.	10 min.	20 min.	30 min.	145 min.	% Organic Iodine (Corrected) 1-2 min.	10 min.	20 min.	30 min.
Plasma	96.65	15.60	15.96	6.55	7.88	28.4	4.68	3.2	1.98	1.9	99.21	95.0	66.8	58.0
Liver	5.39	15.74	29.33	17.67	5.9	1.0	3.31	5.8	3.48	1.36	99.9	91.2	82.	51.5
Kidney	1.95	7.62	9.26♀	4.68	1.72	1.64	4.66	8.55♀	2.6	1.21	99.9	85.0	61. ♀	56.0
Stomach	1.58	3.2	3.9 ♀	4.52	16.4	1.27	1.69	2.12♀	4.63	10.88	77.5	25.5	24.6 ♀	15.0
Sm. Int.	10.44	9.13	8.70♀	28.59	18.3	1.05	0.98	2.04♀	0.96	2.95	60.0	32.4	10.5 ♀	6.0
Adrenal		0.06	0.17	0.02	0.015		0.49	0.73	12.0	0.32			19.0	39.4
Testis		0.04		0.52	0.23		0.31		0.89	0.11		32.4		52.5

♀ - Female

Table 29

Effect of Time after Injection of 0.05 mgm $I^{\eta}P$ in 3 Day Lactating Albino Mice

Organs	% Recovered Dose 5 min.	20 min.	60 min.	Concentration 5 min.	20 min.	60 min.	% Organic Iodine 5 min.	20 min.	60 min.	% Ether Extractable Iodine 5 min.	20 min.	60 min.
Plasma	11.1	7.15	1.75	3.66	2.13	0.05	98.0	82.6	70.0	2.1	1.5	6.6
Liver	36.3	15.25	27.0	7.16	2.68	4.2	78.8	62.5	26.2	10.2	10.9	1.4
Kidney	37.3	2.75	10.65	8.23	2.88	10.6	68.0	49.5	66.5	5.0	7.3	5.0
Mamm. Gl.	7.32	10.9	12.85	1.79	1.87	1.6	18.2	11.2	12.7	21.7	14.9	3.2
Thymus	0.51	2.13	0.03	1.19	2.01	0.24						
Spleen	0.67	0.47	0.11	1.62	0.82	0.38						
Adrenal	0.12	0.06	0.02	3.23	1.82	0.22						
Ovary	0.19	0.04	0.03	1.82	0.54	0.31						
Thyroid	0.12	0.17	0.70	0.73	2.64	10.70						

the organs had risen probably in proportion to their content of perivascular extracellular spaces. These, of the organs investigated, seemed to be greatest in liver, kidney, and small intestine. This process had continued at twenty minutes (the animals used for this part of the study received a dose of $I^{*}P$ with 2 times as much iodine on each molecule of prolactin - It had previously been shown that such diffuses less well.) with some shift from organs not here investigated back into these organs, for the blood total had not changed noticeably, while liver and kidney seem to have added considerably to their totals. The amount in the small intestine had not changed much. What was in the small intestine 10 minutes earlier probably now had moved on to the liver via the portal vein, while new material had taken its place from the systemic circulation and, in turn, had moved out into the perivascular spaces of this large capillary bed. By thirty minutes, the level in the blood had again dropped by half and was now down to about 7%. The amount found in the liver had passed its peak and was beginning to move out again, while that in the kidney certainly had. The stomach continued to show an increasing total, since presumably, this organ secretes iodide into its lumen, just as it does chloride. Apparently, at this time, insufficient iodide had been readsorbed or passed on to the lumen of the small intestine to counteract this excretory, or rather secretory, function of the stomach. The amount in the small intestine was now nearly 3/10 of all that which was injected. It probably contained, in its lumen, some from the stomach, some in

its capillary beds, and some in its lymphoid accumulations, all of which together account for this surprising total. By two hours, only stomach and intestine still showed large amounts of activity.

The concentration figures for the 1-2 minute animal indicated a more or less random distribution everywhere examined except in the plasma and kidney. The greater-than-random distribution of the kidney may be attributed to the large capillary bed. By ten minutes, the concentration in kidney equaled that of blood; that in the liver was not far behind; while that in the stomach was already greater-than-random, a fact not true for the small intestine at this time. At 20 minutes, kidney and liver were greater than plasma in concent ration, and stomach and small intestine were twice random, while adrenal had not quite reached random distribution. At 30 minutes, plasma was twice random; kidney was rapidly approaching that of blood; intestine and adrenal were now random, or slightly greater; liver and stomach showed considerable concentrations still. At 145 minutes, blood had not much changed; liver and kidney were now back to nearly random; stomach was very high; and intestine was three times random, probably a spill-over from stomach; adrenal was down to a third of a random distribution.

When the % organic iodine figures are studied, the most obvious finding wasthat by 30 minutes half of the injected material had been destroyed; only 50% - 60% of it could be recovered as organically-bound activity, and in the stomach and small intestine,

the break down was marked. (The figure for the adrenal is not reliable). This break down had apparently been orderly but continuous, as study of the figures indicates.

When the figures for the three day lactating animals are examined (See Table 29); similar trends are noted. Here, however, the whole process seems to have been speeded up, for, by 5 minutes, only a little more than 10% of the activity was still in the plasma. The peaks seemed to be at five rather than twenty minutes as before, with a large accumulation in the liver at that time, which continued and diminished a fourth by 60 minutes. The high kidney finding at 5 minutes was followed by a considerably lower one at 20 minutes, and a high one again at 60 minutes, probably representing iodide excretion at this latter time. The total in the mammary gland continued to rise slowly but steadily. Thymus had a peak at 20 minutes, but all the other organs here investigated showed a steady downward trend in total activity contained, except thyroid, which showed a steady rise.

The concentration figures reflected the changing total contained-activity of these organs. Kidney and thyroid showed a peak at 60 minutes. The concentration in mammary gland was maintained. (The animals were not being suckled and so this accumulated activity was not being lost through the milk; rather it seemed to be being stored there.)

% organic iodine figures indicated that mammary gland and liver were either breaking up the injected iodinated protein or were accumulating the iodide removed from the material in breakdown elsewhere. Surprisingly, the plasma and kidney had high organically-bound iodine figures. Could these indicate some degree of synthesis of the freed iodine into some other compound, such as thyroxine?

The per cent ether-extractable iodine shows high figures for liver and mammary gland for 5 and 20 minutes, with a maintained figure for kidney and a sudden elevation in plasma at 60 minutes. At that time, the same figures for liver and mammary gland had fallen sharply. Just what these findings mean is not known.

Summary:

1. The total recoverable activity of plasma fell with time in both C_3H and 3 day lactating albino mice, as did the concentration and the % organic iodide. The rate of fall was faster in the lactating than in the male mice.

The total recoverable activity and the concentration of that activity in the liver and kidney rose to a peak at 20 minutes, then fell in the C_3H males, while in the 3 day lactating albinos, these were found to be high at 5 minutes, subsequently fell, at twenty minutes, but rose again by 60 minutes. The % organic iodine fell

to 50% in the C_3H males, and in the 3 day lactating females values of 62.5% for the liver and of 49.5% in the kidney were found at 20 minutes. At an hour, these values were modified to 26.2% for the liver and to 66.5% for the kidney in the latter series. 10% of the activity was ether-extractable in the liver for the first two time intervals studied in the 3 day lactating females, with approximately half that in the kidney at the earlier, and three-fourths that value at the later time interval, i.e., twenty minutes. At an hour, these values were 1.4 and 5.0% for the liver and kidney respectively.

The stomach and small intestine in the C_3H mice showed a random distribution at 5 and 10 minutes, an amazing finding in the light of the fact that these specimens contained the contents of these organs as well as the organs themselves. This condition continued for the small intestine, while the stomach showed an increasing concentration with time to the high figure of 10.88 at 2 hours. The concentration of the small intestine had also increased to 2.95 at this same time. By thirty minutes, practically all of this activity was as inorganic iodide.

In the C_3H males, the adrenal showed negligible total activity but a high concentration of 12.0 at 30 minutes. The per cent organic iodide was always low. In the lactating animals, the adrenal showed a falling content and concentration, as did the

ovary. The thyroid showed an increasing content and concentration indicating the extent of breakdown.

From these time distribution studies, it seems that minimally iodinated $I^{\alpha}P$ injected intravenously rapidly diffuses out of the vascular tree into the extracellur spaces, from which it is either carried back to the blood stream by the lymphatics, or trapped in lympoid accumulations, By thirty minutes, some 50% has been destroyed, if it is injected into C3H male mice; the exact time for a comparable breakdown in 3 day lactating albino mice has not as yet been elucidated.

In the mammary gland of the lactating animals, there was a progressive increase in the total contained activity with a maintained concentration of about 1.75. Only small amounts (about 14%) of the contained activity can be recovered as organic iodine, while at 5 minutes, a fifth of the activity was ether-extractable. At twenty minutes this had fallen to 14.9%, and by one hour, to 3.2%. From these findings, it was surmised that $I^{\alpha}P$ is taken to, and collects in, the lactating mammary gland; there it is held for excretion into the milk, probably as iodide. The intermediaries of the presumed catabolism in the mammary gland are as yet unknown, as they are for other sites, which have not as yet been definitely established.

When $I^{\alpha}P$ was found to concentrate in lactating mammary

glands, a study of the metabolism of $I^{\alpha}P$ in this tissue was undertaken.

Metabolism of $I^{\alpha}P$ in Lactating Mammary Gland Tissue

Effect of Size of Dose on Fractionation of Radioactivity in Mammary Gland Tissue

This experiment consisted of a comparison of the time after injection and size of dose of $I^{\alpha}P$ given to 3 day lactating albino mice on the fractionation of the $I^{\alpha}P$ contained in the mammary glands.

Experimental:

Two 3 day lactating albino mice were given 0.05 mgm of $I^{\alpha}P$ (1.5 I_2 atoms/molecule prolactin) at 5:30 P.M. and 5:10 P.M. and sacrificed 5 and 10 minutes later. The mammary glands were lyophilized at -20°C for four days, extracted with ether, and fractionated with Somogyi zinc sulfate reagents.

Two 3 day lactating albino mice were given 0.25 mgm of $I^{\alpha}P$ (1.5 I_2 atoms/molecule prolactin) at 4:45 and 5:10 P.M. respectively. The animals were sacrificed 5 and 10 minutes later. The mammary glands were treated as described above.

The results are recorded in Table 30.

Table 30

Effect of Time and Dose on Fractionation of Radioactivity in Mammary Glands of 3 Day Lactating Albino Mice Given $I^{\alpha}P$ Intravenously

Treatment	% Recovered Dose	% Concentration	% Inorganic Iodide	% Organic Iodide	% Ether Soluble Iodide
5 min. 0.05 mgm	3.98	0.58	68.8	17.55	13.85
5 min. 0.25 mgm	4.83	0.91	70.0	21.3	8.8
10 min. 0.05 mgm	5.8	0.94	85.4	7.2	7.3
10 min. 0.25 mgm	2.6	0.66	79.3	11.1	9.6

Discussion:

From the above table, though the % recovered dose and concentration are not remarkable, the % inorganic iodide found indicated marked breakdown, an average of 75.5% for these four glands. In fact, only about 30% is not accounted for as inorganic iodide at 5 minutes, while only 17.7 % is not so accounted for at 10 minutes, or nearly half of what had not been broken down at 5 was by 10 minutes. The size of dose did not make much difference at 5 minutes, while at 10 minutes, more of the small dose had been destroyed. The amount found in the ether-soluble fraction was greatest with the smallest dose, at the shorter time interval, and least with the smaller dose, at the longer time interval. The latter was a little more than half the former. With the five times

larger dose, the differences do not appear in this ether-soluble fraction. Under these controlled conditions, these ether-soluble percentages are small.

This study was extended to longer time intervals, and to a series of mice in full, rather than early lactation.

Effect of Time on Distribution of $I^{\alpha}P$ in Mammary Glands of 17 Day Lactating Albino Mice

This experiment consisted of the comparison of the fractionation findings for a series of 17 day lactating mouse mammary gland tissues sacrificed at increasing time intervals after injection of $I^{\alpha}P$ intravenously.

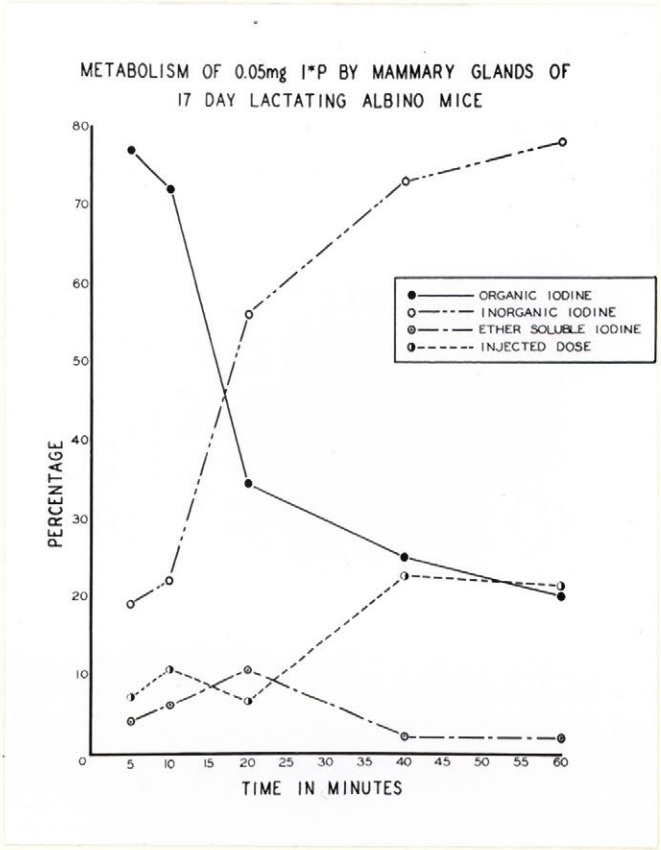
Experimental:

> Five 17-day lactating albino mice were given 0.05 mgm of $I^{\alpha}P$ (1.56 I_2 atoms/molecule of prolactin) at 11:37 A.M., 12:10 P.M., 2:12 P.M., 2:05 P.M. and 2:00 P.M., and were sacrificed at 5, 10, 20, 40, and 60 minutes respectively. The mammary glands were lyophilized at -20°C and 4 mm Hg for 4 days, extracted with ether, and subjected to Somogyi zinc sulfate fractionation. The results are recorded in Figure 3.

Discussion:

From Fig. 3 , the per cent injected dose in the mammary glands rose steadily, with time, to a high of 23% at 40 minutes, and then fell slightly, to 21% at 60 minutes. The % organic iodine recoverable at 5 minutes was 73% here, which by 20 minutes had

Fig. 3. Curves showing percentages of, organic iodine, inorganic iodide, and ether soluble iodine in mammary glands of 17 day lactating mice sacrificed at various time intervals after intravenous injection of 0.05 mgm of $I^{*}P$. A curve showing the per cent of the original injected dose present in the glands at these various time intervals is added also.


METABOLISM OF 0.05mg I*P BY MAMMARY GLANDS OF
17 DAY LACTATING ALBINO MICE
PERCENTAGE
TIME IN MINUTES
ORGANIC IODINE
INORGANIC IODINE
ETHER SOLUBLE IODINE
INJECTED DOSE
0
10
20
30
40
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60
70
80
5
10
15
20
25
30
35
40
45
50
55
60

3

fallen to 33%, and continued to fall to 20% at the end of one hour. The per cent inorganic iodide rose correspondingly. The % ether-extractable iodine at 5 minutes was 4%, but rose to 11% by twenty minutes, then fell off again, so that by 40 minutes it was down to 2.5%.

The breakdown here had not occurred as rapidly as in the former experiment, where, it will be remembered, that at 5 minutes, there was already a 70% catabolism of the material.. The explanation for this may lie in the fact that these animals are in full rather than early lactation, and hence, the avidity of the gland for prolactin is less, or its capacity to destroy the material is less.

Since the rise in % inorganic iodide paralleled the rise in total contained activity in the gland, and since the impression had been gained that the mammary gland was a locus for catabolism of the iodinated hormone, investigation of in vitro catabolism was undertaken.

Study of In Vitro Catabolism of $I^{\alpha}P$ by 17-Day Lactating Mammary Gland.

This experiment consisted of the comparison of the fractionation findings for 17 day mammary gland tissue homogenates to which 0.05 mgm $I^{\alpha}P$/had been added and which were then incubated for varying lengths of time.

Experimental:

To the mammary gland tissue of a 17-day lactating albino

mouse divided into 4 equal aliquots following homogenization in normal saline, 0.05 mgm $I^{n}P$ (1.5 I_2 atoms/molecule of prolactin dissolved in 0.1 cc of 7.6 phosphate buffer) was added. The mixture was incubated in an automatically controlled 37°C oven for 5, 10, 20, and 40 minutes respectively. Subsequently, the incubates were lyophilized at -20°C and 4 mm Hg for 96 hours, extracted with dry ether, and subjected to Somogyi zinc sulfate fractionation. The findings are tabulated in Table 31.

Table 31

Effect of incubation on 220 mgm of mammary gland tissue from a 17-day lactating albino mouse to which 0.05 mgm $I^{n}P$ (1.5 I_2 atoms/molecule prolactin) had been added, following lyophilization, ether extraction, and Somogyi zinc sulfate fractionation.

Time incubated	% Organic iodine	% Inorganic iodine	% Ether extractable iodine
5 minutes	70.5	3.77	25.6
10 minutes	78.0	5.3	16.7
20 minutes	75.0	5.25	19.8
40 minutes	73.0	4.73	22.15
Average	74.1	4.76	21.06

Discussion:

Incubation had no effect whatsoever on the added $I^{n}P$ activity. Homogenates of mammary glands from a 17-day lactating albino mouse do not have the capacity to break the carbon-to-iodine bond.

This finding did not of course prove that mammary gland slices might not have had the ability to catabolize $I^{n}P$. A more likely explanation is that $I^{n}P$ is broken down under living conditions

only. This finding, however, opened the question as to whether the iodide found in the lactating mammary gland really did arise from catabolism within the gland, or whether it was the result of catabolism elsewhere in the body, with transport of the freed iodine here. Much more work needs to be done on this question.

All that can be said from these experiments is that a large amount of the injected activity does collect in the lactating mammary gland; that the form of that activity does change with time; but that the reasons for these observed changes are not as yet understood.

General Conclusions

It has been shown that such factors as amount of protein, degree of iodination of the protein, time of day, sex, physiological state, and time after injection all affect the distribution of the minimally iodinated protein hormone. The physiological factors back of these differences have been pointed out and an attempt has been made to analyze the interactions of these forces. An attempt has been made to study the metabolism of the material in the lactating mammary gland, but the question of its catabolism there or elsewhere has not been settled. It has, however, been shown that the material is 50% catabolized by 30 minutes in C_3H male mice, and that by two hours, its behavior so closely resembles iodide as to make interpretation difficult. Such a destruction is surprising

and suggests that the endogenous hormone must exert its action rapidly on its various end organs, i.e. mammary gland, adrenal, and ovary.

AUTOGRAPHIC RESULTS:

Introduction:

Beta rays emitted by I^{131} will effect photographic emulsion in the same way light rays do. Tissue sections containing radioactivity can be coated with photographic emulsion and, after a suitable exposure time, developed as an ordinary photograph. In such tissue sections, wherever I^{131} had been, the overlying photographic emulsion will contain silver grains, minute particles of metallic silver due to the conversion by the beta rays of the photolytic silver granules of the emulsion to silver granules which become grains upon development. By use of sufficiently thin sections to which a layer of photographic emulsion has been applied closely, this phenomenon can be used to make visable the cellular localization of materials containing radioactive isotopes. For use in this investigation, the retention of prolactin within such histological preparations proved a problem. The following technique was therefore evolved.

Autographic Technique:

Fixation:

Tissues from freshly sacrificed animals were plunged into formalin or orth fixatives, modified by addition or substitution of ammonium ions, and adjusted to the isoelectric point of $I^{\eta}P$. Such modifications--the first to enhance polymerization of the protein (Fraenkel-Conrat and Mecham, 1949), and the second to aid rapid precipitation of the material in situ--used on tissues from animals given large (0.5 mgm) doses of $I^{\eta}P$ intravenously only a short time (7 and 20 minutes) before sacrifice, resulted in the autographs on which the following description is based. After fixation for 12 to 24 hours, the tissues were subjected to the usual histological and autographic procedures described immediately below:

Histological Techniques:

The fixed tissues were washed in running water for 24 hours, trimmed, embedded in paraffin, cut into 5-10 μ sections, mounted on glass slides with the aid of an albumin adhesive, and dehydrated preparatory to ataining with hematoxylin and eosin (half of any set of tissues were left unstained). The slides were then coated with colloidin which formed a protective film less than 1 μ thick. This eliminated chemical reactions of the emulsion.

Autographic Technique:

The prepared slides were counted* to determine the contained activity as a guide to time of exposure required, coated with melted photographic emulsion (according to a modification of the coating technique developed by Belanger and Leblond (Leblond et al, 1948)), and stored in light-tight boxes for exposure at -18°C. Samples were removed in pairs, developed as in routine photography, and mounted in Canada Balsam. NT_4 emulsion was used. The resulting autographs were studied and representative areas photographed.

In the preparation of such tissue sections, all inorganic ions, fat, small organic molecules and easily soluble material was removed. Polysaccharides of large molecular size and insoluble proteins only remained.

Autographic Findings:

The autographic distribution of I^{131} was as follows:

All organs studied showed some "autographic reaction"--hereafter called simply "reaction"--the degree of which varied in intensity (Fig. 4). This general reaction was traced to perivascular concentrations and to slight but definite reactionsin all tissue spaces.

In decreasing order of magnitude, specific concentrations

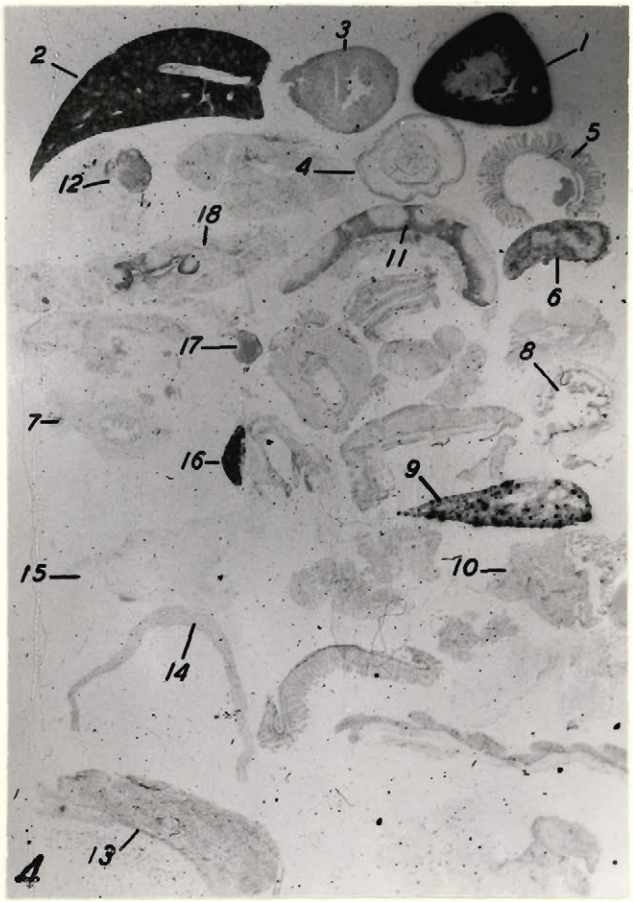
* by Geiger Muller Tube.

Fig. 4. Unstained coated autograph of the orth fixed tissues of a 3 day lactating albino mouse given an intravenous injection of 0.5 mgm of I^{n}P containing 0.3 mc of I^{131}.

X 4.5

1) Kidney
2) Liver
3) Heart
4) Eye
5) Jejunum
6) Spleen
7) Uterus
8) Urinary Bladder
9) Lung
10) Pancreas
11) Mesenteric Lymph Node
12) Ovary
13) Mammary Gland
14) Muscle
15) Brain
16) Thyroid
17) Ovary
18) Salivary Gland

This picture shows the relative intensity of reaction obtained in the various organs.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were found in the 1) kidney, 2) the thyroid, 3) the liver, 4) the lung, 5) the lymphatic organs such as the spleen, 6) the interstitial parts of the entire gastrointestinal tract and other hollow viscera such as the urinary bladder, 7) the endocrine organs, 8) the fatty tissues from the mesentery, 9) the muscle and muscular organs such as the heart, 10) glandular tissues such as the mammary and salivary glands, 11) the skin, and 12) the brain.

1) Kidney.

The intense reaction found in the kidney was limited to the periphery of the organ. It was a discrete response (Fig. 6) forming wiggly lines or segments thereof. Comparatively little reaction was found between these intense concentrations. On high power microscopic examination, these were found to be definite streaks which took various shapes--often separated one from another by wide areas of apparently unreacting tissue (Fig. 7). Examination of these made it possible to assign the reaction to: 1) the space between the visceral and parietal layers of Bowman's capsule surrounding glomeruli, and 2) to the proximal segments of some proximal convoluted tubules. More distally, the reaction was found to overlie the apices of the cells surrounding the proximal convoluted tubules.

2) Thyroid.

An intense reaction was found over the apices of the

Fig. 5. Unstained coated autograph of the liver of the same animal.

X 12

This picture indicates that the reaction in the liver is diffuse but tends to concentrated over the sinusoids.

Fig. 6. Stained coated autograph of the kidney of the same animal.

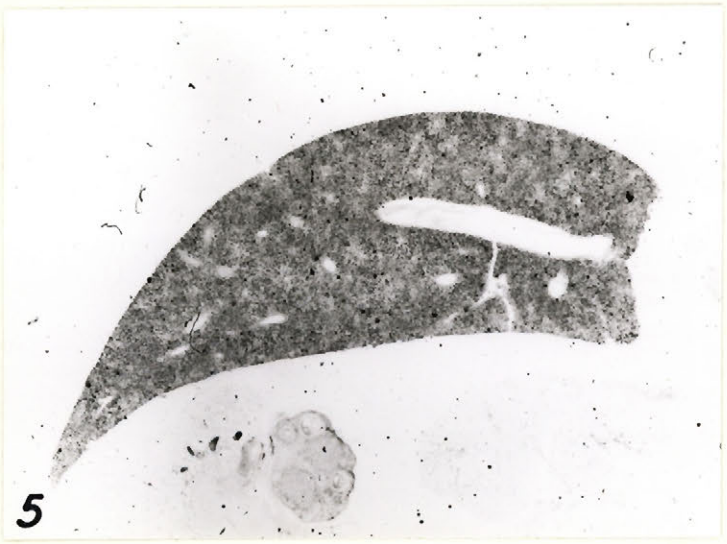
X 12

The reaction here is discrete and limited to the cortex.

Fig. 7. Stained coated autograph of the same kidney as that in Figure 6.

X 237

The reaction here is also around the glomerulus in Bowman's capsule and throughout a length of convoluted tubule. Note the large non-reacting areas of kidney cortex.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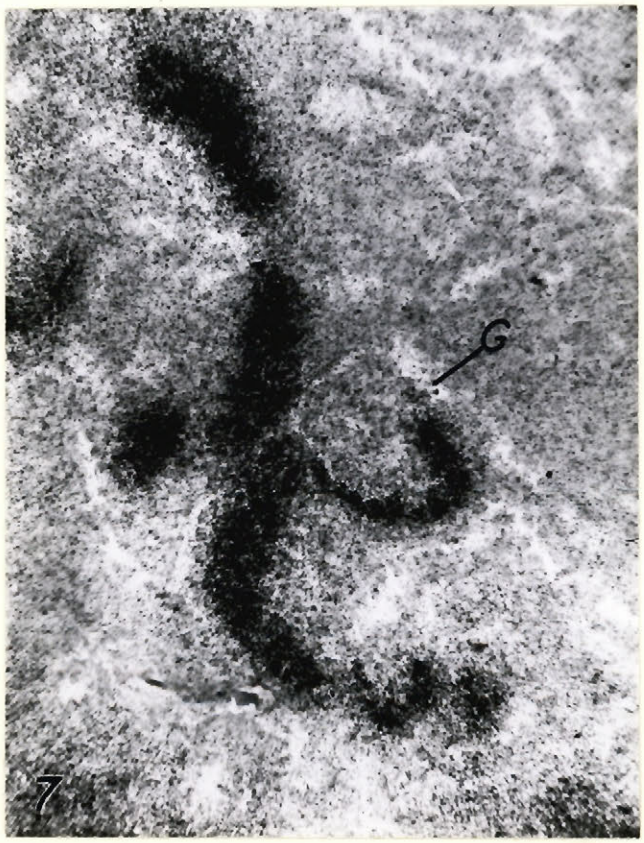
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follicular cells of the thyroid gland. This was thought due to the incorporation of freed iodide from the $I^{\alpha}P$ rather than to the $I^{\alpha}P$ itself. (Fig. 4)

3) Liver.

The reaction in the liver was diffuse throughout. Only the large blood vessels were relatively unreactive. On careful study, slightly more reaction was found over the sinusoids, and especially over the Kupffer cells, than over those of the liver parenchyma itself. (Fig. 5)

4) Lung.

An apparently random patchy distribution of intensely heavy accumulations was found. These were traced to small blood vessels, and gave the appearance of discrete masses plugging these vessels. Between such, the lung parenchyma was surprisingly free of reaction (Fig. 4).

5) Spleen and Lymph Nodes.

The reaction in the spleen (Fig. 8) neatly outlined the vascular channels. There were accumulations in the sinusoids between the lympnatic nodules of the white pulp.

Along a collection of lymphatic nodules in the mesentery, a definite reaction was found surrounding the central lymphatic

Fig. 8. Unstained coated autograph of spleen of the same animal.

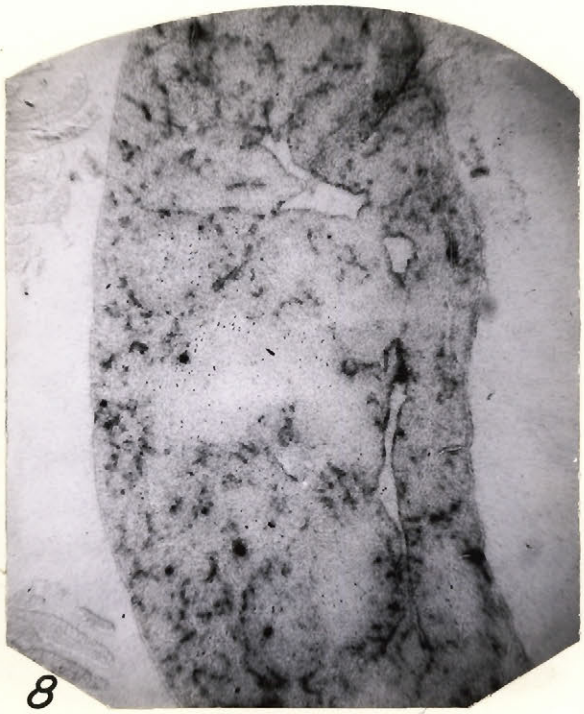
X 40

The reaction here is perivascular and about the periphery of the white pulp nodules.

Fig. 9. Unstained coated autograph of Mesenteric lymph nodules from the same animal.

X 27

The reaction here is perinodular i.e. in the sinusoids between the central nodules.



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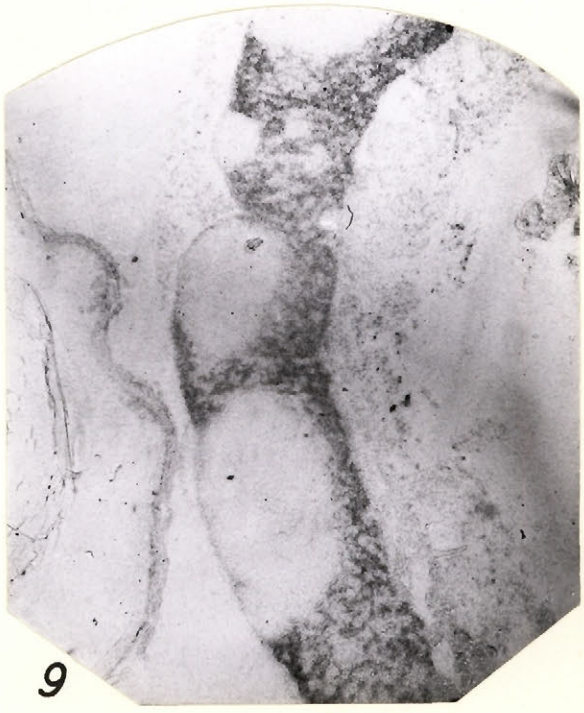


9

nodule. This was heaviest on the mesenteric root side and nearly absent on the serosal side. Between such nodules, the reticulum enclosed heavy reactions within the sinusoids. (Fig. 9).

Gastrointestinal Tract and Other Hollow Viscera:

The submucosal areas of the gastrointestinal tract, urinary bladder, fallopian tube, and uterus stood out in sharp contrast to the mucosa itself in unstained slides. All the interstitial spaces showed a uniformly diffuse reaction with concentrations along the blood vessels. (Fig. 4) In the gastrointestinal tract, especially in the intestine, (Figs. 10 and 11), this reaction extended into the cores of the villi, and was intensified at the tips. There was a definite reaction between the two muscle coats as well as a less promounced subserosal reaction. In the villi, the reaction tended to diffuse out from the core itself to overlie the bases of the epithelial cells. (Fig. 11) The spread of reaction, however, was such as to raise the question as to whether this was due to the spread of reaction obtained with any I^{131}-containing substance. (The energy of the beta particles of I^{131} is such as to cause a somewhat diffuse reaction rather than the sharply localized reaction obtained with C^{14} whose beta particles possess less energy.)

7) Endocrine Glands.

The adrenal showed a diffuse reaction throughout the

Fig. 10. Stained coated autograph of duodenum from the same animal.

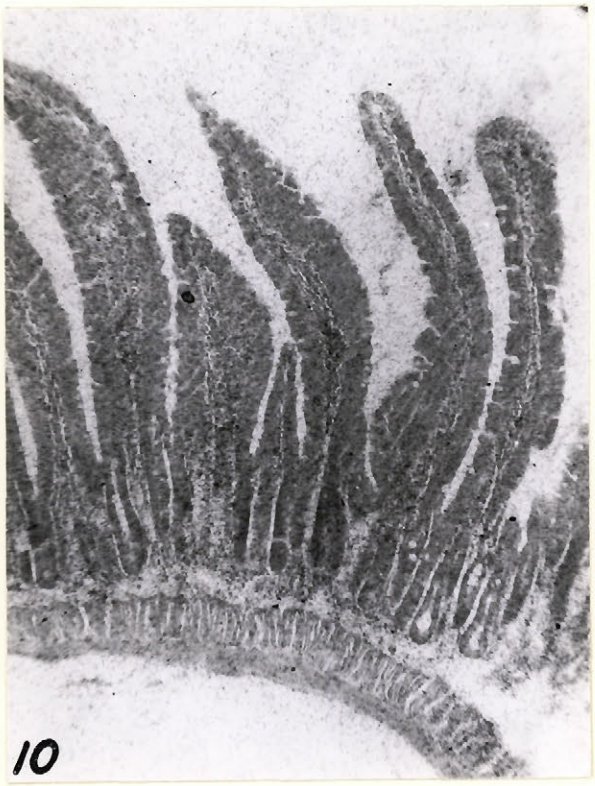
X 108

The reaction is most pronounced in the core of the villi and in the submucosa. The reaction between the muscle coats is less distinct here.

Fig. 11. Unstained coated autograph of jejunum from a 3 day lactating albino mouse given 0.5 mgm of $I^{n}P$ containing 0.3 mc of I^{131} 7 minutes prior to sacrifice

X 108

This shows the reaction in the core of the villi and along the line between the two muscle layers. Note that the reaction is slightly more intense at the apex of the two central villi.



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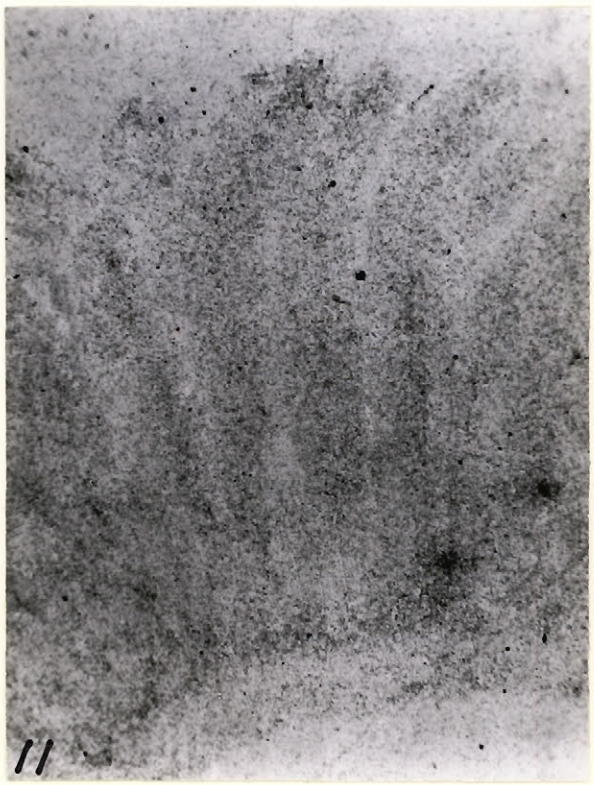


11

cortex which was intracellular. No areas or zones of increased reaction were found. The medulla apparently did not react.

The ovary (Fig. 12) showed a striking reaction throughout the corpora lutea. This was dense, diffuse, and intracellular. The rest of the ovarian tissue was relatively free of reaction except for the larger blood vessels which also showed a heavy ring-like reaction in the perivascular tissue. In one follicle, a reaction was found in the liquor folliculi.

8) Fatty Tissue.

Throughout a section of mesenteric fat, a diffuse reaction was observed. (Fig. 4).

9) Muscle and Muscular Tissues.

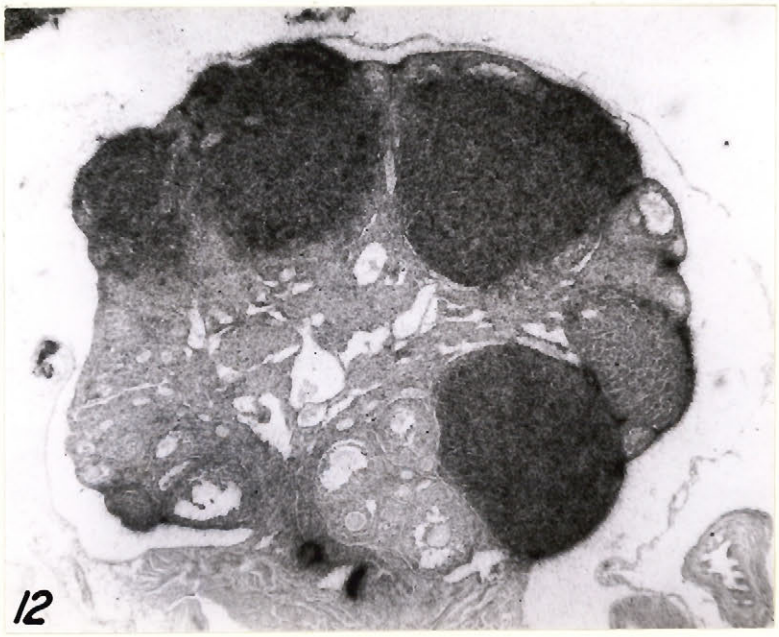
In a section of skeletal muscle there was a slight reaction limited to the interfibrous spaces and the usual accumulations about the more obvious blood vessels. The muscle fibers themselves seemed relatively free of reaction. (Fig. 4).

The heart showed a similar reaction; one got the impression of a negative image of a familiar object for all the interstitial spaces stood out and none of the muscle fibers, which were themselves relatively free of reaction. The pericardial adventitia and the endocardial intima also showed reaction. (Fig. 4).

Fig. 12. Stained coated autograph of ovary of same animal.

X 43

There is an intense reaction over the corpora lutea.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10) Glandular Reactions.

The pancreas and salivary glands showed a diffuse reaction between the acini, with definite concentrations along the blood vessels in the adventitia and septa of each. No other special localizations were found in these organs (Fig. 4).

In an unstained slide, over the mammary gland (Fig. 13 and 14), there were numerous juxtaposed angular, but nearly circular, figures, which corresponded to the outlines of the alveoli. The reaction overlay the bases of two adjacent sets of alveolar cells and faded out into background fog as the centers of the alveoli were approached. (Fig. 14). There were the usual accumulations about the blood vessels (Fig. 13).

11) Skin.

The reaction here was interstitial and showed no particular concentrations. It was diffuse throughout except for definite concentrations along the blood vessels.

12) Brain.

A perivascular reaction was found in the epicerebral and His' perivascular spaces. Cerebral tissue itself showed little or no reaction except in the immediate environs of these reacting blood vessels.

Fig. 13. Unstained coated autograph of mammary gland of same aninal.

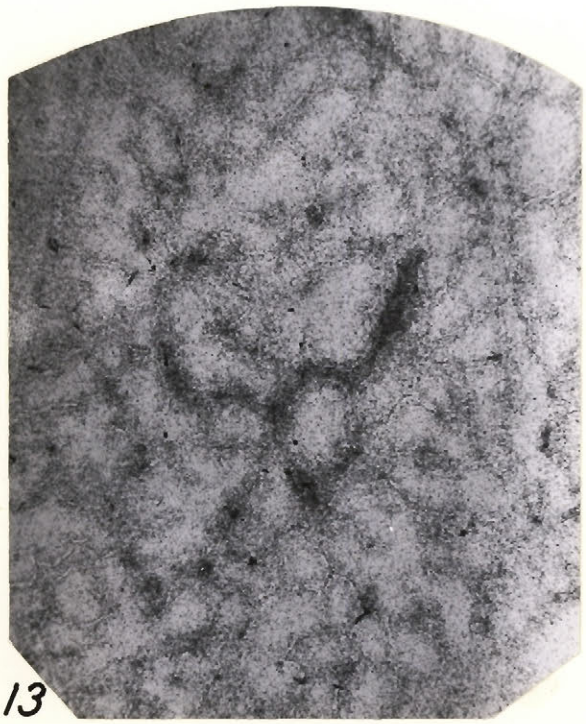
X 98

This shows a blood vessel and surrounding alveoli. The reaction is located over and between adjacent alveoli and gives the impression of a chicken wire pattern.

Fig. 14. Unstained coated autograph of mammary gland of same animal.

X 373

The reaction is seen to overlie the bases of the cells lining two adjacent alveoli.



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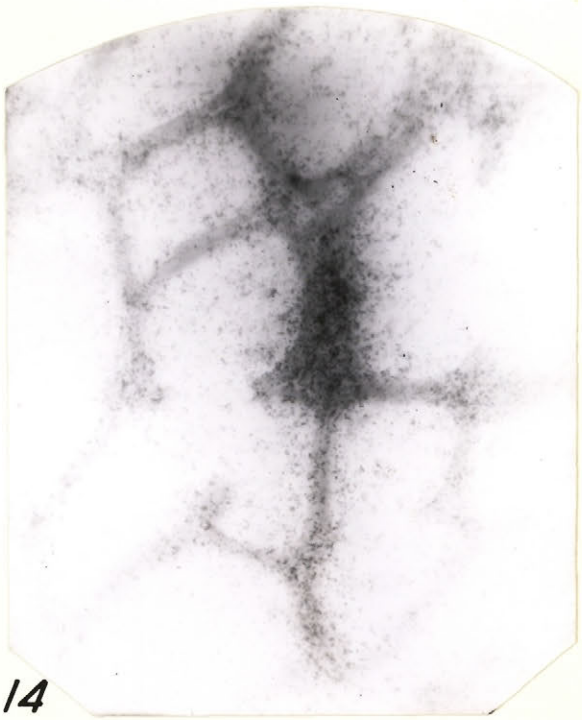

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Summary

There were diffuse intercellular reactions in all organs studied (Fig. 4), with greater accumulations of silver grains along, but outside of, the smaller vessels of the organs.

Intense reactions were found in the proximal convoluted tubules of the kidney, (Fig. 7),over the apices of the follcular cells of the thyroid, (Fig. 4), diffusely throughout the liver (Fig. 5), intracellularly throughout the corpora lutea of the ovary (Fig. 12), intracellularly in the cortex of the adrenal, in the sinusoids of the spleen (Fig. 8), and lymphatic accumulations (Fig. 9), and over the bases of the alveolar cells of the mammary gland (Fig. 13 and 14), as well as between them.

Diffuse but definite reactions were found along the intestine, in the cores of the villi (Fig. 10 and 11), and in the submucosal and intermuscular layers, as well as subserosally, in the interstitial tissue of pancreas and salivary glands, fatty tissue (Fig. 4), and in the loose connective tissues of the skin and lamina propria of the hollow viscera (Fig. 4).

DISCUSSION OF DISTRIBUTION OF I*P IN THE ANIMAL BODY

The ease with which diffusion of I*P out from the plasma into, and mixing in, the extravascular spaces takes place is affected by several factors; such as the amount of protein initially injected; and the degree to which it is iodinated; the time of the day when the injection was made; and the state of the animal into which the material was injected. The more material which has to be so mixed, the longer it takes--by twenty minutes the concentration of 0.1 mgm in the plasma as compared to that of 0.05 mgm was 7.64 as compared to 4.24 (Tables 21 and 22) while the same amount of protein iodinated to different extents, gave concentration of 4.24 for that iodinated to 7.1 I_2 atoms per molecule as compared to 3.16 for I*P iodinated to 2.1 I_2 atoms per molecule (Table 22) (of the latter a lot was already iodide). Diffusion at 20 minutes out of the blood stream was faster in the afternoon than in the morning, (4.24 as compared to 1.42 at 4:00 in the afternoon - (Table 23)), was faster in the female (3.16 as compared to 2.17 (Table 24)), and increasingly rapid in the postpartum and lactating states (1.6 for the postpartum state and 0.47 for the lactating state (Table 25)). These findings would be expected on theoretical grounds, for the more molecules which have to diffuse, the longer it will take; the bulkier or more distorted the molecule (provided the addition of iodine atoms to a prolactin molecule does so distort it), the harder it will be

for it to diffuse across, and the more sluggishly diffuse through, extracellular fluids in order to come to a complete state of mixing, though at the same time the more conspicuous it will be in the population of molecules already there, and hence, presumably, the faster it will be reduced to undistinguishable material by the scavanger cells in these very same extravascular spaces. The increasingly rapid removal from the circulation in the increasingly demanding physiological states may be just an expression by the body of its need for this material, and its avidity to convert it into moieties which fit its needs. Or it may represent an increased vascular permeability which, in turn, allows freer exit to the body from the vascular system. In the afternoon when the metabolic rates are slowed, the greater stasis may make diffusion faster through the capillary walls, due to the lessening rapidity of flow which might otherwise sweep such molecules along before they could diffuse out into the extracellular fluids.

Since $I^{131}P$ diffuses out of the vascular system from a higher to a lower concentration, so freed iodide will diffuse back into the blood stream from a higher to a lower concentration, and from there it will be distributed and concentrated in those organs which have a special affinity for it, i.e. thyroid and stomach. Other molecules will be distributed uniformly throughout the extracellular fluids in the skin and gastro-intestinal tract especially where high totals are found as late as 2 hours after

the initial injection of I*P (Table 26). Those organs which have a special affinity for iodide will continue to remove it from the blood and in time return it in the same or a different form. But the blood probably is only a passive vehicle for it; it may attach it onto one of its proteins in order to reduce the number of molecules which it carries along, and thus maintain its own osmotic pressure.

The liver, on the other hand, at 20 minutes does not distinguish between the degree to which the protein material is iodinated for it had a concentration of 5.6 and 5.8 for 0.05 mgm of I*P, one sample of which was iodinated to 7.1 and the other to 2.1 I_2 atoms per molecule (Table 22). Both were injected about 9:45 A.M. However, a sample of the more heavily iodinated material injected in the afternoon resulted in a concentration of only 3.42 (Table 23), while a female injected at noon with less heavily iodinated material, resulted in a concentration of 8.35 (Table 24). A postpartum and a lactating animal injected later in the afternoon with a still less heavily iodinated sample of I*P resulted in concentrations of 2.9 and 1.5 (Table 25). From these figures, the concentration in the liver appears to depend only upon the rate of diffusion from the blood. The usual per cent of recoverable organic iodine in this organ at this time, i.e. 20 minutes, was 82.8%, which, in the light of its sizable concentration, may be indicative of breakdown. Meites (personal communication) said that addition

of prolactin to liver slices in vitro resulted in loss of biological potency, thus indicating breakdown. The catabolism so indicated may, however, not be the same as that which would result in release of iodide. (This needs investigation). The 17.5% (Table 25) of inorganic iodide recoverable from liver at 20 minutes may simply indicate breakdown elsewhere, with carriage to the liver by the blood stream. The amount of iodide found in liver has been shown to increase with time, so that by 30 minutes, in C_3H male mice, 50% is in that form (Table 28). In three day lactating mice, an ether-extractable portion, 10% of concentrations of 7.2 and 2.7 at 5 and 20 minutes (Table 29) was found. The meaning or the nature of these fractions has not been elucidated. From the data at hand, the liver acts like a protein collector. The autographs indicate that the distribution is diffuse at 7 minutes (Fig. 5), and that the Kupfer cells are slightly more active than the rest of the liver tissue.

The kidney, like the liver, is a protein collector and does not distinguish, in the first instance, between degrees of iodination of the protein collected. Its concentration also is a reflection of that of the blood (Fig. 16), though it is higher than the blood except at the very earliest time intervals studied. Thus, at 20 minutes in C_3H male mice given 0.05 mgm at 9:30 A.M., it has a concentration of 5.18 (Table 23), while the sample of $I^{\eta}P$ injected in the afternoon, resulted in a concentration at the same time of only 2.98 (Table 23). The female injected at noon

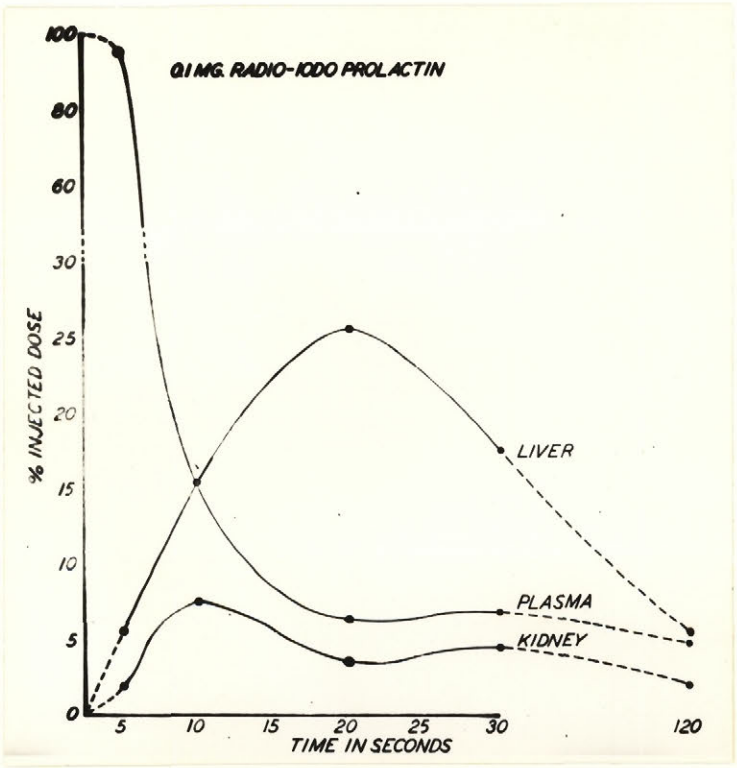
with a less heavily iodinated I*P had a concentration of 8.35, (Table 24), and the postpartum animal injected at 5:00 P.M. had a concentration of 3.8 (Table 25), a concentration higher than that of the male injected only an hour earlier. A 3 day lactating mouse injected at 5:30 P.M. led to a concentration (Table 25) of I*P in the kidney of 1.25. The kidney, unlike the liver, does make a differentiation between heavily and lightly iodinated prolactins (Tables 23 and 25), for in the noon-injected animal, where the concentration is highest, the recoverable organic iodine was only 61%, while that of the 9:30 A. M. injected animal was 81.5% (in the latter case, there was far more iodide to be recovered of course which may account for the higher % of recoverable organic iodine). In the 5 P.M. injected postpartum animal (Table 25), only 50% of the material present was organically bound.

In Fig. 15 the per cent injected doses of 0.1 mgm I*P (7.6 I_2 atoms/molecule prolactin) given to a series of C_3H male mice are plotted against time. As the activity in the plasma fell, that in the liver and kidney rose. In the liver the total activity found crossed that of the plasma at ten minutes then continued to rise to a peak of 25% at twenty minutes, while that of the kidney never rose as high as that of the plasma, but apparently came into equilibrium with it shortly after ten minutes, then followed it (Fig. 15).

From the autographs, (Fig. 6 and 7), this I*P is

Fig. 15. Curves of per cent injected dose at successive time intervals after intravenous injection of 0.1 mgm of $I^{*}P$ to C_3H male mice for plasma, liver, and kidney.

Note that the curve for liver crosses that of plasma at 10 minutes and rises to a peak of 25% at 20 minutes, while that of kidney approaches and then follows that of plasma.


0.1 MG. RADIO-IODO PROLACTIN
% INJECTED DOSE
100
80
60
30
25
20
15
10
5
0
LIVER
PLASMA
KIDNEY
5
10
15
20
25
30
120
TIME IN SECONDS

15

initially in the glomeruli capillaries and then in the proximal convoluted tubules. By twenty minutes after injection it can actually be seen being reabsorbed by these cells for the line of silver granules which, higher up in the tubule was one, splits to form two lines which overlie the inner surfaces of the cells lining the proximal convoluted tubules. Furthermore, it is interesting to observe that only a few of the many glomeruli of the kidney seem to work at any one time. (a fact which has been known for quite a while (Boyd 1944) and which is strikingly demonstrated in these autographs). In fact, such a protein, iodinated in such a way, should provide a useful tool to study renal physiology.

A rising concentration was found in those animals whose stomach wall and contents were studied, starting with a concentration of a little over 1 and rising to one greater than 10 by two hours (Table 28). The per cent of recoverable organic iodine falls from a high of 77.5% to a low of 15.0% by thirty minutes (Table 28). The initial high % organic iodine probably was either contained blood or iodide trapped within cells and there precipitated. The stomach is known to concentrate iodide ions in the same way it concentrates chloride ions (Leblond 1951) and to excrete them into its lumen. They may be converted there by the acid medium into I_2, which could then react with the proteins in the stomach contents.

The small intestine has one of the largest capillary beds in the body (Franklin, 1951), hence large amounts of injected

material are found there. A concentration, vascillating from 1-2, was found in a time series study done on C3H male mice (Table 28), The initial high per cent recoverable organic iodine fell rapidly to one of 6% by thirty minutes (Table 28). Autographs of this tissue show, at 7 minutes, anywhere along it, a uniform diffuse reaction throughout the cores of the villi (Figs.10 and 11) with a slight concentration at the tips and along the vascular bed, between the inner and outer muscle coat. At this time, in the lymphatic accumulations (Figs. 8 and 9) also, one side and the distal and proximal ends of individual nodules show a reaction which fades as the center of the nodule is approached. This indicates that the lymphatic channels drain into these nodules, all on one side, and that that side is toward the root of the mesentery in which they were found (Fig. 9). The continuing high concentration probably is due to these accumulations in the lymphatic nodules for the capillary beds must fill rapidly and then empty, the flow in must equal the flow out, for the concentration remained surprisingly constant for sometime (Table 28). Undoubtedly this is one of the sites of breakdown both in the lymphatic nodules and in the extravascular spaces throughout the cores of the villi. Inorganic iodide secreted in the stomach must in time find its way into the lumen of the gut and help account for the high iodide content at the later time intervals. Any iodinated protein from the stomach lumen may be rebroken down here. An entero-hepatic circulation of iodine is believed to exist (Leblond, 1951) as a

protective mechanism for animals deficient in iodine. This would allow reabsorption and reuse of a scarce element essential to the body economy. It has been shown that in animals with an adequate iodine intake, thyroxine iodine is excreted in the feces, whereas physiological thyroxine iodide is secreted, via the kidney, in the urine (Gross, 1949), and that thyroxine, or its conjugates or metabolites, is excreted by the liver into the bile. (Leblond, personal communication). Hence the need for this entero-hepatic circulation of iodide.

The muscle at 20 minutes has a higher concentration if 0.1 mgm of material rather than 0.05 mgm has been injected (0.51 as compared to 0.33, (Table 21)), and a higher concentration in the morning-injected than in the afternoon-injected animal (0.33 as compared to 0.18 (Table 23)). This probably is a reflection of what is happening in the blood.

The thymus and spleen in 3 day lactating animals show reasonably high concentrations (Table 29). These are probably due to the lymphoid accumulations contained therein. The autographs of these two organs indicate such is the case (Figs. 8 and 9)

The story in the adrenal is less clear. A large dose of heavily iodinated prolactin given at 10:30 A.M. to a C_3H male mouse resulted in a high concentration of 12.6 (Table 21), while a smaller dose with nearly the same degree of iodination, resulted

in a 2.06 concentration only (Table 21), The same size dose iodinated to 2.1 instead of 7.1 I_2 atoms per molecule also given at 9:50 in the A.M. led to a concentration of only 0.73 (Table 22), while a more heavily iodinated I*P sample given in the afternoon resulted in a concentration of 1.24 (Table 23). Considerable breakdown appears to have taken place, for the corresponding percentages of recoverable organic iodines were 95, 75, 19, and 69.5% (Tables 21, 22 and 23) which correspond generally with the observed concentrations. Therefore the lower the concentration, the more obvious the breakdown, which is more apparent inthe lightly iodinated sample.

A female injected with a similarly lightly iodinated prolactin at noon showed a concentration of 7.18 and a breakdown of nearly 90% by 20 minutes (Table 24), while a postpartum and lactating female, injected in the late afternoon had concentrations of 1.3 and 2.9 (Table 25), and all of the activity was recoverable as organically-bound. By autography, it is evident that there is an intracellular localization of prolactin at 7 minutes in a three day lactating animal. It is an intense reaction, diffuse throughout the cortex but not affecting the medulla. Whether there is contamination of the original material with ATCH (which has been iodinated and hence shows this reaction) cannot be said; also it cannot be ruled out, though the methods used to purify the prepared I*P were believed to help remove any contaminating material originally present in this sample of prolactin.

In the time series run on the three day lactating animals (Table 29), a concentration of 3.23 was found in the adrenal at 5 minutes, a concentration of 1.82 at 20 minutes, and one of only 0.22 by 60 minutes. If prolactin does have a specific effect on the adrenal, it must be an extremely rapid one, and the material must be destroyed in the process. In the C_3H male mouse time series (Table 28), a rising concentration to 30 minutes, with a high of 12, was found. By two hours it was back to 0.32.

In the ovary in the female C_3H mouse injected at noon with the 2.3 I_2 atoms/molecule of prolactin, a concentration of 1.03--i.e. one equal to random distribution--was found (Table 25), while in the 3 day postpartum female injected late in the afternoon, there was concentration of 1.6 with only 40.9% of it recoverable as organically-bound iodine. (Table 25). The three day lactating female injected at the same time of day with a similar dose of prolactin (Table 25) showed only a 0.4 concentration, and 95% of it was recoverable as organically-bound iodine. In the time series done of 3 day lactating albino mice (Table 29), an initial concentration of 1.82 fell to 0.31 by 60 minutes. On autography, a strong reaction was found over the corpora lutea of a 3 day lactating mouse injected 7 minutes prior to sacrifice (Fig. 12) There was an intracellular reaction present throughout the corpora lutea. In one follicle, a reaction was found in the liquor folliculi. During the course of this work, a paper appeared by Sonenberg et al (1951)[b] of

Sloan Kettering Institute. This group had prepared a similar I$^{\alpha}$P (they had trace-labelled their material rather than minimally iodinated it as was done here). They found autographic evidence of corpora lutea concentration and concentration in follicular fluid in one ovary. Their test animal was the rat.

In the mammary gland of a 3 day postpartum mouse given 0.05 mgm I$^{\alpha}$P containing I I_2 atom per molecule, a concentration of 0.69 with only 38.8% recoverable organic iodine was found, while in a 3 day lactating albino mouse given a similar dose of I$^{\alpha}$P similarly iodinated, a concentration of 3.5, with an 77% breakdown to inorganic iodide, was found at 20 minutes (Table 25). In a time series on three day lactating mice, a concentration of about 1.8 was maintained for an hour, with a falling % of recoverable organic iodine and ether extractable iodine as well. (Table 29) Autographically, at 7 minutes (Figs. 13 and 14) a chicken wire-like reaction was found over the alveolar cells of the lactating gland. Since the gland is a large organ, and since the % recoverable organically-bound activity is so small so soon, demonstration of the presence of I$^{\alpha}$P in the mammary gland is difficult. It requires a large dose which has been rendered highly active, and use of a fixative which promotes polymerization of the protein material so that it is held in the tissue sections during preparation of the slides. Since all but protein materials containing I^{131} would be washed out in such an autographic preparation, it seems quite safe to say that in the mouse, at least, it is possible to demonstrate

the presence of $I^{*}P$ in the lactating mammary gland. In the rat, Sonenberg et al did not find it.* At 20 minutes, the thyroid of the animal given 0.1 mgm $I^{*}P$ (containing 7.6 I_2 atoms/molecule) had a concentration of 3.95 at 20 minutes (Table 21) while a similar animal given a small dose (0.05 mgm) similarly iodinated (7.1 I_2 atoms/molecule) at a similar time af day (9:30 A.M.) had a concentration of 2.07. When more lightly iodinated (2.1 I_2 atoms/molecule) prolactin was likewise given to a C_3H male at a similar time of day (9:50 A.M.) the concentration was 7.7 (Table 22). This was taken to indicate that smaller more lightly iodinated doses of prolactin led to faster or more evident dehalogenation breakdown/with consequent pickup of more iodide by the gland. Of the last two concentrations, 99 and 90% were organic iodide - indicating rapid incorporation of the I^{131} into thyroglobulin (Table 22). When the more heavily iodinated prolactin was administered in the afternoon (4:00 P.M., Table 23) a concentration/comparable (3.30) to that obtained with the more heavily iodinated larger dose given in the morning was obtained (3:90 Table 21). This was taken to indicate that removal of freed iodine to the thyroid by the blood was slower in the afternoon.

In the three day postpartum animal injected with 0.05 mgm $I^{*}P$ (1.0 I_2 atoms/molecule prolactin) at 5:00 P.M., a concentration of 6.67 was found, while a similar dose was given to a three day lactating animal at a similar time of day (5:30 P.M.), a concentration of 22.4 was found (Table 25). These very much increased

* Paragraph

concentrations were thought to be due to the increased metabolic rates of these increasingly stressful physiological states. In a time series run on 3 day lactating albino mice, the thyroid concentration was found to rise with time to 20 minutes but to have fallen at 60 minutes (Table 29). This was interpreted as a rapid pickup, rapid incorporation of I^{-n} into thyroglobulin, and a rapid release of freed thryoxine to the circulation.

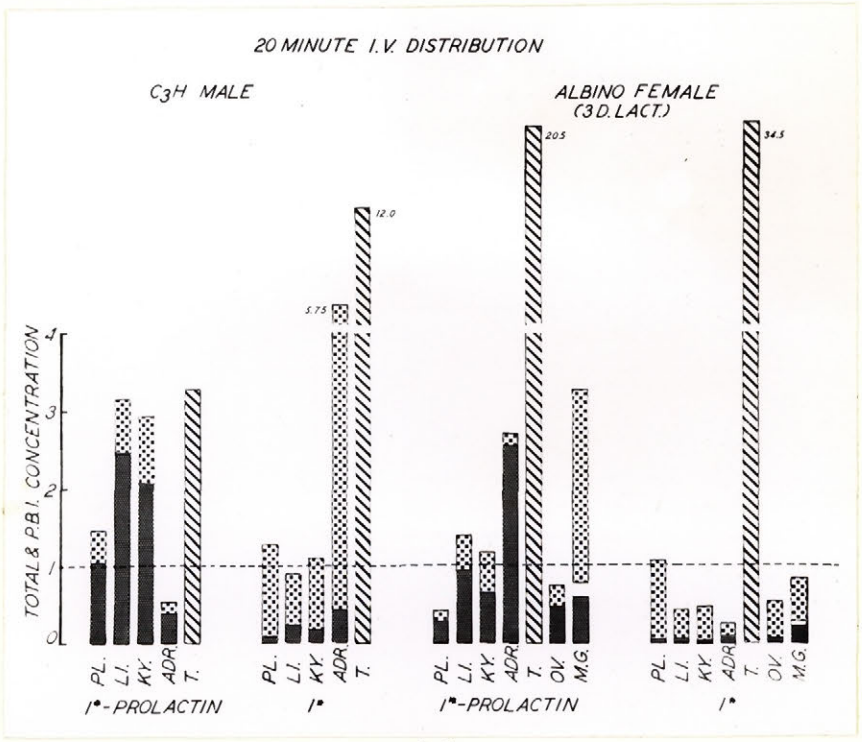
In Fig. 16 the concentration and per cent organically-bound iodine, as found for a C_3H male mouse injected with 0.05 mgm of I^nP (7.1 I_2 atoms/molecule prolactin) at 9:30 A.M., is compared with that of its I^n control, and a 3 day lactating albino mouse given 0.5 mgm of I^nP (1.0 I_2 atoms/molecule) at 5:30 P.M. is compared with its I^n control. From the graph (Fig. 16), in the C_3H male mouse the activity accumulates in the liver, kidney, and thyroid, and of that in the first three organs - plasma, liver, and kidney - a large per cent was still organically-bound. (This was a heavily iodinated sample; it takes longer for the iodine to be freed from a heavily than from a more lightly iodinated prolactin sample.) In the control animal, the plasma concentration is not too different, though a bit lower, while that of the liver and kidney are considerably less. Here the organically-bound iodine is negligible. In the lactating animals, there is less in the liver and kidney at this time, with more in the adrenal and a lot more in the mammary gland, which more or less resembles the findings for liver in the male. Note that the ovary

Fig. 16. Graphs of the distribution at 20 minutes of 0.05 mgm $I^{*}P$ (7.1 I_2 atoms/molecule prolactin) injected intravenously at 9:30 A.M. into a C_3H male mouse and its corresponding I^{-*} control and of the distribution at 20 minutes of 0.05 mgm $I^{*}P$ (0.77 I_2 atoms/molecule prolactin) injected intravenously at 5:30 P.M. into a three day lactating albino mouse and its I^{-*} control.

The concentration for the various organs depicted is represented by the dotted colums, superimposed on which are the per cent organic iodine recoverable as dark gray columns. The cross hatched columns represent the total iodine concentration for the thyroid gland.

The organs represented are: plasma (PL.), liver (LI.), kidney (KY.), adrenal (ADR.), thyroid (T.), ovary (OV.), and mammary gland (M.G.).

The explanation is to be found in the text.


20 MINUTE I.V. DISTRIBUTION
C3H MALE
ALBINO FEMALE
(3D. LACT.)
TOTAL & P.B.I. CONCENTRATION
0
1
2
3
4
5.75
12.0
20.5
34.5
PL.
LI.
KY.
ADR.
T.
OV.
M.G.
I¹³-PROLACTIN
I¹³
I¹³-PROLACTIN
I¹³

16

showed a less than random distribution. Already, at this time interval, considerable breakdown had occurred, as evidenced by the large concentration in the thyroid and mammary gland. The iodide control values in this case, were all at a lower level. More was in the thyroid.

An attempt has been made to study the metabolism of the I^{n}P in the lactating gland. So far, no answer has been found to the question of whether the breakdown takes place in the gland, or whether it becomes anavid iodine concentrator when it takes on the lactating state. The latter, from the figures obtained, seems hardly probable. But in vitro homogenization in saline, with subsequent incubation in the presence of I^{n}P, led to no breakdown of I^{n}P after 40 minutes.

SUMMARY

From the findings presented,it seems reasonable to postulate the following theory of the factors governing the distribution of I^{n}P. From the blood, to which it is added in the jugular vein, it goes to the heart where it is mixed with the systemic venous blood, thence to the lungs, where any undissolved protein particles are filtered out by the small capillaries (Fig. 4), thence back to the left heart,and from there out to be distributed to the body generally. Probably it is contained within the vessels until it reaches the various capillary beds where it diffuses out, first into the perivascular (adventitial),and then into the extracellular spaces. Here it is carried either by lymphatics to the various lymphatic nodules

such as those in the mesentery (Fig. 9), or by extracellar fluid current into contact with cells of the various organs. Those organs which have a special affinity for it-- the liver, kidney, adrenal, corpus luteum, and mammary gland-- specifically pick it up and it becomes intercellular. Autographically (Figs. 12 and 14), in all these organs but the mammary gland, the reaction is intense, whereas in those organs which have no special affinity for it, it diffuses in and out-- thus giving rise to the weak, diffuse, overall reactions found in all other tissues studied (Fig. 4). Some cells catabolize or dehalogenate it, thus releasing its contained I^{131} which, in turn, diffuses out of the cells and is carried by the lymph and/or blood back to the circulation. Here it is carried to the thyroid where it is concentrated and incorporated into thyroglobulin and gives rise to "ring reactions" (Fig. 4). The blood also may carry it to other organs where, on biochemical analysis, it probably accounts for the inorganic iodide fraction found at the later time intervals. The presumed sites of breakdown are the mammary gland, muscle, kidney, liver, and reticulo-endothelial system. In the kidney, $I^{*}P$ seems to be filtered out through the glomeruli into the proximal convoluted tubules where it is either reabsorbed as such or freed of iodide, or it is excreted as such, depending upon the needs of the body. The iodide so freed, here or elsewhere, is excreted as such, or mixed with the iodide pool of the body. Exactly what happens to $I^{*}P$ in the organs of special affinity-- adrenal, ovary, mammary gland especially, and possibly the reticulo-endothelial system-- is, as yet, unknown. Once the iodine label has been removed, the hormone can no longer be followed by the methods here employed.

GENERAL DISCUSSION

Prolactin, for purposes of this discussion, is defined as that substance(s) which causes a characteristic pigeon crop gland response. Such a biological definition indicates nothing of the chemistry or physical chemistry of the substance(s) which presumably is protein in nature, for a simple protein--one containing only amino acids--has been isolated from pituitary tissue (White et al, 1937) and shown to cause this characteristic pigeon crop gland response. The amino acid content of this protein hormone has been elucidated by Li (1949) who has shown also that certain contained radicals are necessary for this characteristic response (Li et al, 1941a, Li and Kalman, 1946 and Li and Fraenkel-Conrat, 1947).

The need for a label for Prolactin:

Due to its chemical inconspicuousness, the contained amino acids are those common to most proteins, prolactin is not discernable in the mass of protein material of any given organ. As yet, the distribution of the endogenous hormone cannot be studied because methods for its chemical or physical isolation are either not available or not sufficiently developed; furthermore, the assays available are too gross to detect the minute amounts present in the tissues of single small laboratory animals. Therefore, a label was needed to trace, by physical means, this chemical entity (s) in the animal body. The most desirable label would have been

endogenously incorporated C^{14}, or even S^{35}, since synthesis, as yet, is out of the question bedause the intimate chemistry of the hormone, or its biologically active moiety(s), is unknown. By charring, pile conversion to its radioactive isotope of sulfur contained within the purified protein would have resulted in destruction of the protein itself in all probability.

Desirability of I^{131} as a protein label:

For these reasons, an exogenous label, I^{131}, chemically incorporated onto a purified preparation of this protein hormone, was used, thus rendering the material non-physiological. I^{131} was chosen because of its availability and because, to quote Dixon et al (1951 a), "The I^{131} label is desirable because:

1. It can be attached to proteins in traceable amounts without measurably altering their immunological specificity.

2. Available evidence indicates that I^{*3} protein bound is a stable chemical link which in vitro resists wide changes in temperature and pH, salt exchange dialysis, prolonged storage and enzymatic action... . In vivo it appears that the iodine remains attached to the protein as long as the latter is immunologically detectable.

3. Prompt excretion of the I^{*} label liberated by antigen metabolism can be accomplished by iodine prefeeding to saturate the iodine-utilizing tissues.

4. The I^{*} liberated by protein metabolism is not appreciably incorporated either by synthesis or interchange into the rabbit's own proteins as determined by activity measurements of plasma protein fractions after administration of I^{*} labeled homologous globulin. Furthermore, I^{*}

injected as inorganic iodide into iodine-prefed animals is rapidly excreted unchanged in the form of iodide. In contrast, the injection of I^{n} attached as a protein label is followed by the excretion of non-protein organic combinations of I^{n}, probably di-iodo-tyrosine and perhaps other amino acid forms, as well as iodide, suggesting actual protein degredation prior to liberation of the iodine label. This difference in excretion forms was determined by paper partition autoradiograms....."

3. I^{n} herein after refers to I^{131}."

Quoted from The Effect of Sensitization and X-Radiation on the Metabolism of I^{131} Labeled Proteins, F. J. Dixon, S. C. Bukantz and G. J. Dammin. Science, March 9, 1951, Vol. 113, No. 2932, p. 274.

Incorporation of iodine (I_2^{127}) onto the tyrosyl radicals of this protein converted it from an undistinguishable entity to one that could be followed, for as little as 0.01 gamma of iodine can be detected by the highly sensitive ceric arsenious catalysis method (Gross 1949). However, certain detection of such amounts added to the relatively sizable pool of circulating iodide in the form of iodo-prolactin would have required either hugh doses of minimally iodinated prolactin or more moderate doses of maximally iodinated prolactin which would have been biologically inert (Li, et al, 1941[a]) and hence, only a protein foreign to the animal body. By use of I^{131} as a label for the iodine label, minimally iodinated prolactin in relatively minute doses could be administered and the labelled compound easily detected, so long as the label remained attached to the protein in all the organs of the animal body.

Effect of the I^{131} Label on Prolactin

Chemically

Chemically, iodo-prolactin is different by its very iodine content, However, this chemical difference was presumably limited to the tyrosyl radicals (Li, et al a 1941) to which the iodine was attached. To a compound containing only carbon, hydrogen, oxygen, sulfur, and nitrogen, all small atoms, a few very large atoms had been added. In the mass of small elements making up a protein, one, two or even several iodine atoms do not add much in weight, but, at the points of attachment, such iodine atoms must have altered the local electrical balances and steriochemistry considerably. If such an added iodine atom is also radioactive, alteration in local atatus quo certainly occurs at the moment, if not before, of disintegration with its concomitant emission of a beta particle and gamma ray and transmutation of an iodine atom into the inert gas atom, zenon. The recoil of such a disintegration must strain the local chemical bonds to, if not beyond, the breaking point. Though the total effect of one such disintegration cannot currently be measured, the local disturbances caused must influence local energy interchanges and hence chemical reactions, which in turn influence physiological and eventually morphological behavior, currently grouped under the term "radiation damage". A good example of such is the interference with the production of antibodies in previously irradiated rabbits given I^{n} bovine gamma globulin intravenously by Dixon et al (1951 a).

When, in molecules of iodo-prolactin containing one I^{131} atom, the isotope disintegrates, the resulting "radiation damage may prepare the way for abnormalties of tissue reaction to other iodo-prolactin molecules, while the effect in the disrupted iodo-prolactin molecule cannot be followed longer. When, however, a similar disintegration occurs in a molecule of iodo-prolactin containing more than one I^{131} atom, the resulting disruption not only affects the surrounding tissue, but also the entity being traced and probably gives rise thereby to abnormal molecules whose behavior may affect the results obtained. These "radiation damage" factors, whether measurable or not, must not be forgotten in any isotopic work.

Biologically:

Iodination of prolactin must alter the biology of this hormone, for Li et al, a (1941) has shown that the tyrosyl radicals are essential for biological behavior as measured on the pigeon crop gland. That a number of such radicals have to be iodinated for this difference in biological behavior to become evident can mean either that only some tyrosyl radicals are essential, or that the biological assay methods used are dependant either on the more difficultly iodinated tyrosyl radicals, or are not sensitive enough to distinguish between other than minimal and gross iodination. The latter would appear to be true for a difference was found with prolactin iodinated so that 3 or more tyrosyl radicals were in the di-iodo-form, as compared to samples which were iodinated so that only one

tyrosyl radical had been iodinated (see page 62 and Tables 10, 12 and 13). Furthermore, the distribution work done in mice indicated that the animal body handles more heavily iodinated prolactin differently from minimally iodinated material (see page 102 and Tables 22 and 25). This handling may be due to interference with its permeability through vascular walls, its diffusion through extra-cellular spaces, and/or the increased load placed on the intercellular enzyme systems in either stripping it back to its native form or catabolizing it to free iodide.

The very presence of iodine atoms may also render those points of attachment, (Scatchard, 1952) or the immediate enviorns, unaccessible to other enzyme systems which, in turn, may have to act before the protein moieties can react with other metabolic chains. The end results of these are the characteristic biological actions as interpreted by modifications of morphological or physiological function expressed as specific secretion, such as prog. esterone from the corpus luteum. Here again, ability to detect such biologic differences is limited by the sensitivity of the methods available, for they may be masked by the presence in a given sample supposedly homogeneous, of traces of native material, for minimal iodination of a prolactin sample is a statistical phenomenon where the probability but not the certainty, due to the laws of chance, that most of the molecules of a given species will be effected (Latta, 1951), exists. Such minimal iodination, therefore, provides

no guarantee that all protein molecules will react even when subjected to conditions which presumably induce most molecules to do so. Knowledge of just where and to how great an extent this minimal iodination affected this particular protein was not investigated; rather, it was assumed that preferably one tyrosyl radical per molecule would react with the available iodine, thus being changed to either the mono- or di-iodo- form before a second radical of the same protein molecule would react. The validity of this supposition was not investigated. In fact, the homogeneity of the prolactin molecule population itself, or even the homogeneity of a heterogeneous prolactin molecule population cannot be claimed for the preparation used. There is disagreement among the purifiers and users of prolactin as to what potency, in terms of the International Standard, a mgm. of purified hormone should be. Li (personal communication) claims a prolactin of 35 I. U. per mgm while Folley (personal communication) claims that prolactin preparations are never better than 24 I.U. per mgm. This problem cannot be settled with the present state of knowledge, for the criteria of protein homogeneity breakdown under scrutiny. How can 100% homogeneity ever be substantiated before the intimate chemistry and morphology of the substance under question has been thoroughly elucidated and universally accepted?

Despite all these possibilities, minimally iodinated prolactin can induce a pigeon crop gland response indistinguishable from that caused by a similar amount of untreated prolactin, or of prolactin subjected to the chemical manipulations here employed

to iodinate and subsequently purify $I^{\alpha}P$ (see page 62). Intracellular autographs have been obtained in the proximal convoluted tubules of the kidney (Fig. 6), liver (Fig. 5), corpora lutea of the ovary (Fig. 12), adrenal, and alveolar cells of the mammary gland in lactation (Figs. 13 and 14). Iodination does not disturb the antigenic activity of bovine gamma globulin (Dixon et al, 1951[a]), or hormonal action of insulin (Reiner et al, 1942 and 1943).

Strength of the Carbon Iodine Bond:

If an isotope is to be a useful tool as a label for a substance, it must be so incorporated into the material to be studied as to become an intimate part of it, and it must stay with it long enough to serve its function as a label. Therefore, the strength of the carbon-to-iodine bond is important. Fine and Seligman brominated (1943), and later iodinated (1944), plasma protein, and found that of the amount of bromine incorporated, only 3% could be liberated by in vitro or in vivo hydrolysis, and that, therefore, 97% was firmly incorporated into one or more amino acids, presumably tyrosine or possibly tryptophane (Wormall, 1940). Trypsin hydrolysis at 37°C at pH 8 for 6 hours released only 4% of the incorporated I^{131} from the protein into the non-protein fraction--apparently the supernatant from trichloroacetic acid precipitated protein--while acid hydrolysis with 25% H_2SO_4 at 90°C for 6 hours resulted in 40% of the total activity appearing in the non-protein

fraction. They therefore claimed that the stability of the carbon-iodine bond was similar to that of the bromine linkage (from which, they had previously claimed, bromine would be liberated by nothing less than destruction or degradation of the aromatic ring containing the bromine atom) for alkaline hydrolysis, but that the bond was weaker in strong acid. 40 hours of dialysis loosed only 2% of the incorporated iodine so that it appeared in the non-protein fraction. Warren and Dixon (1948) partially iodinated bovine gamma globulin and found that, after 24 hours at room temperature in aqueous solution at a pH of 10.5, only 5% had split off, while at pH 5, 1.3% had been released. 24 hours in absolute alcohol, dioxane, ether, and paraffin removed 3% or less; they therefore claimed that the I^{131} protein linkage was stable under their experimental conditions--conditions similar to those used in this investigation. Here the experience has been that considerable chemical manipulation can be done without apparently disrupting this carbon-iodine bond. Thus, from the data presented, it is assumed that the carbon-to-iodine bond is reasonably strong, and that <u>in vivo</u> freeing of iodine from $I^{n}P$ is the result of catabolism of the $I^{n}P$ itself. Banks et al, (1949) found no activity in the protein free filtrate of dog plasma, though in the total plasma, there was considerable activity after subcutaneous injection of radioactive iodinated plasma proteins. They concluded that the I^{131} was still bound to the protein initially

injected. Dixon et al, (1951) summed up this general consensus of opinion in their four points regarding the desirability of the I^{131} protein label, as quoted earlier.

Behavior of I^{n}P

The question of whether such a minmally iodinated prolactin truly portrays the behavior of the endogenous hormone can be answered only partially. It has been shown that it does cause a pigeon crop gland response, and that it does give rise to intracellular autographs. However, as has already been pointed out, prolactin may be several hormones in one, the various protein moieties each being responsible for one of these responses, but not necessarily for another. Li has shown that not only tyrosyl (Li et al, 1941) but also amino (Li and Kalman, 1946) and carboxyl radicals (Li and Fraenkel-Conrat, 1947) are essential for biological behavior. Each of these radicals in particular combinations of amino acids may actually be the active moieties, and the iodination of one or more tyrosine radicals would not interfere therefore with the biological behavior of the various parts of the molecule. That iodinated tyrosyl radicals are present in these various biological target organs is evidenced by the autographs, but that they are a necessary part of that particular hormonal activity cannot be claimed from the data available. Certainly, the particular tyrosyl

radical bearing the I^{131} cannot be the key group in these particular loci. Or, if they are, then some other part of the tyrosyl radical is active, not the positions ortho to the OH.

Action of the Body on Intravenously Injected I$^{\eta}$P

Diffusion of I$^{\eta}$P from Blood.

0.05 mgm of I$^{\eta}$P in 0.1 cc was routinely injected into the jugular vein of a mouse. This represents the addition of 10% more fluid, less than 0.1% more protein by weight, and the addition of about 1×10^{18} molecules to the blood of the animal injected (A mouse's plasma volume is about 1 cc, and is presumably a 6% protein solution (Best and Taylor, 1943)). Since the injection was made into the jugular vein, the material would go first to the right heart where it would be mixed with the venous return from the systemic circulation and then be pumped out into the lungs. From the work on autography, it is known that there any particles of protein not dissolved, too large to get through the fine capillaries of this organ, were filtered out and held, giving rise to the peculiar patchy areas of intense reaction found in coated autographs of lung (Fig. 4). After this purification by filtration, the material would be returned via the pulmonary veins to the left heart and from there would be pumped out into the great

vessels, which would distribute it to the general body. Since the heart rate of a mouse is of the order of 350 beats per minute normally, the time required for this initial wide distribution must have been only seconds in duration. From there it could not have taken long for it to have diffused out through the pores along the intercellular lines of the vascular endothelium (Pappenheimer et al, 1951), for by seven minutes, it is found extravascularly wherever it has been sought. Perfusion into, and diffusion throughout the extracellular spaces must have been very rapid; the concentration in the blood fell from a high of 28.4 at 1-2 minutes after injection to 4.68 by 10 minutes, and from a total content of 96.65% radioactivity to 15.6% in the same time interval (Table 28). Albert and Keating (1951) injected 1-2 mgm of di-iodo-tyrosine whose initial concentration in 85 cc of blood/kg fell in two hours to one that corresponded to a volume of 530 cc/kg. From these findings, it is assumed that the plasma volume represents about 16% of the extracellular volume, and hence, presumably, a similar state existed in the mouse with respect to $I^{\alpha}P$ by 10 minutes after injection. In this time of exit from the blood, the recoverable organic iodine in that tissue was 95%. From such findings it is assumed that the blood, i.e. plasma, had little or no catabolic effect on the material, that it acted merely as the transporting medium of the material distributed to the body generally.

The distribution of $I^{\alpha}P$ as here studied indicates that

by 30 minutes after injection, not even half of the intravenously injected organically-bound iodine (as a dose of 0.05 mgm of $I^{\alpha}P$ containing 2.0 or less iodine atoms per molecule) can be recovered in the organic form by the Somogyi zinc sulfate method as here employed. A similar destruction independently has been observed with trace labelled iodo-prolactin (Sonenberg et al, (1951), and also with trace labelled iodo-adreno-cortico-trophic hormone (Sonenberg et al, 1951 b). Different results were found for such iodinated proteins as serum protein, albumin, and globulin injected into normal dogs (Fine and Seligman, 1944), or rabbits (Dixon et al, Dixon and Talmage, 1951
1951 a and). The reasons for such differences have been sought.

Certain obvious facts come to mind; the relative size of the molecules involved, and the species of test animals used. The albumins and globulins are relatively large molecules; albumin has a molecular weight of 69,000, and gamma globulin one of 90,000. Both have molecular diameters of 35-40Å. Scatchard (1952) claims that these are the smallest molecules which can be retained by the capillary walls. On pure size alone, auch a difference of removal from the blood can be explained, for prolactin has a molecular weight
though
of 33,300 (Li, 1949), and that of the adreno-cortico-trophic hormone used by Sonenberg et al, (1951 a) is not specified; from its behavior, however, it must be relatively small also.

Mechanics of Diffusion from Blood:

The albumins and globulins, however, do diffuse out of the vascular system, for Dixon et al (1951)[a] showed that by the end of 24 hours, all but 20% of the injected dose of rabbit gamma globulin given to normal rabbits had so diffused. Thereafter, the rate of removal was considerably slower. This initial fall in blood concentration was thought due to a mixing process by which the injected protein came into equilibrium with the extracellular space fluid from the more limited serum space fluid. In the work here reported on $I^{m}P$, less than 20% of the injected dose could be recovered from the blood by 10 minutes.

This work was done on much smaller animals, mice rather than rabbits or dogs; it is not surprising that the whole process seems speeded up. Such a speed-up may reflect other factors than mere size. Pappenheimer et al, (1951) have offered an explanation for this rapid removal from the blood in their evaluation of the pore size available for diffusion from the vascular system. They have shown that there is a virtual pore size gradiant with a means pore diameter of 24 $\mathring{A} \pm 12 \mathring{A}$, and that these pores which allow filtration of water and non-lipoid soluble materials from the vascular system are limited to about 0.2% of the histological surface of the capillary endothelium, which they suggest may represent the intercellular areas. They studied a series of substances graded by molecular size, the largest of which was insulin, and

were able to establish; that the rate of filtration from the blood of such non-lipoid soluble substances depended upon both the range in osmotic pressure due to the added material along the length of the capillaries of the hind limb of cats and the concentration gradiant of the added substance across the capillary membranes, and also on the molecular size as well. They pointed out that in this process of filtration out from the blood stream, there was not only filtration out/of the added material but diffusion in of the surrounding extra-cellular water, the latter in an attempt to re-establish the normal osmotic pressure level within the vascular system. A falling plasma concentration of injected material early after injection may be partly spurious. Blood hydration would render subsequent vascular filtration slower due to the smaller concentration differences on the two sides of the endothelial membrane involved. Furthermore, Pappenheimer et al found that the diffusion rate of some substances, i.e. oxygen, could not be explained on the basis of pore size only; from experimental trial, lipoid soluble substances had to have a much larger diffusing surface available-- in fact, the whole capillary endothelium. Such substances, these workers claimed, could dissolve in the plasma membrane of the endothelial cell and thus, by crossing the cell, reach the extravascular spaces. $I^{131}P$ also may be soluble in fatty materials; its rapid diffusion may entail this type of transfer.

Once outside the capillaries, and from the autographs $I^{131}P$

moves through the capillary walls rapidly (by seven minutes it was found wherever it was sought (Fig. 4) along blood vessels), it rapidly diffuses through extravascular spaces (evidenced by the rapid accumulations of it in the mesenteric lymph nodes for example) - where by seven minutes, it is seen beginning to percolate through the sinusoidal spaces of such nodules (Fig. 9). Krieger et al (1950) showed that within an hour, in dogs injected with labelled plasma proteins, considerable amounts of such proteins appeared in the thoracic duct lymph. The material could have gotten there only by filtration into and diffusion through the extravascular spaces, with subsequent collection by the lymphatics and hence, transport into the large lymphatic channels, thus establishing flow currents in the extravascular fluids.

Once out of the blood stream into the extravascular spaces, such an iodinated material presumably can permeate into and out of all the cells of the body. Here, it has been found that it permeates into the cells of the liver (Figs. 5 and 4), proximal convoluted tubules of the kidney (Fig. 7), the corpus luteum of the ovary (Fig. 12), adrenal, and mammary alveolar cells (Figs. 13 and 14) specifically. What forces are at work in such intracellular concentrations? Ussing, (1949) points out that materials, ions in particular, concentrate in cells by being carried there in some relatively stable complex--as would be the case here, for it is the I^{131} that is being measured--and that either the ion so carried,

or the carrier, is held within the cell by some constituent of the cell itself, or collected there due to gradients of electrical and/or chemical potentials. It would be of interest to know what specifically causes this material to collect in such cells.

The degree of concentration found in other places eventually may be traced to blood flow rates to different parts of the body (Franklin, 1951). For instance, why do some, but not all, glomeruli, with their juxtaposed proximal convoluted tubules, show intense pickup? Are some deprived of blood flow by the Trueta shunts, and do these shunts work rhythmically? Can such shunts explain differences found from time to time in such organs as the lung and skin?

Sonenberg et al (1951 b) also have studied the distribution of radio-iodo-prolactin.

50-300 gram rats of the Spraque-Dawley strain were injected intracardially with 1-1000 gamma of prolactin iodinated such that 6 molecules contained 1 iodine atom, or 1 molecule contained 1.6 iodine atoms per animal. Gamma ray emission in the tissues, which had been subjected to prolonged mild hydrolysis, was measured by use of a multiple center-wire counter and contact autography. The results were calculated as per cent of the injected I^{131} per gram of organ and by the ratio of that "concentration" to that of blood. Their controls were radio-iodo-bovine serum albumin and radioactive inorganic iodide. They reported findings for 94 females--a part of

the 200 animals studied--summarized in one table. No details are given as to the animal or experimental conditions used other than that the animal weights were uniform for a given experiment.

It is impossible to compare their findings with those obtained here inasmuch as rats rather than mice, 1-1000 rather than 50 gamma of rather differently iodinated prolactin, nembutal rather than ether anaesthesia, and intracardial rather than the intravenous route, were used. Superficially, their findings resemble those obtained here except for the differences in mammary gland concentrations.

The problem of the rapid catabolism of this radio-iodo-prolactin has not as yet been considered.* One technical possibility exists. The lung autograph indicated that not all of the protein was in solution, for particles of apparently undissolved material were unable to traverse the capillary bed there. The autographs of liver, spleen, and lymph nodes suggest a fairly active pickup by the cells of the reticulo-endothelial system whose function is to remove particulate matter from the circulation. Against this particulateness of the injected material are the diffuse autographic patterns found throughout the organs studied and the definite intracellular localizations obtained. However, since the reticulo-endothelial system is so widespread, the possibility does exist that the 50% breakdown observed at 30 minutes is the result of catabolism of undissolved material which has been picked up by this

* See page 192

system. Dixon and Talmage (1951) have shown that in immune rabbits, 60% of injected radio-iodo-bovine gamma globulin is catabolized in 6 hours, while in non-immune animals, the catabolism is only 7% in the same time. They postulate that the presence of the antibody causes an intravascular formation of antibody-antigen-complexes which is picked up by the reticuloendothelial cells, which then rapidly catabolize the material, with liberation of the I^{131}. They do not state the size of such antibody-antigen complexes, but obviously they would be larger than single molecules of either, and this very size factor may provide the necessary impetus for phagocytic uptake with consequent catabolism. The necessary size may be such as to explain the results obtained here, though this seems unlikely.

As to the function of prolactin in the animal body, Meites (personal communication) has shown that prolactin added to liver and lactating mammary gland slices in vitro results in loss of biological activity of the prolactin. No other tissues apparently have this effect. And Folley (1951) has shown in in vitro studies on the metabolism of the mammary gland that prolactin added to tissue slices of mammary gland taken after the young were born, raised the respiratory quotient, which in full lactation is 1.6 to an even higher figure. This finding suggests that prolactin has some role in the production of mammary gland fat. It is noted in this work (page 95) that radio-iodo-prolactin was ether extractable when added to homogenates of mammary gland from 3 or 17

day lactating mice, and it was postulated from these findings that it was soluble in mammary gland fat.

The findings so far obtained in pursuit of this study have contributed to the general problem of lactation in that a high concentration of prolactin has been found in the lactating glands of mice soon after injection, and that a large breakdown of prolactin takes place in the mammary gland has been postulated. This high concentration in the mammary gland has not as yet been extended to mammary tumors, and hence the value of prolactin as a therapeutic tool in mammary cancer, has not been assessed. On this point, however, the large concentrations found in such organs as liver, kidney, adrenal, and ovary would render such use dangerous. From the standpoint of pure research, it appears that radio-iodo-prolactin soon after injection behaves like a protein in its rapid removal from the blood and in its initial distribution in the extravascular spaces and in liver and kidney, like prolactin itself in its intracellular localizations in the corpora lutea of the ovary and mammary gland, and possibly in the adrenal as well, though this latter localization may be due to A.C.T.H. contamination. The high concentrations in such organs as thyroid, stomach, intestine, and skin are probably only the reflection of its contained, and subsequently freed, iodine. Insofar as this work has shed light on what such a protein hormone does immediately after injection, the general knowledges of biology have been advanced. However, much more work is needed to round out the many defects and queries that have arisen.

The author wishes to reiterate the fact that the biological work was done on single animals. The validity of the results obtained and the conclusions drawn therefrom must be assessed with that in mind. For instance, in the matter of catabolism, a low per cent organically-bound iodine was found in the plasma of the C_3H male mouse given 0.05 mgm I^*P (2.1 I_2 atoms/molecule prolactin) in the experiment cited regarding the effect on distribution of prolactins of different degrees of iodination. This particular finding in this particular animal seemed proper in that a high thyroid concentration was also found. However, in other experiments with similarly lightly iodinated prolactins, considerably higher per cents of organically-bound iodine were found in plasma though the corresponding concentrations were usually lower, and the thyroid concentrations higher (suggesting more rapid removal of freed inorganic iodide from the blood). Since such a low % organically-bound iodine coupled with a high thyroid concentration suggested rapid catabolism of the protein (the same carbon-iodine bond in other proteins is so stable that free inorganic iodide is interpreted as catabolism of the protein chain rather than simple disruption of the C-I bond), the implication is that the more nearly physiological the iodinated material, the more rapidly it is destroyed. Such would be out of line with Dixon's work on iodinated bovine serum albumin, and the suggestion that highly iodinated prolactin molecules would be so conspicuous in the extracellular population that they would be rapidly catabolized. Protein hormones, however, may have quite

different catabolic characteristics than other proteins, such as those of the plasma. Of course, it is, also possible that pure dehalogenation is taking place, and that a minimally iodinated protein simply allows that phenomenon to become evident.

GENERAL SUMMARY

The protein pituitary hormone, prolactin, was iodinated with labelled iodine in such a way that fewer than three of the known nine tyrosyl radicals, to be contained in each molecule of prolactin were converted to the di-iodo-form. The material was proved to be truly iodinated to the extent of 43.7% of the initial radioactivity, since all but this amount of radioactivity was removed by dialysis in 24 hours. When iodide was mixed simply with a like amount of prolactin (0.8 mgm), only 3.2% failed to be removed by dialysis in the same time. Routinely, the iodinated prolactin was freed of unattached iodide by isoelectric (pH 5.4 or 5.7) precipitation and reprecipitation. It was shown that all but 0.26% of radioactivity mixed with 1 mgm of prolactin as I^{-n} was removed. Microkjeldahl analysis showed that 50% of the protein nitrogen of 1 mgm of prolactin-- iodinated and so purified-- was lost in this process. In iodo-prolactin only tyrosine radicals were iodinated, for addition of acid to remove iodine attached to histydyl radicals after prolactin iodination made no difference in the extent of iodination.

$I^{n}P$ was found to be biologically active by Lyon's "intradermal" pigeon crop gland assay. Samples of material iodinated to various degrees showed that iodination just sufficient to convert one tyrosyl radical to the di-iodo-form per molecule caused no detectable loss of biological activity, whereas the use

of two and a half times as much iodine caused a decrease of 25% in the area of crop gland response and 12% in opacity. The technique of iodination as used had no effect on the biological activity when the loss of 50% protein in purification was taken into account. No irrative or toxic reactions were observed.

80 to 85% of organically-bound iodine added as 0.05 mgm $I^{\eta}P$ to 3 day lactating mouse tissues could be recovered in the precipitate fractions of tissues sujbected to the modified Somogyi zinc sulfate fractionation method. (The technique involved homogenization of 500 mgm of wet tissue in Somogyi's acid zinc sulfate reagent No. I, subsequently neutralized to pH 7 by addition of 1 cc of 0.75N NaOH (Somogyi's reagent No. II). Precipitation in these reagents proceeded at 4°C for 24 hours. The precipitates were washed 3 times with 3 cc of Na_2SO_4, isotonic with the initial supernatant. Any residual precipitate was recovered from the pooled supernatants and washes by a second precipitation at 4°C for 24 hours.) These modifications were worked out on the large organs of rats, i.e., liver, kidney, and plasma, from which 96.4% of 0.1 mgm of $I^{\eta}P$ could be recovered in the precipitate fraction, and all but about 2% of a similar amount of inorganic iodide (2 gamma) could be removed. Since the recoveries obtained with the smaller mouse tissues to which smaller amounts of $I^{\eta}P$ had been added were only 80-85%, it was therefore thought necessary in this case to apply a 20% corrective to the recovery findings obtained with such organs.

This was done routinely. Mammary gland tissue had to be lyophilized at -20°C. and 4 mm Hg for 96 hours before satisfactory recoveries of organically-bound iodine could be obtained. 30% of small amounts of added $I^{n}P$ (0.025 mgm) were found to be ether-extractable under these conditions, whereas, of similar amounts of added iodide, only small percentages, less than 5%, were ether-extractable from mammary gland tissues.

Intravenous injections of 0.1 and 0.05 mgm of purified $I^{n}P$ given to C_3H male and albino female mice provided a way to study the distribution of the iodinated protein at time intervals up to 2 hours. It was found that the distributions were affected by such factors as amount of protein injected, time of day of injection, sex, physiological state, and time after injection when the tissues were examined. Iodide controls also were studied. It was found that the larger the dose, the slower it diffused out of the circulation into the extracellular spaces, and the more slowly it was catabolized to inorganic iodide.

A time series at 1-2, 10, 20, 30, and about 145 minutes was run on C_3H male mice. This indicated that removal of $I^{n}P$ from the blood was rapid at first and then slower. Furthermore, as the plasma concentration fell, that of the organs (liver, kidney, gastrointestinal tract, muscle, spleen, skin, mammary gland, and endocrines) rose. In the kidney, a peak of radioactivity was reached earlier than in the liver where the peak was obtained at 20 minutes. The catabolism of $I^{n}P$ proceeded

at a rate such that 50% of the injected dose was reduced to inorganic iodide by 30 minutes. By two hours, the distribution obtained in mice given 0.05 mgm I*P (2.0 or 1.0 I_2 atoms/molecule prolactin) corresponded so closely to that obtained in one C_3H male mouse given a similar amount of radioactivity in the form of "carrier-free" I^{131} that no conclusions could be drawn other than that considerable catabolism had taken place.

A similar time series run in 3 day lactating female albino mice at 5, 20, and 60 minutes after injection indicated that the removal of I*P from the blood was even more rapid; that the uptake by the organs was faster in consequence; and that catabolism also was more rapid. The peak of activity here seemed to be at 5 rather than 20 minutes. The total contained activity in the lactating mammary gland rose with time, while the concentration was maintained or even fell slightly at the latest time interval. A high inorganic content was found even at 5 minutes and this increased with time, indicating a rapid catabolism. More than 20% of the activity in the gland at 5 minutes was ether-soluble, while by 60 minutes the same fration represented only 3.2% of the contained activity. Likewise, mammary glands of 17 day lactating albino mice given 0.05 mgm of I*P (0.77 I_2 atoms/molecule prolactin) showed a rapid catabolism of the material with a fraction soluble in ether which rose for the first twenty minutes, and then fell (Fig. 3). However, <u>in vitro</u> incubation of I*P with mammary gland

tissue indicated no breakdown whatsoever.

Autographs of organs obtained from a 3 day lactating mouse 7 minutes after an intravenous injection of 0.5 mgm of hidhly radioactive $I^{\Pi}P$ indicated that, in all organs studied, there was at least a weak diffuse reaction throughout the extracellular spaces and definite perivascular concentrations. Amounts of radioactivity of decreasing concentrations were found in many organs: kidney, thyroid, liver, gastro-intestinal tract, endocrine glands, lymphatic organs, and mammary gland. Intracellular localizations were present in the proximal convoluted tubules of the kidney, throughout the liver, adrenal cortex, andcorpora lutea of the ovary, as well as over the bases of the alveolar cells of the mammary gland. The autographic reactions in the first four organs mentioned above were intense and unmistakable, even on naked eye inspection, while that in the mammary gland was definite but not intense. In the mammary gland, the high content of inorganic iodide and ether-soluble $I^{\Pi}P$ at this time precluded an autographic localization of an intensity similar to that found in the kidney, for such a reaction depends upon the contained protein-bound iodine rather than on the contained activity.

The high concentrations found in the stomach, skin, and especially the thyroid at the later time intervals are a measure of the catabolism of $I^{\Pi}P$, or of its dehalogenation. That intense autographic reactions found over the thyroid follicules at 7 minutes in 3 day lactating mice signifies how rapid both the breakdown of

the injected material was and how rapidly the thyroid picked up and incorporated the released iodide into its own protein-- thyroglobulin. Such an intense reaction suggested in this state at least the turnover of endogenous prolactin must be very rapid.

The intense concentration both biochemically and autographically in the liver and kidney suggests that I*P here behaves like a protein only, and that possibly these organs are excreting or catabolizing it. The indifferent biochemical concentrations and autographic reactions found so generally throughout the body suggest also that it is simply behaving like a protein, passively at that. But the biochemical and autographic findings in the adrenal, corpora lutea, and mammary gland suggest that in these organs at least, it is exhibiting its hormonal nature. The autographic reactions in the first two of these three organs is so definately and unmistakably intracellular, so intense so soon after injection, and so uniform throughout, that the avidity of these organs for this material cannot be doubted. In the mammary gland, where the material also collects in large quantities, it is so rapidly catabolized on the other hand, that the autographs give a misleading picture. This very catabolism here, when in other organs there seems to be relatively so little, may be another expression only of a similar avidity. This suggests another field for discriminate exploration.

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APPENDIX

COUNTING

Two methods of detecting the presence of I^{131} were used in this work, the Geiger Muller Tube and Photographic Emulsion. The Geiger Muller Tube works via the ionization of the few gas molecules left within the evacuated tube caused by the passage through them of a beta ray emitted by a single disintegrating nucleus of I^{131}. Such bursts of ionization fire off a series of amplifiers which in turn fire off a series of electrical light circuits which are "counted" by a scaler on an electrically driven meter. A clock is connected into the circuit in such a way that the time required for a given number of disintegrations to occur is recorded simultaneously. The burst of ionizations resulting from the passage of a single beta particle through the partially evacuated G.M. tube is "quenched" by the presence of a vapor, such as alcohol, which, by absorpting the ionization, is bfoken up into smaller fragments. Hence with continual use, the the G.M. Tube eventually loses its "quenching" power and the ionizations set up become a continuous rather than single bursts. The high voltage applied acfoss the G.M. tube facillitates the rapid piling up of the ions upon either the anode or cathode of the tube, and its pickup from there by the electric amplifying circuit. With such an arrangement it is possible to obtain a

record of the individual disintegrations in the sample which resulted in a sufficiently energetic beta particle emission to escape from the plate, traverse the intervening layer of air, penetrate the thin mica end window of the G.T. tube, and so set up ionizations within the G.M. tube itself. These in turn are picked up by the electric circuit outlined above, and hence recorded.

From what has just been said, it will be realized that the "count" obtained represents only a fraction of the total number of disintegrations actually taking place in the sample in a given time. The fraction recorded depends upon several factors: (1) the amount of solid present in the sample (solid causes self-absorption); (2) the position of the mica window (only those beta rays hitting and penetrating the window can be recorded); (3) the distance of the sample from the mica window (the greater the distance, the fewer the beta particles with enough energy to escape, cross the air interval, penetrate the window, and set up ionizations); (4) the thickness of the mica window (the thinner the window, the lower the energy the beta particles require to traverse it); (5) the ease of ionization within the G.M. tube, a function of the number and size of the gas molecules to be ionized; (6) the ease of collecting the ionized particles on either the anode or cathode of the tube; (7) the threshold of the electrical pick-up circuits; (8) the effectiveness of the quenching gas (the more rapidly the bursts of ionization are quenched, the more rapid the escape of

individual effective beta particles can be and still be individually recorded; (9) the background of the apparatus; (10) and the amount of water present in the sample, (water acts as a self absorber).

Of the 10 factors sited above, the following were constants:

1. The amount of solid present, 1 cc of 2N NaOH per plate
2. The position of the mica window;
3. The distance of the sample from the mica window. This could be varied, but the same distance was always used for any one experiment or corrected so that it would be the same;
4. The thickness of the mica window;
5. The ease of ionization within the G.M. Tube-- for any one given tube only of course;
6. The ease of collecting the ions on the anode or cathode (The same voltage was used for any one experiment).
7. The threshold of the electrical pick up circuits;
8. The effectivness of the quenching gas; (Tubes had a relatively long life in comparison to any one experiment).

The above factors with the exception of (1), (3), (9) and (10) were characteristics of the apparatus and hence were presumed constants.

In regard to the amount of solid per plate, a minimum of the equivalent of 1 cc of 2N NaOH was always used. Slight to moderate variations were undoubtedly present as between plates made from very dilute aqueous solutions and tissue digests. However, the amount must have been negligible in comparison to that attributable to the NaOH routinely used.

The distance of the sample from the mica end window of

the G.M. tube was a factor that could be varied. If a set of plates was found to be very high in activity, they were counted at a greater distance from the mica window than plates which were very low in activity. However, all samples of any one experiment were either counted at the same distance from the mica window, or, the counts obtained were multiplied by a suitable correction factor determined by counting the same standard at the two distances.

Care was taken by the entire group using these counters to maintain the background at the lowest possible figure. Background was always determined at the beginning and end of any counting period, and if that period was long, in the middle as well. These backgrounds were averaged and subtracted from the actual counts obtained.

The state of function of the apparatus was determined each time it was used by counting the Uranium Standard at the beginning and end of the period. Uranium has such a long half-life that the same sample counted day after day in an apparatus that is functioning uniformly changes to only an undetectable extent. Any variation in the count of the Uranium Standard, therefore, was always taken to indicate trouble in the apparatus. One ten-day period during which a large experiment was counted was found to show wide variation in the performance of the apparatus. ~~(See figure)(Graph)~~ This trouble was subsequently traced back

to variations in incoming voltage due to line variation and was later compensated for by the installation of a constant voltage transformer. Therefore all counting of any given experiment was limited to approximately the same time of day.

Furthermore, duplicate counts of every plate were made as a check on the performance of the apparatus. Each plate was counted twice routinely, and if there was much discrepancy between the two, counting was continued until two duplicate figures were obtained.

Since the efficiency of the machine was best in the 2000-8000 range per 100 seconds, fast plates were either counted at a greater distance from the mica end-window of the tube or were held until some of their activity had disappeared.

It was routine to count a given plate for not less than a 100 seconds and 1000 counts. In other words, if 1000 counts did not require 100 seconds, sufficient counts to occupy at least 100 seconds were counted. When a plate was very, very slow, a plate was counted for two successive counts of 500. Background was counted in this way.

Since radioactive disintegration is a random phenomenon, the rate at which it takes place is not a constant in the mathematical sense of the word. However, if a sufficient number of disintegrations are counted, a statistical rate can be established.

Thus, the average of a large number of disintegrations occupying a given time is a truer measure of rate than the number taking place in a short interval. The Standard Error has been formalized as follows:

$\rho = K\sqrt{M}$, where K equals 1.960 for a 95% probability, and M is the number of counts counted, and ρ is the % error. For 1000 counts, ρ was found to be 6.2.

As an example, consider the case where the count was 250 per 100 seconds and 1000 counts counted. The gross error is 250 x 6.2% = 15.5, so the gross count is 250 $\pm$ 15.5. If the background was 20 counts / 100 seconds and two times 500 counts were taken (i.e. 1000 counts), then 20 x 8.8 (% error for 500 counts) equals 1.76, so the background is 20 $\pm$ 1.76 counts.

The net count equals the plate count, minus the background count, plus and minus the square root of the background percentage error, plus the plate percentage error both squared, or

$$250 - 20 \pm \sqrt{(15.5)^2 + (1.24)^2}$$

$$250 - 20 \pm \sqrt{(240) + (3.09)}$$

$$230 \pm \sqrt{243.09}$$

$$230 \pm 15.6$$

Therefore, the true count has a 95% chance of being between 214 and 246 counts. This represents an error of $\pm$ 6%.

Low counts have relatively large statistical errors.

Highly active plates entail another type of error, namely, coincidence. In a highly active sample, the chance that two disintegrations will take place simultaneously increases as the activity increases. Thus, samples showing an activity of 800 counts per minute have a coincidence of 0.25%, or, in other words, not 800 but 802 disintegrations have actually taken place. If 8000 disintegrations appear to have taken place in the same length of time, actually 8200 disintegrations have taken place.

The application of these correctives to some actual counts done during the course of this work are illustrative.

Two fractions from a particular sample of plasma were counted and found to contain 100 counts and 19,515 counts per 100 seconds respectively. The coincidence factors were 0.05 and 4.5% respectively. The coincidence corrected counts were therefore 100.05 and 20,780. These two factors were counted on May 8th, but the experiment was being calculated as if it had been counted on May 10th and only 84.05% of the activity would then be present. These two counts would then have been 84 and 17,045 respectively. The first of these was counted for 1000 counts and the second for 20,000 counts. The gross error for the first was 6.2% or $\pm$ 5.2 and the gross error for the second was 1.35% or $\pm$ 235.5. The background that day was 21 and its gross error was $\pm$ 1.76. Therefore,

the true count for the first was 84 - 21 ± 6.96 or 63 ± 6.95--- an over all error of 11%, whereas the second was 17045 - 21 ± 237.26 or 17024 ± 237.26, or an error of 1.4%. In other words, there was a 95% probability that the first count was true to within 11% while in the second there was a 95% probability that the true count was within 1.4% of 17024/100seconds. Counts in the 1000 or less per 100 second range entailed an overall counting error of about 5%, those in the 1 - 10,000 range an error of about 2.5%; those in the 10,000 up range a counting error of less than 2%. In general then, the average error of counting was about 3%.

FACTORS INVOLVED IN CALCULATION OF AMOUNT OF IODINE NEEDED TO IODINATE PROLACTIN

	Prolactin			Tyrosine					Iodine										
	Type	Mol. Wt.	% by wt.	Proportion by wt. in 1 mole prolactin	Mol. wt.	No. of radicals/molecule of prolactin	Wt. of 4 g. atoms 126.92x4[&]	Wt. needed for complete iodination of 1 mole of prolactin	Wt. needed for complete iodination of 1 mgm of prolactin	% tyrosine to be iodinated	Wt. needed to iodinate % tyrosine in 1 mgm prolactin	Wt. prolactin to be iodinated	% yield (theoretical)	Amt. I_2 to be released	Vol. of final sol. containing I_2	Vol. of KI & KIO_3	KI conc.	KIO_3 Conc.
Units		(gm)	(%)	(gm)	(gm)		(gm)	(gm)	( )	(%)	( )	(mgm)	(%)	(%)	(cc)		mgm/100cc	mgm/100cc
Date:																		
1/48	AP3004	35000	5.5	1925	181.19	10.6	507.68	5392	154	10	15.4	10		100		0.133	83	21.4
1/48	"	16000	5.5	883	181.19	4.87	507.68	2479	155	10	15.5	10		100		0.133	83	21.4
2/48	Schwenk	35000	4.5	1575	181.19	8.67	507.68	4410	126	10	12.6	6		75		0.10	83	21.4
5/48	"	35000	4.5	1575	181.19	8.67	507.68	4410	126	10	12.6	3		37.8		0.05	83	21.4
5/49	"	35000	4.5	1575	181.19	8.67	507.68	4410	126	10	12.6	5	40	157.5	1	0.1	175	50
4/50	"	33300	4.5	1498	181.19	8.29	507.68	4240	126	10	12.6	5	20	31.5	1	0.1	322	86
12/50	"	33300	4.88	1630	181.19	9.0	507.68	4575	137.5	11.1	15.1	5	40	184	1	0.1	196	50
3/51	"	34700	4.7	1630	181.19	9.0	507.68	4575	131.5	11.1	14.65	1	100	14.65	0.2	0.02	79.75	20.25
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII

[&] Hawk and Bergeim - Practical Physiological Chemistry, 11th Ed., P. Blakiston's Son & Co., Inc., Philadelphia; p. 936, 1937.

1) **To get column III** multiply Column I by decimal form of column II.
2) To get column V divide column III by column IV.
3) To get column VIII multiply column V by column VI.
4) To get column VIII divide column VII by column I.
5) To get column X multiply column VIII by decimal form of column IX.
6) To get column XIII multiply column X by column XI and that by factor to make column XII up to 100. i.e. 40 x 2.5 = 100
7) To get columns XVI and XVII, use

$$\underset{5(166)}{5KI} \;\&\; \underset{214}{KIO_3} \;\&\; 6HA \qquad 6KA \;\&\; 3H_2O \;\&\; \underset{762}{3I_2}$$

when $3I_2$ = figure in column XIII and column XVI =

$$\frac{\text{XIII}}{762} = \frac{x}{830} \quad \text{and or} \quad \frac{830\ (\text{figure of column XIII})}{762} = \text{fig. of column XVI}$$

Column XVII

$$\frac{\text{XIII}}{762} = \frac{x}{214} \quad \text{or} \quad \frac{214\ (\text{x figure of column XIII})}{762} = \text{fig. of column XVII}$$

Sample calculation 3/51

$$\frac{34700 \times 4.7 \times 10^{-2}}{181.19} \times \frac{507.68^{gr}}{34700} \times \frac{1.11 \times 10^{-2} \times 1 \times 1}{1000} = 14.65 \times 10^{-6} \text{ grams } I_2/\text{mgm prolactin}$$

$$\frac{830 \times 14.65}{762} = 15.92 \qquad \text{and} \qquad \frac{214 \times 14.65}{762} = 4.12$$

$15.9 \times 5 \times 100 = 79.75$ mgm $\qquad 4.12 \times 5 \times 1000 = 20.25$ mgm

Note

$$\%\ \text{tyrosine} \times \%\ \text{iodination} \times \frac{I_2}{\text{mol. wt. tyrosine}} = 2.8 \times \text{no. mg prol.} \times \text{yield factor} = \text{amt. } I_2 \text{ to be released}$$

$0.047 \times 0.111 \times 2.8 \times 1 \times 1 = 14.65$

Table 9

FACTORS AFFECTING YIELD OF RADIO-IODO-PROLACTIN

EXP. NO.	PROLACTIN mgm	cc	pH	CARRIER IODINE amt mg.P	cc	HCl N	cc	RADIO ✱ IODINE State	conc.	cc	NEUTRALIZATION NaOH N	cc	When	IODINATION CONDITIONS Volume Final cc	cc/ mg.P	pH	Time min	Temp. °C	ACIDIFICATION HCl N	cc	pH	ISOELECTRIC PURIFICATION WITH 0.2N ACETATE BUFFER ppt. vol. cc	Wash cc	cc	NaOH N	cc	ppt. vol. cc	Wash cc	NaOH N	cc	PER CENT YIELD	EXP. NO.
FACTORS CAUSING LOW YIELDS																																
13	1.0	7.6	12.6	0.1	2% HAc	0.05	D	nbd	0.1					1.15	0.4	7.6	30	20														
21	0.2	7.6	12.2	0.04	1	0.02	"	bd	0.14	0.5	0.04	A		0.44	0.44	?	hours	"				ppt.	c̄	0.1 cc	5% HAc.	Wash	1 x	c̄	0.05 cc	0.1% HAc			1.6	1
33	1.0	7.6	12.6	0.1	5	0.07	ND	"	0.15					1.25	0.42	7.2	30	"				5.4	0.2	0.05	0.05	0.5	0.03	0.1	0.1	0.5	0.03	7.72	2
41.5	0.5	7.6	62.5	0.1	1	0.05	D	nbd	0.25	0.5	0.1	"		0.69	0.46	10.	120	"				"	1.2	"	"	0.03	0.65					1.19	3
56	1.2	7.6	12.6	0.2	5	0.2	"	"	1.0						0.4	4.	30	"				"	0.7	0.25	0.25	0.05	0.4	0.4	0.4	0.25			8.5	4
																						"	2.5	0.5	0.5	0.02	0.7					4.7	5
FACTORS NOT AFFECTING YIELDS																																
61	0.2	7.6	14.7	0.04	1	0.02	"	"	0.14	"	0.04	"		0.44	0.44	7.6	60	"	1	0.02	5.7	0.5	0.1	0.1	0.5	0.03	0.2	0.1	0.5	"	10.4	6
7 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	"	"	"	"	5.4	"	"	"	"	"	"	"	"	"	"	10.5	7
8 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	"	"	"	"	5.7	48°	"	"	"	"	"	"	"	"	"	10.4	8
9 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	"	"	"	"	"	48°4°	"	"	"	"	"	"	"	"	"	10.15	9
10 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	3	"	"	"	"	0.5	"	"	"	"	"	"	"	"	"	9.15	10
11 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	20	"	"	"	"	"	"	"	"	"	"	"	"	"	"	10.4	11
12 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	30	"	"	"	"	"	"	"	"	"	"	"	"	"	"	9.65	12
13 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	210	"	"	"	"	"	"	"	"	"	"	"	"	"	"	10.15	13
14 "	"	"	"	"	"	"	"	"	"	"	"	"		"	"	"	24°	"	"	"	"	"	"	"	"	"	"	"	"	"	"	10.3	14
15 "	"B	"	"	"	"	"	"	"	"	"	"	"		"	"	"	60	"	"	"	"	"	"	"	"	"	"	"	"	"	"	10.3	15
16 "	"A	"	"	"	"	"	"	"	"	"	"	B		"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	10.8	16
17 "	"A	"	"	"	5	"	"	"	"	5.0	"	"		"	"	"	24°	"	"	"	"	"	"	"	"	"	"	"	"	"	"	9.21	17
FACTORS CAUSING HIGH YIELDS																																
18 0.75	0.25	"	15.7	0.05	1	0.25	"	"	0.10✱	"	0.05	A		0.47	0.63	"	60	"				5.4	"	0.2	0.2	0.25	0.5	0.5	0.2		0.05	31.1	18
19 "	"		7.85	"	"	"	"	"	" ✱	"	"	"		0.46	0.62	"	"	"				"	0.2	"	"	0.05	"	0.75	"	4N	0.5	33.6	19
20 2.	0.4	"	31.5	0.08	"	0.04	"	"	0.28	"	0.08	"		0.88	0.44	"	5.5°	"	"	0.04	"	1.0	0.1	0.1	0.1	0.03	0.4	0.5	0.1	0.5	0.5	43.9	20
21 10.	1.	13.2	7.5	0.2	HAc	0.05	"	"	0.2					4.35	0.44	5.5	---	"				48°4° already a ppt. washed c̄ 1 and 0.5 cc of 1% HAc										38.2	21

✱ I_2 releasing mixture stood for 1/2 hour before addition of prolactin solution.

c̄ = with

48°4° = 48 hours at 4°C.

Table

Effect of Protein Precipitants on I^{n}P with or without tissue proteins

SAMPLE	Vol. cc	Amount protein mgm	Degree Iodinated I atoms/ molecule	I^{131} Counts/ minute	REAGENTS Vol. cc	Chemical	Fraction Studied	% Activity Found
1. 10% TCA								
I^{n}P	0.5	3.0	1.1	28,500	2.5	10% TCA	supernatant	36.8
					0.5	5% "	"	4.8
					0.5	" "	"	4.3
					1.0	2N NaOH	Dissolved protein	54.1
2. 10% TCA + Tissue Proteins								
I^{n}P plus	0.2	0.75	1.1	4,768	65	10% TCA	supernatant	79.0
Liver Homogenate	5.0	2735.			13	5% "	"	5.5
					6	2N NaOH	Dissolved protein	15.5
I^{n}P plus	0.2	0.75	1.1	2,505	27	10% TCA	supernatant	48.5
Kidney Homogenate	5.0	368.			8	5% "	"	3.2
					2	2N NaOH	dissolved protein	48.7
3. TCA neutralized to pH 5.4								
I^{n}P						10% TCA + 2N NaOH to 5.4	dissolved protein	98.0
I^{n}P plus	0.25	0.75		3,152	2	10% TCA		
Liver Homogenate	2.5	1525.				2N NaOH to 5.4	supernatant	25.3
					4	2N NaOH	dissolved protein	74.7
4. 5% Acetic Acid								
I^{n}P plus	0.2	0.75		3,398	11	5% HAc	supernatant	42.7
Liver Homogenate	2.5	1525.			5	2N NaOH	dissolved protein	57.8
5. 10% TCA buffered to pH 5.4								
I^{n}P	0.45	0.5	2.0	1,329	0.5	10% TCA (5.4)	supernatant (murky)	39.4
					0.5	95% Alcohol	"	28.8
					0.5	Ether	"	1.2
					1.0	2N NaOH	dissolved protein	31.0
6. Carrier IP								
I^{n}P	0.2	0.42	1.0	334	0.7	10% TCA (5.4)	supernatant	9.0
IP	0.5	5.0	1.0		0.5	95% Alcohol	"	28.5
					0.05	Ether		
					1.0	2N NaOH	dissolved protein	62.5
7. 0.2N NaAc-HAc, pH 5.4, buffer								
I^{n}P plus	0.4	0.2	2.0	21,051	1.0	5.4 buffer	supernatant	34.3
Liver homogenate	1.0	160.			0.6	H_2O		
					0.4	0.1N NaOH	dissolved protein	78.4
					1.0	5.4 buffer	supernatant	4.3
					1.0	2N NaOH	dissolved protein	47.1
8. I^{n}P plus	0.4	0.2	2.0	18,060	1.0	5.4 buffer	supernatant	36.1
Mammary gland homogenate	1.0	60.0			0.6	H_2O		
					0.4	0.1N NaOH	dissolved protein	42.0
					1.0	5.4 buffer	supernatant	5.6
					1.0	2N NaOH	dissolved protein	35.3
9. $Cu(OH)_2$ precipitation								
I^{n}P	0.2	0.1	2.0		1.0	5.4 buffer	supernatant	25.5
H_2O	1.0				0.1	2% $CuCl_2$	"	25.5
					0.5	0.5N NaOH	precipitate	74.5
I^{n}P plus	0.2	0.1	2.0		1.0	5.4 buffer	supernatant	
Plasma	1.0				0.1	2% $CuCl_2$	"	50.0
					0.5	0.5N NaOH	precipitate	50.0
I^{n}P plus	0.2	0.1	2.0		1.0	5.4 buffer	supernatant	
Liver Homogenate	1.0				0.1	2% $CuCl_2$	"	3.5
(200 mgm)					0.5	0.5N NaOH	precipitate	96.5

Table 15

INVESTIGATION OF SOMOGYI ZINC SULFATE METHOD OF FRACTIONATION OF ORGANICALLY BOUND IODINE FROM INORGANIC IODINE

Sample	Wt. of Tissue (mgm)	Wt. of Prolactin (mgm)	Form of added activity	Wt. of iodine	Amt. of $ZnSO_4$ (cc.)	Amt. of 0.75N NaOH (cc.)	Wash Solution Chemical	Vol. 3 X (cc.)	pH	No. of Samples	% Activity in precipitate	RANGE
Effect of Tissue Types												
Plasma	0.5 cc	0.1	$I^{n}P$	2.1	4.0	0.5 plus	H_2O	3	8.2	4	79.27	(79.0 - 81.5)
"	"		I^{n}	2.6	"	" "	"	"	"	"	1.21	(0.9 - 1.4)
Liver	250.	"	$I^{n}P$	2.1	"	" "	"	"	"	"	67.24	(50.3 - 82.9)
"	"		I^{n}	2.6	"	" "	"	"	"	"	4.28	(2.4 - 5.6)
Kidney	"	"	$I^{n}P$	2.1	"	" "	"	"	"	"	88.86	(86.5 - 91.8)
"	"		I^{n}	2.6	"	" "	"	"	"	"	2.56	(2.1 - 3.2)
Effect of pH												
Plasma	0.3	"	$I^{n}P$	2.65	2.4	0.3	"	"	7.0	"	90.6	(85.7 - 91.8)
"	"		I^{n}	2.86	"	"	"	"	"	"	11.5	(7.4 - 14.4)
Plasma	"	"	$I^{n}P$	2.65	"	"	"	"	9.3	3	86.9	(80.2 - 90.6)
"	"		I^{n}	2.86	"	"	"	"	10.0	"	8.3	(8.0 - 9.2)
Liver	"	"	$I^{n}P$	2.65	4.0	0.5	"	"	7.0	4	92.8	(90.0 - 96.5)
"	"		I^{n}	2.86	"	"	"	"	"	3	9.3	(8.1 - 10.0)
"	"	"	$I^{n}P$	2.65	"	"	"	"	9.3	4	89.4	(79.0 - 95.5)
"	"		I^{n}	2.86	"	"	"	"	10.0	3	15.8	(14.0 - 18.1)
Effect of Tissue Concentration												
Liver	50	"	$I^{n}P$	1.53	"	"	"	"	7.0	2	93.5	(93.4 - 93.6)
"	"		I^{n}	2.86	"	"	"	"	"	"	14.25	(14.1 - 14.4)
"	100	"	$I^{n}P$	1.53	"	"	"	"	"	"	95.0	(94.1 - 95.9)
"	"		I^{n}	2.86	"	"	"	"	"	"	14.25	(15.4 - 15.57)
"	150	"	$I^{n}P$	1.53	"	"	"	"	"	"	94.5	(93.9 - 94.8)
"	"		I^{n}	2.86	"	"	"	"	"	"	13.5	(11.1 - 11.3)
"	200	"	$I^{n}P$	1.53	"	"	"	"	"	"	96.6	(96.8 - 96.6)
"	"	"	I^{n}	2.86	"	"	"	"	"	"	17.7	(13.8 - 22.7)
"	250	"	$I^{n}P$	1.53	"	"	"	"	"	"	97.2	(97.0 - 97.4)
"	"		I^{n}	2.86	"	"	"	"	"	"	13.5	(13.2 - 14.3)
Effect of Cold and Time (Centrifuged, 4°C for 24 hours, centrifuged.) 2 X												
Liver	"	"	$I^{n}P$	1.89	"	"	"	"	"	"	84.6	(33.6 - 49.7) Initial
"	"		I^{n}	2.52	"	"	"	"	"	"	95.42	(94.7 - 96.2) Final
											1.8	(. - .) Initial
											2.65	(2.2 - 2.9) Final
Effect of cold and Time (4°C for 24 hours, centrifuged,) 2 X												
Liver	"	"	$I^{n}P$	1.89	"	"	"	"	"	"	81.0	Initial
"	"		I^{n}	2.52	"	"	"	"	"	"	95.45	(95.2 - 95.7) Final
											1.8	Initial
											2.19	(1.2 - 3.2) Final
Effect of Na_2SO_4 instead of water as washing solution: (centrifuged, 4°C for 24 hrs.) 2 X												
Liver	"	"	$I^{n}P$	1.89	"	"	Na_2SO_4	"	"	"	86.0	Initial
"	"		I^{n}	2.52	"	"	"	"	"	"	96.48	(96.4 - 96.6) Final
											1.6	Initial
											2.25	(1.5 - 2.9) Final
Effect of $I^{n}P$ Concentration (4°C for 24 hours, centrifuged) 2 X												
Plasma	0.5	0.05	$I^{n}P$	0.9	4	"	"	"	"	2	65.0	(63.8 - 66.3)
"	"		I^{n}	0.98	"		"	"	"	"	11.85	(11.6 - 12.1)
MAMMARY GLAND												
Effect of Tissue Concentration												
Mammary												
Gland	50	0.1	$I^{n}P$	1.53	"	"	H_2O	"	"	"	41.66	(33.6 - 49.7)
"	"		I^{n}	2.86	"	"	"	"	"	"	8.48	(7.2 - 9.8)
"	100	"	$I^{n}P$	1.53	"	"	"	"	"	"	53.4	(43.6 - 63.1)
"	"		I^{n}	2.86	"	"	"	"	"	"	11.59	(10.1 - 13.0)
"	150	"	$I^{n}P$	1.53	"	"	"	"	"	"	76.28	(61.0 - 91.5)
"	"		I^{n}	2.86	"	"	"	"	"	"	8.18	(4.9 - 11.5)
"	200	"	$I^{n}P$	1.53	"	"	"	"	"	"	88.36	(82.5 - 91.5)
"	"		I^{n}	2.86	"	"	"	"	"	"	5.42	(5.2 - 5.6)
"	250	"	$I^{n}P$	1.53	"	"	"	"	"	"	59.52	(21.1 - 92.9)
"	"		I^{n}	2.86	"	"	"	"	"	"	7.67	(4.9 - 10.5)
Effect of Ether extraction (centrifuged, 4°C for 24 hours centrifuged) 2 X												
After Zn(OH)2 precipitation												
"	"	"	$I^{n}P$	1.53	"	"	Na_2SO_4	"	"	"	21.4	(14.8 - 28.0)
"	"		I^{n}	2.86	"	"	"	"	"	"	0.31	(0.5 - 0.1)
Before Zn(OH)2 precipitation												
"	"	"	$I^{n}P$	1.53	"	"	"	"	"	"	65.29	(39.4 - 91.44)
"	"		$I^{n}P$	2.86	"	"	"	"	"	"	2.75	(0.7 - 4.75)
Ether Homogenization and Zn(OH)2 precipitation (4°C for 24 hours, centrifuged) 2 X												
"	"	0.05	$I^{n}P$	0.93	"	"	"	"	"	4	85.2	(82.9 - 86.5)
"	"		I^{n}	0.98	"	"	"	"	"	"	12.6	(11.2 - 13.4)