STUDIES ON THYROXINE AND TRIIODOTHYRONINE IN THE CHICKEN

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CHAPTER I

INTRODUCTION

The major physiological actions of the thyroid hormones are well established. Although significant advances have been made in understanding certain of the parameters of thyroid function, the mechanisms involved in incorporation of the hormones by the peripheral tissues and the mechanisms responsible for eliciting their actions are still unknown.

Since the discovery of 3,5,3'-triiodo-l-thyronine (triiodothyronine, T-3), which joins 3,5,3',5'-tetraiodo-l-thyronine (thyroxine, T-4) as one of the natural thyroid hormones, a vast literature has accumulated on the comparative physiological effects of the two hormones. In fact, it has been demonstrated that T-3 has five to seven times the biological potency of T-4 in various mammalian species (7). This difference in potency is maintained over a wide variety of physiological tests of potency involving basal metabolic rate, body growth rate, and antigoitrogenic action. In the chicken, on the other hand, it has been shown that T-3 is no more potent (95) or less potent (75, 74) than T-4 with respect to antigoitrogenic action in thiouracil-treated birds, and less potent in stimulating oxygen consumption of surviving cardiac muscle (76). The only explanation that has been offered to account for the reverse potencies of T-4 and T-3 in chickens as compared to mammals relates to the relative binding of the two hormones to the plasma proteins of these species; this property limits the rate of entry of the hormones into the cells (109).

Recently, doubt has been cast upon this explanation by Balfour and Tunnicliffe (6) and by Ingbar and Freinkel (55).

In view of the foregoing considerations, the experiments in this study were designed to re-investigate the phenomenon of protein-binding of thyroid hormones to chicken plasma by various methods and to see if there were other explanations that could account for the differences in potencies of the hormones in birds. Furthermore, in contrast to the situation in mammals, there is relatively little information available concerning the metabolism of T-4 and T-3 in birds; consequently, it is desirable to obtain basic information of this nature.

CHAPTER II

LITERATURE REVIEW

Biological Half-Life of T-4 and T-3

The biological half-life of any compound is defined as the time required for one-half of the biological activity of the compound to be lost either from the whole animal or any designated organ or tissue. The decline in biological activity with time follows an exponential type of decay; hence when the data are plotted semilogarithmically a straight line should result.

Quantitative comparisons of the metabolism of T-4 and T-3 show that, in man, the rate of loss of I^{131}-labelled thyroxine from the blood gives a half-life of about 6-11 days; for triiodothyronine, the half-life is 2-3 days (99, 23, 54, 12, 100, 82, 11, 83). A more rapid disappearance of T-3 as compared to T-4 has also been found in rats and mice (26, 73, 41, 59), and the dairy cow (77). In chickens, the half-lives of the two compounds have been reported as identical (109).

Although T-3 has a shorter half-life than T-4 in mammals, marked variation in the relative half-lives of the two hormones is found between the various species. In the rat, the values are found to be 16.6-19.0 hours and 9-10 hours for T-4 and T-3 respectively (26, 73). The guinea pig and rabbit occupy an intermediate position between the rat and man with respect to thyroid hormone metabolism; half-life of T-4 varies between 3-8 days for the rabbit and about 3-4 days for the guinea pig (15, 116). The half-life of T-4, as well as T-3, in the chicken is

22.5 hours (109).

Most observations on the disappearance of I^{131}-labelled T-4 and T-3 from blood have been used to determine the rate of distribution through the body's extrathyroidal organic iodine pools and the rate of metabolic degradation of the hormones. Sterling, et al. (99, 100) injected 50 microcuries of I^{131}-labelled T-4 or T-3 into human beings that had various states of thyroid function and withdrew blood daily for 14 days. It was noted that in all cases there was an initial, rapid drop in radioactivity during the first 1.5-2.0 days following injection of the hormones. This rapid drop in radioactivity was attributed to the diffusion of the hormone out of the vascular compartment as it was distributed through the body's iodine pools. After 2 days the radioactivity decreased at a much slower rate which was considered to be indicative of the rate of metabolism of the hormones. In one instance it was noted that a flattening of the curve occurred at the eighth day (99); this was attributed to the reutilization of I^{131} as the T-4 was degraded. This phenomenon did not occur when iodide was fed to the patient. To study factors involved in the rate of egress of T-4 from the circulation, Engbring, et al. (23) determined the half-life of T-4 during the first hour after intravenous administration of the hormone and found it to be 73.6 minutes. The interval from 20 minutes to 50 minutes post-injection was considered to represent the rate of distribution into the body iodine pools. Comparative studies with T-3 were not done.

A semilogarithmic plot of the rate of thyroid hormone egress from the blood does not result in a straight line, which indicates that the curves depend on more than one factor, each having its own rate constant. In all the foregoing studies the curves have been divided into two com-

ponents arbitrarily designated the "fast" and "slow" components. The former component represents equilibration of T-4 into the extravascular compartments; the latter denotes the rate of metabolism and excretion of the hormone. Investigating the role of the gastrointestinal tract in the metabolism of T-4, Albert and Keating (1) postulated at least three rate constants in the disappearance of labelled T-4 from the blood. Component A had a half-life of 36 minutes and represented the equilibration of T-4 with a larger volume of distribution almost entirely within the gastrointestinal tract; component B with its half-life of 15 hours represented the disappearance of T-4 from its initial volume of distribution into the excreta and tissues; component C which had a half-life of 62 hours was taken to represent T-4 leaving the blood by some route other than the urine and feces into some larger equilibrium volume in the body. Although only three components of the curve were described they stated that "such curves actually represent complex biological phenomena having an indeterminate number of exponential components." In this regard, Rall, et al. (82) studied the disappearance of T-4 and T-3 for three weeks following the intravenous injection of the compounds into human beings. Both T-4 and T-3 disappeared at a rate which could be decomposed into five exponential decay rates. In the absence of the thyroid glands this number was reduced to four.

Protein Binding of T-4 and T-3

Reasons for T-4 and T-3 having different half-life values in mammals have been based on the relative binding affinities of these compounds to serum proteins (99). The criterion of binding affinity used was that described by Tata (107) wherein the transient instability of I^{131}-labelled

T-4 and related iodophenols was inhibited by the presence of human serum in aqueous medium, the amount of T-4 "stabilized" being proportional to the T-4 binding properties. By this method Tata found T-3 to be bound less firmly than T-4 in mammals, but bound with equal intensities in chickens and ducks. When binding affinity was determined using a dialysis procedure, mammalian plasma again exhibited a greater affinity for T-4 than T-3 (102). However, the prolonged half-life of T-4 (as in myxedema) may be independent of protein binding (23).

The explanation by Tata and Shellabarger (109) for the difference between the responses of mammals and birds to T-4 and T-3 hinges on the absence of a specific thyroxine-binding protein (TBP) from chicken and duck sera, and the sole binding of both T-3 and T-4 to albumin in these species. According to Balfour and Tunnicliffe (6) this argument does not take into account the marked binding of T-4 by pre-albumin (in the duck) thus possibly invalidating their argument. Observations described by Ingbar and Freinkel (55) also would tend to refute the hypothesis that the absence of specific thyroxine-binding moieties is an adequate explanation for this difference in response. They found that myxedematous patients maintained on constant doses of T-4 displayed no alteration in metabolic status, despite the induction of marked increases in TBP by estrogenic therapy. Concomitantly, hormonal degradation rates remained normal. Normal rates of hormonal degradation also were found in association with normal metabolic status in patients given methyl-testosterone, in whom TBP was markedly decreased, and in patients with idiopathic increases or decreases in thyroxine-binding capacity of this protein. They further stated that while it would appear that binding interactions influence the rate of turnover of hormones, and thereby the quantities

required to effect a standard metabolic response, it seemed unlikely that they influence their intrinsic biological potency. Summarizing the regulation of the peripheral metabolism of the thyroid hormones it was postulated that peripheral turnover of T-4 in vivo could be formulated in terms of two sequential steps: (a) cellular accumulation and (b) cellular disposal. Thus it was postulated that circulating T-4 affixes either to cellular or extracellular thyroxine-binding components, so that the static cellular : extracellular distribution of hormone conforms to a reversible binding equilibrium between these interactants (51, 105). Thereafter, hormone is removed from this equilibrium as cellular T-4 is degraded at a constant, proportional rate. In this regard, Ingbar (53) studied the role of TBP in regulating peripheral turnover of T-4 in a patient lacking the specific TBP. His findings indicated that TBP regulated the rate of removal of T-4 from plasma, presumably by limiting its availability to the cell. Furthermore, thyroid activity was regulated by the delivery of hormone to peripheral tissues, rather than by the concentration of hormone in the circulation. Direct evidence that TBP does in fact influence the rate of entry of T-4 into tissue cells by a simple binding equilibrium has been presented by Freinkel, et al. (31) who studied the penetration or "uptake" of labelled T-4 by surviving slices of liver, heart, and kidney cortex in the presence of enriched human TBP and other serum protein fractions. They have shown that the "uptake" of T-4 by tissue slices was depressed by the addition of thyroxine-binding proteins or serum to the medium; this depression was proportional both to the affinity of the added protein for T-4 and to the concentration of the binding protein; further, T-4 already bound in the tissue could be discharged from it by the addition of TBP to the incubation medium. The

reversibility of this phenomenon demonstrated the existence of an equilibrium between cellular and extracellular binding of T-4. The identification of a specific globular protein in rat skeletal muscle extracts that is capable of binding thyroid hormones (105, 104) suggests that a role for this cellular protein may be the regulation of the intracellular level of thyroid hormones by influencing their availability to enzymes in the cell, for when TBP extract from muscle was added to rat muscle "deiodinase," deiodinating activity was lost, the inhibition being directly proportional to the amount of T-4 bound to muscle TBP.

Another factor regulating T-4 disappearance from the vascular compartment has been described by Roche and Michel (88). Following the intravenous administration of T-4 to dogs it was found that 65 percent of the T-4 was present in the extravascular space in the first hour and more than 30 percent still remained in the plasma space. This distribution was quite independent of the fact that T-4 was strongly bound to plasma proteins. The idea was presented that it was likely that the complex thyroxine-binding proteins cross directly from the capillary wall to the lymph, since the same electrophoretic patterns were found in both fluids. In addition, the rate of intracellular penetration of T-3 and T-4 was studied and found to proceed, in the case of T-3, at a rate five times that of T-4. Thus it was assumed that the ratio between the time necessary for the entering of T-3 and T-4 into the cells could explain, at least in part, the differences in the biological activities of both hormones.

Red Blood Cell Uptake of Thyroid Hormones

The uptake of I^{131}-labelled T-4 and T-3 has been studied exclusively in red blood cells from human beings (20, 18, 19, 45, 46, 32, 33, 34, 17).

Crispell, et al. (20, 18, 19) compared the in vitro uptake of I^{131}-labelled T-4 and T-3 by human erythrocytes by taking a volume of washed cells and incubating them with plasma containing the labelled hormones. In all instances a greater percentage of the T-3 was taken up (or bound) by the red cells than occurred with T-4. Maximal uptake occurred after ten minutes incubation in the case of T-3; after ten to twenty minutes in the case of T-4. The explanation was proposed that the difference in uptake of the two hormones by the red cells reflected relative binding abilities of the plasma proteins. To substantiate this hypothesis the washed red cells were incubated with varying concentrations of plasma; as the plasma was diluted with saline the uptake of T-4 by the cells increased in proportion to the amount of saline added (20). In all cases the increase in uptake was greater for T-3 than T-4. Friis (33) added support to the concept of protein binding being the main mechanism responsible for the red cell uptake of T-3 by showing that the amount of T-3 taken up was increased as the serum was diluted. If T-3 (I^{127}) were added, uptake increased more sharply. Addition of hormone in amounts up to 0.2 micrograms (ug.) had no effect on red cell uptake. Possibly the observed difference in uptake of the hormones by red cells may be due to a greater affinity of the cells for T-3 than T-4. To explore this possibility, T-4 and T-3 were incubated with red blood cell stroma using the same procedure for incubation as was done with the intact cells (18). It was found that the red cell stroma bound both hormones with equal intensities; addition of plasma removed twice as much of the T-4 from the stroma as T-3. Therefore, it was concluded that the difference in binding must be due to plasma proteins. The injection of trypan blue (19) appeared to compete with T-4 for the binding sites on TBP since this compound caused

an appreciable rise in red cell uptake. The nature of the red cell uptake was thought to be a physicochemical phenomenon not directly dependent on enzymatic processes of red cell respiration. These studies were not concerned with T-3 and T-4 metabolism in red cells but were used only as a model of any cell which might bind thyroid hormones. This assumption seems justified inasmuch as similar results have been reported by Freinkel, et al. (31) using kidney, heart, and liver, and by Klemperer (61) using liver mitochondria.

In vivo studies show the amount of T-4 found in the red cell is negligible (117); thus in vitro studies of red cell uptake of thyroid hormones may have no in vivo counterpart. Even though this may be true, red blood cell uptake of I^{131}-labelled T-3 has been, and is being used clinically as a diagnostic test of thyroid function (32, 46, 33, 34, 45). Interpretation of the findings rests on the assumption that the mechanism of red cell uptake is dependent on the binding of T-3 to serum proteins, or in simpler terms, the availability (and affinity) of binding sites for the added T-3. If more of the sites are occupied because of the increased T-4 levels as in hyperthyroidism, more of the added T-3 will "spill" over to the weaker secondary carrier(s) and thus more will be taken up by the cell. The opposite would occur in myxedema in which there are more (or firmer) binding sites available.

Localization of Thyroid Hormones in Subcellular Fractions

Early information relative to the distribution of iodinated compounds in animal tissues has been summarized by Salter (93). More recently, several studies have been made on the distribution of I^{131}-

labelled thyroid hormones or their analogues in the tissues and organs of mammals. Studies on the intracellular localization of these hormones are few; it is noteworthy that these investigations have been confined exclusively to the distribution of thyroxine in the rat liver cell.

Lipner, et al. (68) administered as much as 612 ug. of C-14 labelled T-4 per kilogram of body weight to rats and found an equal distribution of radioactivity in the nuclear, mitochondrial, and microsomal fractions of liver up to 24 hours after the administration of the hormone. It was stated that there was no evidence that T-4 concentrated in any one cellular fraction since dry weight values of the fractions corresponded with radioactivity concentrations. Carr and Riggs (16) compared I^{127} concentrations in the subcellular components of extra-thyroidal tissues in normal and hyperthyroid dogs and observed that I^{127} was localized within the cell at approximately equal concentrations. Similar findings were reported by Tabachnick and Bonnycastle (101). Somewhat at variance with the foregoing studies were those of Lee and Williams (67) who investigated the subcellular distribution of physiological doses of I^{131}-labelled T-4 in rat liver at times varying from five minutes to two hours after injection. Initially, activity was highest in the mitochondrial fraction but after ten minutes it had decreased to a level that remained relatively constant up to two hours. Concomitant with this decrease in mitochondrial activity was the sudden rise in radioactivity in the microsomal and supernatant fractions, which reached a maximal level at ten minutes and remained elevated up to two hours. With the possible exception of this study, no striking selective localization of T-4 has been observed at the subcellular level, although the authors have described the distribution as an active process.

Metabolism of Thyroid Hormones

Six basic reactions characterize the biochemical transformations of the thyroid hormone molecule in tissues; (a) deiodination; (b) oxidation of the phenolic group; (c) conjugation of the phenolic group; (d) rupture of the diphenyl ether linkage; (e) oxidative deamination of the alanine side chain; and (f) decarboxylation (80). Since 1920, when it was shown that thyroglobulin breaks down to iodide, deiodination has been considered the most important reaction in the metabolism of thyroid hormones. When iodotyrosines or iodothyronines labelled with I^{131} were administered intravenously to normal men, most of the labelled iodine appeared, in the course of time, in the urine as inorganic iodide. Evidently there are mechanisms in the body that can remove iodine from the aromatic rings of these physiologically important substances and in the process reduce it to its ionic form (97).

Deiodination

Most investigations of the metabolism of thyroid hormones have been concerned with deiodination. This is due mainly to the fact that only I^{131}-labelled hormones of high specific activity are available; once this is lost, the fate of the rest of the molecule is unknown. For this reason T-4 labelled with C-14 on the carboxyl group also has been investigated (68, 63, 62); unfortunately, the feeble specific activity of the T-4 compelled these workers to use extremely large doses of T-4, thereby making any interpretation of their results in physiological terms almost impossible.

Stanbury (97) has discussed the physical chemistry of the iodine atoms in the phenol rings of iodinated tyrosines and thyronines and

stated that the iodine in the 3 position probably differs little in its intrinsic stability from the iodine of iodophenol which is quite stable. A major factor in the chemical reactivity of any of these aromatic iodine-containing compounds is the easy ionizability of the hydroxyl group. The linkage of iodothyronines is not ionized and, accordingly, there is more stability of the 3 and 5 positions of the alpha ring than there is of the 3' and 5' positions. Thus, one might expect the 3 and 5 positions to be more stable than the corresponding "prime" positions. This expectation was confirmed by deiodination studies in the rat (87). Plaskett (81) synthesized T-4 with I^{131} in the 3:5 or 3':5' positions. Deiodination of T-4 with the label in the latter position was slight (5-20 percent) in contrast to 80-90 percent deiodination when the label was on the 3':5' carbon atoms. An experiment also was carried out in which the samples of T-4 contained the label in varying proportions on the two rings. Results showed a proportionality between the extent of deiodination and the percentage of the label present in the "prime" positions. Modification of the alanine side chain did not influence the lability of iodine atoms in the diphenyl ether moiety; this was shown by Wilkinson (118) who studied excretion rates of tetra- and triiodothyroacetic acid labelled with I^{131} in the 3:5 and 3':5' positions. Such does not appear to be the case with the metabolism of thyroid hormones by the kidney (86) since 3:3' diiodothyronine (T-2) appeared following either the injection of 3:5:3' T-3 or 3:5':3' T-3 into rats. It would appear, therefore, that in the kidney the iodine bound to the 3-carbon and that bound to the 3'-carbon react in the same way. Yamazaki and Slingerland (120) incubated endogenously labelled T-4 with liver slices and identified only iodide as a reaction product; no material with an R_f of 3:5 T-2 or any other compound

identified chromatographically, thereby suggesting that the iodine atoms in both aromatic rings are equally labile.

The problem of deiodination has been approached in many laboratories and from many different points of view. Seemingly discordant results may be attributed to differences in techniques and, perhaps more significantly, to differences in the events being measured. Workers have used the following criteria as being indicative of deiodination: (a) the appearance of T-3 after T-4 administration (4, 58); (b) the appearance of T-2 after T-3 administration (86, 85); (c) identification of iodide in more than one chromatographic system (104, 103); or (d) urinary and fecal excretion of iodide following in vivo administration of thyroid hormones (73, 57).

All organs and extravascular tissues seem to have some ability to deiodinate thyroid hormones. The evidence, although indirect, was obtained by studies in the guinea pig where it was found that those tissues that did not concentrate iodide had a higher radioactive iodide concentration than that of the serum after the injection of I^{131}-labelled T-4 and T-3 (30). This would reflect a breakdown of the hormone after its concentration by the tissue.

Numerous reports have appeared concerning the deiodination of thyroid hormones in vivo. Gross and Leblond (43) injected radioactive T-4 into thyroidectomized rats and found that T-3 was produced (actually labelled as unknown #1 but later shown to be T-3); iodide also appeared in the urine. Pitt-Rivers, et al. (79) injected labelled T-4 into athyreotic human beings and reported T-3 subsequently in the plasma. Hogness, et al. (50) used keiselguhr columns to separate the metabolites of T-4 in kidney, liver, and muscle following the injection of the hormones into

rats. In approximately 20 percent of the analyses, T-3 was identified; fractionation of the tissues into subcellular components revealed deiodination to T-3 in all fractions, the microsomes exhibiting greatest activity. Kallman and Starr (58) identified T-3 in skeletal muscle, heart, salivary gland, and kidney from rats injected with T-4 as well as in the serum of human beings injected with T-4. Kalant, et al. (56) reported the appearance of T-3 in muscle and liver after T-4 was injected into normal and propylthiouracil-treated rats, and Ford, et al. (30) consistently found T-3 in many tissues of the guinea pig after T-4 administration.

It must be noted, however, that several investigators have been unable to identify T-3 as a metabolite of T-4 deiodination in vivo. Roche, et al. (89) injected rats intraperitoneally with T-4 and failed to identify T-3 in either the bile or the urine, but demonstrated the alpha-keto derivatives. They considered oxidative deamination and not deiodination to be the first step in the degradation of these substances. In the hepatectomized dog, T-3 was found only rarely after the injection of T-4 (29). Lassiter and Stanbury (66) were unable to find T-3 in the blood of human beings after giving T-4. They stated that this did not suggest that deiodination to T-3 in extrathyrodial tissues did not occur, but rather that the T-3 which is normally present in plasma is not a deiodination product of circulating T-4.

Many of these investigators have postulated that T-4 undergoes partial deiodination to T-3 at the peripheral tissues before it can exert its action at this level. Indirect evidence suggesting this was based on the effect of the "antithyroxine" drug n-butyl-4-hydroxy-3:5 diiodobenzoate (BHDB) on the overall metabolism of the two hormones; BHDB was found to diminish the output of urinary iodide when T-4 was injected

into rats but did not do so when T-3 was injected. From this work, Maclagan and Wilkinson (119, 96) have interpreted the prevention of deiodination of T-4 to T-3 as the "antithyroxine" mode of action of BHDB.

Studies on in vivo deiodination have been extended to T-3. It has been shown that rat kidney and skeletal muscle can convert this hormone to 3:3' T-2 although this did not seem to be the major metabolic pathway since only minute amounts were formed compared to the acetic acid derivative of T-3 (86, 90). Thus, it appeared that T-3 may have two principal pathways of widely different metabolic implications; one to the metabolically active and perhaps key substance, triiodothyroacetic acid (T-3A), and the other to the biologically useless 3:3' T-2 intermediate (90).

The first studies on deiodination of thyroid hormones by isolated tissues gave negative results; Roche and co-workers (91) found that the iodotyrosine deiodinase present in thyroid, liver, kidney and heart slices or extracts had no action on T-4. Later, however, numerous reports appeared on the in vitro deiodination of T-4 and T-3 during incubation with tissue preparations. Controversial evidence is found in the literature regarding the nature of the products produced following incubation of various tissues with I^{131} substrates. This seems strange inasmuch as most investigators have used thyroid hormones labelled in the 3' and/or 5' position; thus, results obtained by different workers studying the metabolism of T-4 or T-3 in a particular organ should be comparable but such is not the case.

Albright, Larson and co-workers (5, 65) incubated labelled T-4 with surviving kidney slices and detected a radioactive product that, when chromatographed in butanol-dioxane-ammonia, had a mobility corresponding to T-3. More recent work from the same laboratory (4) has confirmed their

earlier findings. They examined enzymic deiodination of T-4 to T-3 by human liver, kidney, heart, and muscle slices. With kidney, the appearance of labelled T-3 was observed regularly; the extent of conversion of T-4 to T-3 ranged from 11-38 percent in six hours. With heart, T-3 was observed on occasions but was not detected in liver and muscle. Cruchaud, et al. (21) employed similar techniques and demonstrated the formation of T-3 in kidney slices incubated with T-4. Becker and Prudden (10) perfused isolated rabbit liver for 12-15 hours with labelled T-4 and T-3 and found it capable of producing T-3 from T-4 in the majority of studies. Essentially the same results were obtained by Glitzer, et al. (38) who perfused T-4 through isolated rabbit kidneys. Although no T-3 was observed in the perfusate, it was nevertheless shown in extracts of the organ after completion of the perfusion.

In contrast to the above reports, several investigators have been unable to identify T-3 in tissues incubated with T-4 even though a considerable amount of the T-4 was deiodinated to iodide (120, 103, 104). Etling and Barker (24) failed to observe any T-3 produced from T-4 incubated for 3 days with kidney slices. Plaskett (81) using T-4 labelled in the 3 position, found that the product of deiodination by liver homogenates was a compound easily hydrolysed to diiodotyrosine. Yamazaki and Slingerland (120) found that deiodination of endogenously labelled T-4 was complete; accordingly, they concluded that no significant amounts of diiodotyrosine or any compound labelled in the 3 position was formed as an intermediate. This is in accord with the findings of Lissitzky, et al. (71) who identified thyronine as the end product of deiodination of T-4 by liver slices.

Albright, et al. (2, 3) have studied the metabolism of T-3 by rat

mitochondria from various tissues. Two types of reactions were observed: deiodination and deamination. Deiodination occurred most actively in kidney, liver, heart, spleen and brain. Mitochondria from intestine showed very little ability of deiodination. Deamination was observed mainly in the kidney, although a small amount of T-3A was observed regularly with liver. Sonic disruption of the mitochondria resulted in a loss of the deiodinating activity and an accentuation of the deaminating activity by kidney, liver, heart and brain mitochondria. Other work with rat liver homogenates (72, 92) and human leukocytes (64) have shown that iodide was the only product observed when T-3 was used as substrate.

Tata, et al. (104, 103, 110) have performed extensive studies on the deiodination of T-4 and T-3 by muscle and brain homogenates and slices from rats, chicks, and dogs. It was found that no noteworthy differences were observed in deiodination by slice or homogenate preparations. In the brain preparations, deiodination and deamination occurred, the former reaction occurred chiefly in the soluble subcellular fraction, whereas deamination was confined principally to the mitochondria. Because no T-3 was formed from T-4, Tata (110) tentatively formulated a scheme of preferred degradation through the deaminated analogues prior to deiodination in brain tissue. In a later work Tata (108) suggested the idea that since no labelled T-3 was produced from the 3':5' labelled T-4, the dehalogenase catalysed the simultaneous removal of both 3':5' iodine atoms. Homogenates of muscle have yielded a deiodinase similar to brain, but there was evidently no deaminase; this has suggested that direct deiodination of the iodothyronines is the only metabolic process occurring in this tissue (103).

Stanbury (97) and Stanbury, et al. (98) have done extensive studies

on T-4 deiodination by a microsomal preparation requiring ferrous ions, oxygen, and cysteine or glutathione. Curiously, deiodination by whole homogenates of liver or microsomal suspensions was greatly enhanced by heating the preparation to 100°C. for a few minutes, but prolonged heating destroyed all activity. Precipitation of the microsomes by perchloric acid did not destroy the deiodinating ability of the microsomes. Iodide was the only observed deiodination product when microsomes were prepared by perchloric acid precipitation which suggested rupture of the phenolic ring of T-4 following removal of the iodine. Galton and Ingbar (35), studying metabolism in liver and kidney homogenates of sevaral vertebrates, found that several products appeared. Although in tadpole homogenates the major metabolite was iodide, moderate amounts of material which remained at the origin during chromatography in organic solvents also were seen. In homogenates of mouse and rat kidney, traces of tetraiodothyroacetic acid (T-4A) were usually observed.

In the many reports on deiodination of thyroid hormones, little attention has been paid to the relative rate of deiodination of T-4 as compared to T-3. In those instances where a comparison was made fundamental differences have been observed. T-3 was deiodinated faster and to a greater extent than T-4 in the organs of intact animals, the criterion of deiodination was the rate of excretion of inorganic iodide after administration of the hormones (73, 57). In isolated mammalian tissue preparations the rates were reversed, T-4 being deiodinated to a greater extent than T-3 (81, 108, 104, 120, 103, 110). Other investigators (24, 72) have produced evidence that T-4 and T-3 are deiodinated by mammalian tissues at equal rates. Pitt-Rivers and Tata (80) are of the opinion that different enzymes are not involved. The difference could be explained

on the basis of a more rapid diffusion of T-3 into mammalian tissues in vivo, because of its weaker binding by the thyroxine-binding proteins of serum. If this were the case, the slight difference in the affinity of the deiodinating enzyme for T-4 and T-3 would not be the rate-limiting factor in the intact animal.

Whether or not the deiodination of T-4 and T-3 is directly related to their physiological activity has yet to be discovered (80). By investigating the effects of reserpine, serotonin and metabolites of tryptophan on the degradation of T-4 and its derivatives, Galton and Ingbar (35) presented evidence to support the view that deiodination may be closely associated with its biological action.

Deamination and Conjugation

The only other reactions of importance in the metabolism of thyroid hormones are oxidative deamination of the alanine side chain and esterification of the phenolic group.

Pitts-Rivers (78) assayed the acetic acid analogues of T-4 and T-3 by the goitre-prevention test in rats and found them to possess considerable biological activity. She suggested that these deaminated analogues might arise during peripheral metabolism of the hormones by oxidative deamination and decarboxylation of the alanine side chain of the parent amino acid. In support of this hypothesis, Roche, et al. (89) found that rats injected with large doses of T-3A or T-4A excreted the corresponding pyruvic acid analogues in their urine and bile. Traces of T-3A have been detected in kidney and muscle of rats given small doses of T-3 (85, 86, 90), and T-4A was found in the serum of dogs injected with I^{131}-labelled T-4 (29). T-4A and T-3A have been detected in rat kidney homogenates

and cell-free extracts (115) as well as in mitochondrial and tissue slice preparations from various tissues after incubation with the parent thyronines (4, 2, 3). Tata and co-workers (103, 110, 104) obtained evidence that brain and muscle slices can convert T-4 and T-3 into their respective acetic acid analogues. To demonstrate that the deaminated analogues of T-4 and T-3 were normal metabolites and not formed only after the administration of unphysiological doses of the hormones, Galton and Pitt-Rivers (37) injected radioactive iodide into mice and showed that the thyroacetic acids were formed from endogenous thyroid hormones. Whether these deaminated compounds are important in the thyroid hormone cycle is not yet clear; they may merely represent breakdown products of T-4 and T-3 which can later undergo conjugation and deiodination like the parent compounds (80).

Just as oxidative degradation of thyroid hormones is common to most tissues, the conjugation process also can be considered a general one since Flock, et al. (29, 27, 28) detected the glucuronides and sulfate esters of T-4 and T-3 in plasma and urine after the administration of these hormones to hepatectomized dogs. However, the formation of glucuronides takes place chiefly in the liver after injection of T-4 (89, 111). Galton and Pitt-Rivers (36) identified the glucuronide of T-4 in kidney following the injection of inorganic iodide-I^{131}. No conjugate was found in the blood or urine. The sulfate ester of T-3 has been shown to occur in the bile of thyroidectomized rats receiving I^{131}-labelled T-3 (88). Gross and his colleagues (42) have suggested that the sulfate ester may be the transport form of T-3 which is synthesized by the liver and kidney. However, Roche, et al. (84) consider the sulfoconjugate of T-3 as the storage form of T-3 which the cells can use

after hydrolysis of the ester bond. Thus, it appears as though the injection of T-4 results chiefly in excretion of the glucuronide ester of T-4 by the bile, whereas the injection of T-3 results in excretion of the sulfate ester (28, 88).

From this review of data pertinent to the present problem, it can be seen that little information is available in regard to thyroid horomone metabolism in birds.

CHAPTER III

MATERIALS AND METHODS

The experimental procedures used herein were designed to compare the in vivo and in vitro effects of T-4 and T-3 in chickens in order to formulate an explanation(s) for the observed difference in potencies of these hormones in this species.

Experimental Chickens

Six to sixteen-week old White Leghorn cockerels were used throughout these experiments. They were fed ad libitum on a standard commercial chick ration and had continued, free access to water.

I^{131}-Labelled T-4 and T-3

Commercially prepared* I^{131}-labelled T-4 and T-3 with average specific activities of 34.1 and 26.4 millicuries per milligram respectively were used as substrates. On receipt of the compounds (0.5 or 1.0 millicuries), they were placed in the refrigerator to minimize radiodecomposition. This procedure, however, did not prevent the spontaneous liberation of I^{131} but was successful in slowing down the process. Galton and Ingbar (35) diluted the concentrated radioactive solution with one percent human serum albumin and reported measurable success in reducing

*Abbott Laboratories, Oak Ridge, Tennessee.

radiodecomposition. The material as received showed various degrees of impurity. Typical chromatogram scanner records of the propylene glycol solutions of T-4 and T-3 on the day of arrival are shown in Figure 1. Contaminating iodide and T-4 or T-3 combined rarely exceeded 12 percent of the total radioactivity. Since the iodide contamination increased at the rate of approximately 4-9 percent per week, neither hormone was used after one week without further purification by paper chromatography.

When chromatographing the original radioactive material or any subsequently diluted samples, it was noted that quite often unusually high amounts (20-50 percent) of radioactivity corresponding to inorganic iodide occurred unless a small amount of plasma (0.05 ml.), serum albumin or protein (0.01 mg.) was added before spotting the material on the chromatogram (Figure 2). It was evident that protein had a stabilizing effect on the radioactively-labelled position on the molecule, a phenomenon observed by Tata (106).

Half-Life Studies

Five groups of six to sixteen-week old cockerels were injected intravenously with 0.75 - 1.0 ug. of I^{131}-labelled T-4 or T-3. Group I had blood withdrawn into heparinized syringes every minute until 20 minutes post-injection; group II, 3 ml. of blood withdrawn every 2 minutes from 20 minutes post-injection until 50 minutes post-injection; group III, 5 ml. of blood withdrawn at 3, 6, 9, 12, and 24 hours post-injection; group IV, 10 ml. of blood withdrawn once daily for 4 days. Group V consisted of birds decapitated at the following time intervals: 1, 3, 6, 9, 12 and 24 hours, at which times the hearts were removed.

The radioactivity in all blood samples and cardiac tissue was

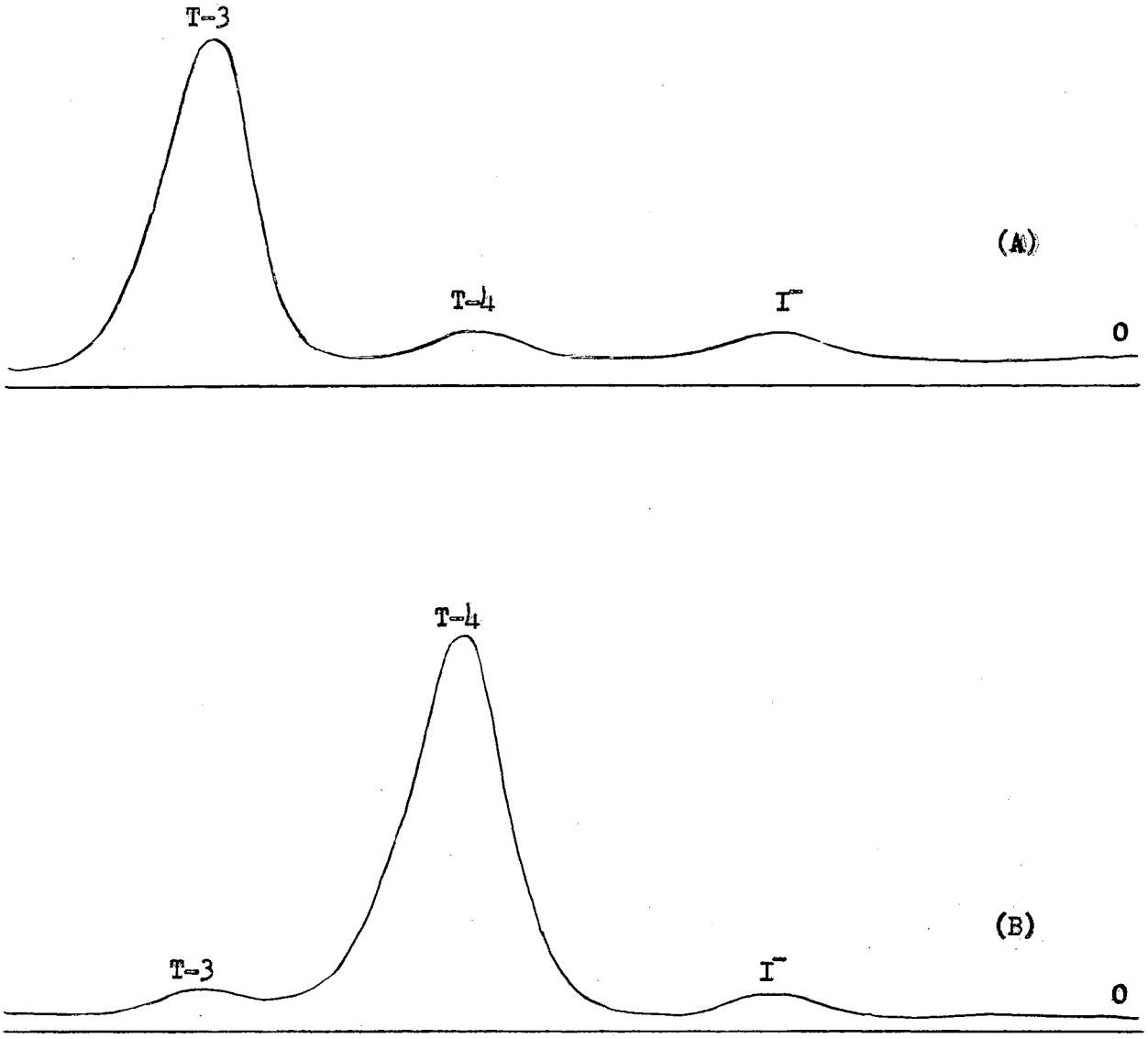

Figure 1. Chromatogram Scanner Records of the Propylene Glycol Solutions of T-3 (A) and T-4 (B) as Obtained from Abbott Laboratories, Oak Ridge, Tennessee. Solvent: tertiary amyl alcohol saturated with 2N ammonium hydroxide. 0 = origin. Solvent front not shown.

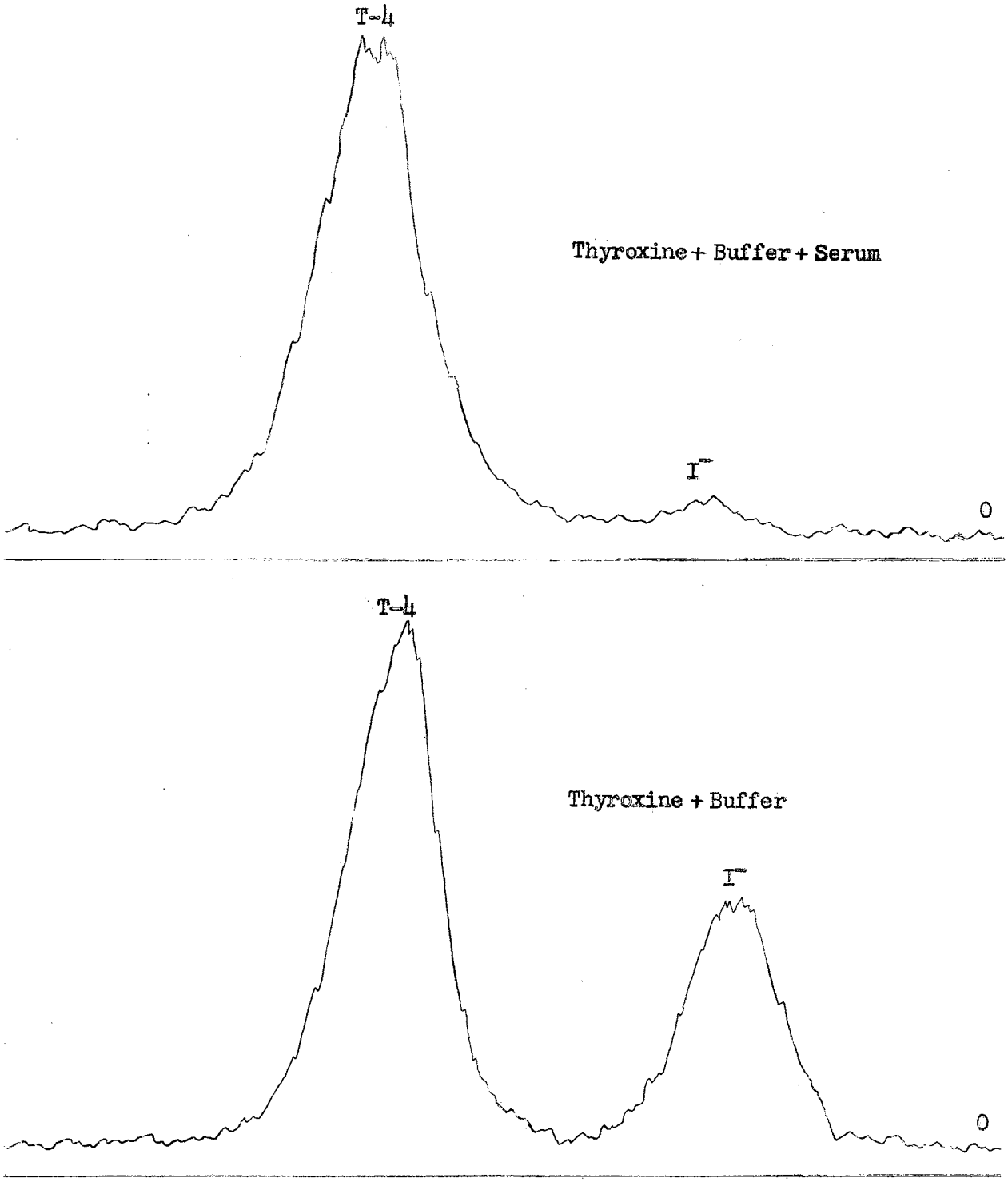

Figure 2. The Effect of the Presence of Chicken Serum on the Stability of I^{131} Labelled Thyroxine in Phosphate Buffer, pH 7.4. 0 = origin. Solvent front not shown.

measured in a well-type scintillation counter. In the first two groups, 0.1 ml. of plasma was counted, whereas in groups III and IV, 1.0 ml. and 3.0 ml. of plasma respectively were counted. All samples were corrected for decay and background at the time of counting. Resulting data (counts/minute or percent of the injected dose) were plotted on semilogarithmic paper and the half-lives calculated. With group V, the results were expressed as counts/minute/gram of tissue. This enabled the calculation of the half-life of the two hormones in cardiac tissue.

Protein-Bound Iodine

An ion exchange resin* was used for this phase of the experiments. Commercial Dowex-2, X8, 50-100 mesh, in the chloride form was taken through several cycle changes from base to acid, etc., and then suspended in 0.19 percent benzyl alcohol to inhibit bacterial growth.

Six-week old chickens were injected intravenously with 0.75 ug. of I^{131}-labelled T-4 or T-3. Five to ten milliliters of blood were drawn at various time intervals and counted in a scintillation counter. The resin columns were prepared as follows: into polyethylene cylinders (53 mm. X 10.5 mm.) were placed small pieces of filter paper. Sufficient "Ioresin" was added with a dropper to give columns 1.5 - 2.0 centimeters (cm.) in height and the excess fluid was allowed to drain through. Two milliliters of saline were then added to each tube and allowed to drain through, the excess was expelled by gentle air pressure from a rubber bulb. The plasma samples were added gently to the column, taking care not to disturb the resin bed. Air pressure was again applied to expell

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*Ioresin, Abbott Laboratories, North Chicago, Illinois.

all the plasma. Two milliliters of saline were added, as a wash, to the tubes that contained the plasma samples and then added to the column, followed by another one milliliter of saline. The radioactivity in the effluent was counted as described above and the percent protein-bound iodine calculated as follows:

$$\frac{\text{counts/minute in effluent from column}}{\text{counts/minute in original plasma sample}} \times 100$$

An aliquot (0.01 ml.) of the effluent from the column was subjected to paper electrophoresis in 0.075 M barbiturate buffer, pH 8.6 (180 volts, 5 milliampers, 14 hours) and by locating the radioactive areas with an automatic recording chromatogram scanner* it was possible to determine whether or not the effluent was associated with any plasma protein component.

Red Blood Cell Uptake of I^{131}-Labelled T-4 and T-3

The technique described by Hamolsky, Stein, and Freedberg (45) was used for this study. Two milliliters of whole blood were placed into test tubes. Immediately following the addition of I^{131}-labelled T-4 or T-3 (0.005 ug.), the tubes were incubated at 37°C with continuous shaking for 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 hours. At each time interval, one or more tubes were removed and the total activity in each tube measured. The blood was then centrifuged at 2500 rpm for five minutes and the plasma discarded. This was followed by washing the red cells twice in five milliliters of isotonic saline and discarding the washings. Radioactivity remaining in the cells was counted in a well-type scintilla-

*Scanogram II, Atomic Associates, Valley Stream, New York.

tion counter. The percent red blood cell uptake of T-3 or T-4 expressed in terms of a corrected hematocrit of 100 was calculated from the following formula:

$$\frac{\text{Net counts of washed R.B.C. X 100 X 100/hematocrit}}{\text{Net counts of whole blood}}$$

When the effects of various plasma dilutions on red cell uptake of T-4 and T-3 were studied, the technique was modified to a slight extent. Three milliliters of whole blood were placed in the desired number of test tubes and the plasma removed by centrifugation. The red cells were washed twice with isotonic saline and the washings discarded. Appropriate dilutions of the plasma were made in a total volume of two milliliters with isotonic saline and added to the washed red cells. After gentle, but thorough mixing, the tubes were incubated in a water bath at 37°C. for four hours. Following incubation, the cells were washed as previously described and the uptake expressed as a percent of the total activity added to each tube.

Distribution of T-4 and T-3 in Subcellular Fractions of Cardiac Tissue

Approximately 0.75 ug. of I^{131}-labelled T-4 or T-3 were injected intravenously into six-week old cockerels which were then killed by decapitation at 1, 3, 6, 9 and 12 hours post-injection. A total of 10-15 birds were injected with each compound and autopsied at each time interval although no more than three birds injected with one of the compounds were autopsied at any one time interval. At the time of autopsy, the heart was removed from each bird and immediately homogenized in 0.25 M sucrose using a mortar, pestle and a small amount of acid-washed sand. The homogenized tissue was diluted with sucrose to make a 15 percent

homogenate (w/v) and the subcellular fractions (nuclei, mitochondria and microsomes + supernatant) were isolated by differential centrifugation according to the method of Schneider (94). Less than five minutes elapsed from the time of decapitation until the tissue was ready for centrifugation.

The relative amounts of I^{131}-labelled T-4 or T-3 in each fraction was determined by the use of a well-type scintillation counter. Each sample was counted for that length of time required to give a count rate of at least ten times background. All counts were then converted to counts per minute (c/m) and expressed as a percentage of the total activity in the heart.

Mitochondrial Uptake of I^{131}-Labelled T-4 and T-3

Mitochondria from chick cardiac tissue were isolated by the method previously described (94) and incubated in vitro with I^{131}-labelled T-4 or T-3 according to the method of Albright (2). Each incubation tube contained an amount of mitochondria present in 2.0 grams of fresh tissue. Several tubes were prepared for the incubation of each hormone for each of the following time intervals: 15, 45, 90 and 180 minutes. Incubation was carried out at $37^{o}C$. At the appropriate time intervals, the tubes were removed from the water bath and centrifuged at 10,000 x g for one minute. The supernatant was decanted and the tubes inverted for five minutes to allow the excess supernatant to drain. The mitochondria were resuspended in a volume of phosphate buffer equal to the supernatant volume and both fractions were counted in a well-type scintillation counter. From these values the percentage uptake of the hormones

by the mitochondria was calculated.

Metabolism of I^{131}-Labelled T-4 and T-3 by Cardiac Tissue

This series of the experiments was composed of both in vivo and in vitro investigations.

I^{131}-labelled T-4 or T-3 (0.75-1.0 ug.) were injected intravenously into 6 to 8-week old cockerels which were sacrificed by decapitation at 1, 3, 6 and 9 hours later. The heart was removed from each bird and straightway chilled on ice then homogenized in a Potter-Elvejhem teflon homoginizer with 3 ml. of water. Two volumes of ether were added to the homogenate, mixed, then decanted. This procedure was repeated once. The lipid-free homogenate was adjusted to pH 1-3 with 5N hydrochloric acid and extracted five times with two volumes of n-butanol saturated with either 2N ammonium hydroxide or acidified water (pH 3). Approximately 70-85 percent of the radioactivity was removed. The butanol extract was reduced to a small volume in vacuo in a water bath at 55-60°C. and under nitrogen after the addition of two milligrams of thiouracil to prevent the breakdown of thyronines (113). Fifty microliters (ul.) of the viscous, highly pigmented residue were placed along a thin line of Whatman No. 1 filter paper, chromatographed and quantitatively assayed according to the procedure reported in the ensuing section.

I^{131}-labelled T-4 and T-3 were incubated in vitro with the following cardiac tissue preparations: total homogenate, mitochondria, microsomes, microsomes + supernatant and supernatant. Incubation of an amount of homogenate or subcellular component equivalent to one gram of fresh tissue was carried out with continuous shaking at 37°C. in Warburg flasks

under atmospheric air for 1, 2, 4, 8 and sometimes 12 hours. At the end of each time interval, 250 ul. of the suspension were removed from each flask, centrifuged, and 25-100 ul. of the clear supernatant were applied directly, along with non-labelled carrier T-4, T-3, T-4A, T-3A or iodide as reference compounds, to a thin line on Whatman No. 1 filter papers for chromatographic analyses in tertiary amyl alcohol saturated with 2N ammonium hydroxide. Spotting the reaction mixture relatively soon after incubation is of utmost importance. In preliminary studies it was noted that freezing or refrigerating the mixture for 24 hours or more resulted in almost complete deiodination of the labelled hormones. Additions to each flask were as follows: 2.5 micromoles of diphosphopyridine nucleotide (DPN), 50 micromoles of potassium phosphate buffer (76), pH 7.4, and two ml. of the tissue preparation. Control flasks were prepared by either adding boiled tissues, adding buffer instead of tissue, or taking a sample of the incubation mixture at zero time or within five minutes after adding the labelled substrate. One or more of these controls were run concurrently with each group.

Chromatographic Procedures

Ascending, one-dimensional chromatography was used throughout the experiments except for an occasional two-dimensional chromatogram for purposes explained in a later section. Of the several solvent systems investigated, tertiary amyl alcohol saturated with 2N ammonium hydroxide in an atmosphere of concentrated ammonia was found to be most effective in separating T-4, T-3 and their deaminated analogues. Butanol-ethanol-2N ammonium hydroxide (5:1:2) and butanol saturated with 2N ammonium hydroxide occasionally was used but did not resolve the deaminated

analogues very effectively, although good separation of the parent thyronines and iodide was achieved. R_f values of various iodinated compounds in the teriary amyl alcohol system are shown in Table I.

With the exception of the ammonium hydroxide, all solvents were redistilled before use. The saturated tertiary amyl alcohol was prepared by vigorously shaking 250 ml. of the alcohol with 100 ml. of 2N ammonium hydroxide. This was done for 30 seconds every five minutes for 30 minutes.

TABLE I

R_f VALUES OF VARIOUS IODINATED DERIVATIVES IN TERTIARY AMYL ALCOHOL SATURATED WITH 2N AMMONIUM HYDROXIDE

Compound	Roche (84)	Albright (3)	Present Investigations
T-4	0.28	0.20	0.28 ± 0.14
T-4A	0.40	0.30	0.42 ± 0.14
T-3	0.46	0.40	0.48 ± 0.18
T-4"	0.55	0.56	0.61 ± 0.22
I-	0.18	--	0.19 ± 0.03

Finally, when the excess ammonium hydroxide had settled to the bottom of the flask the saturated alcohol was decanted off. Under the prevailing atmospheric conditions in the laboratory 23 ml. of 2N ammonium hydroxide usually would saturate 100 ml. of the alcohol. This always was prepared immediately before use. The source of the ammonia atmosphere

was a beaker containing concentrated ammonium hydroxide. This latter step was an important factor in obtaining adequate separation of the iodinated compounds used in these experiments.

Rectangular, pyrex chromatography jars measuring 53 cm. X 29 cm. X 8 cm. served as containers for development of the filter paper strips. Large sheets of filter paper covered three sides of the jars and were saturated with the developing solvent. This was found to aid separation of the compounds since it provided for more complete saturation of the air inside the jars and prevented the solvent from evaporating from the chromatographic strips. In cases where this was not done the R_f differences between the different compounds were considerably less due to the progressive slower movement of the compounds as they migrated up the paper. It was most desirable to use fresh solvent each time a set of chromatograms was run, but in any case no more than three runs were made without changing the solvent. A small amount of thiouracil (five milligrams per 100 ml. of solvent) was added to each batch of solvent to prevent spurious oxidation of the iodide during chromatography. Stopcock grease was used to produce an air-tight seal for the lid to prevent solvent evaporation.

Tapered Whatman #1 filter paper (Figure 3) was used in most of the experiments since it was found that as the compounds migrated they did not spread out as readily as when standard 35 millimeter-wide strips were used, i.e., each compound was confined to a relatively narrow band. Potentially, this could be an important factor in chromatography where two compounds differed only slightly in their R_f values. During the course of the experiments in this study T-3 and T-4A were found to have R_f values of 0.48 and 0.42 respectively but were not separated too well

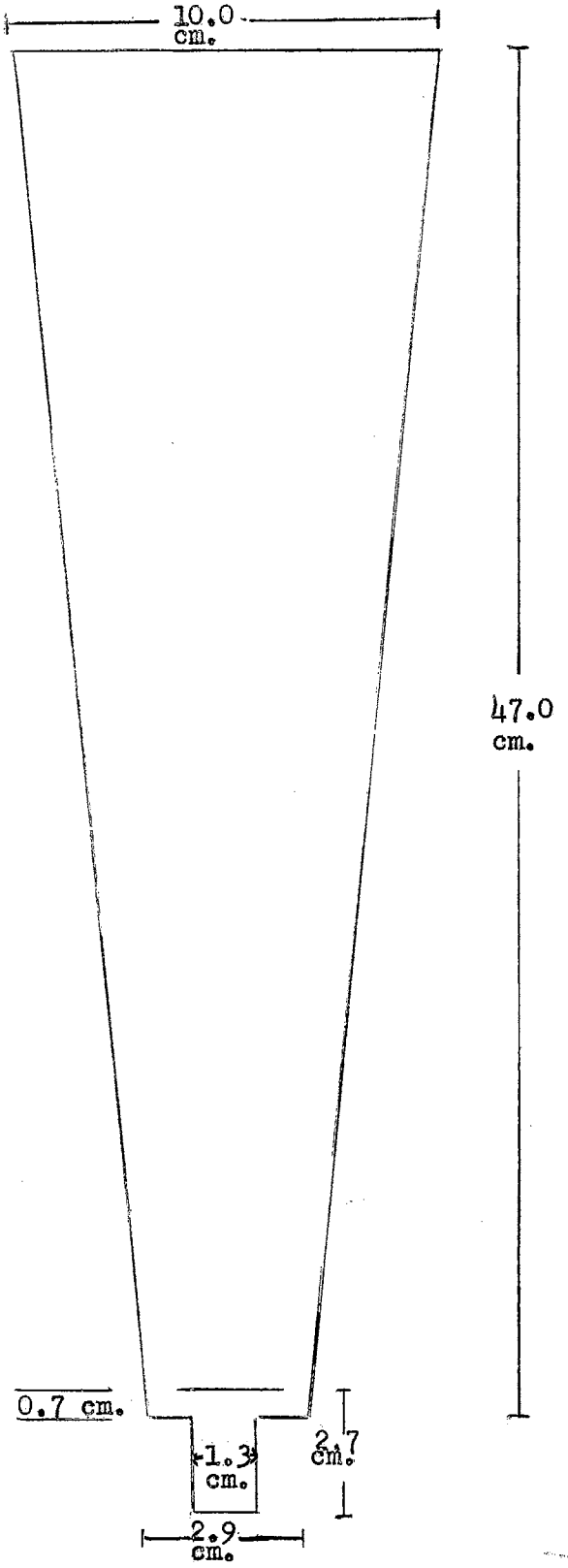

Figure 3. Single Dimensional Chromatographic Strip

from one another unless the tapered paper was used.

Adequate separation of various compounds in a mixture by paper chromatography does not depend wholly on the choice of solvent but also on the extent to which the material spreads out as it is spotted on the paper. For this reason the samples were spotted, five ul. at a time, along a two cm. line near the bottom of the paper (Figure 3) and allowed to dry before the next five ul. were applied. Drying was enhanced by allowing the warm air from a hair dryer to blow on the paper. By following this procedure a line of consistent width (0.5 cm.) was made at the origin of the chromatogram. Under most circumstances it was not possible to spot more than 50-100 ul. of the experimental material since there was a tendency to "overload" the paper resulting in (1) failure of the compounds to separate due to extensive streaking, or (2) failure of the compounds to migrate any appreciable distance.

Along with all chromatograms 25-50 ug. of unlabelled carriers were spotted with the experimental material. However, only two or three different carriers were spotted on any one chromatogram. Routinely, the chromatograms were developed for 36 hours during which time the solvent usually ascended a distance of 35-40 cm.

Detection of Radioactivity

Prior to staining, the radioactivity on the chromatograms was determined by counting them in a continuously recording chromatogram scanner and quantitating the areas under the curves by planimetry.* Radioautographs were prepared by placing the chromatograms in apposition to either Kodak or Ansco no-screen X-Ray film for a period varying from

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*Lasico planimeter Model #700, Los Angeles Scientific Instrument Co.

several days to three weeks, depending on the amount of radioactivity added. Chromatograms containing radioactivity less than 5000 c/m were exposed for three weeks while those with activity above 20,000 c/m necessitated exposure for only two or three days. The films were developed by placing them in Kodak X-Ray developer until the radioactive areas turned black, but never longer than four minutes. They were then placed in Kodak X-Ray fixer for eight minutes after which they were rinsed in water and dried. For identification of the radioactive areas, the films were superimposed on the stained chromatograms. All chromatograms were not radioautographed. Random chromatograms in each experiment were radioautographed as well as in those instances where there may have been the possibility of more than one radioactive band located under one peak on the chromatogram scanner record. In those cases where two bands were located under one peak, the respective areas were cut out, counted in a scintillation counter and the percentage of each compound recorded. Occasionally, chromatograms were assayed for radioactivity by both methods.

Staining of Carriers

Following chromatography and radioautography the chromatograms were stained with one of the following reagents:

A. 4-Aminoantipyrine reagent (4): the chromatograms were dipped in 0.2 percent 4-aminoantipyrine made up in distilled water followed by dipping in 2.0 percent potassium ferricyanide made up in 2.0 percent sodium carbonate. The tetraiodinated thyronines produced a lavender, triiodinated compounds red, and diiodinated an orange color. The color was stable for only a few months so appropriate areas were outlined in pencil to preserve their localization.

B. Ferrichloride-ferricyanide-arsenious acid reagent (39): Solution A-2.7 grams of ferric chloride (6 H_2O) dissolved in 100 ml. of 2N HCl; Solution B - 3.5 grams of $K_3Fe(CN)_6$ dissolved in 100 ml. water: Solution C - 5.0 grams of $NaAsO_2$ dissolved under cooling in 30 ml. N NaOH followed by 65.0 ml. 2N HCl added under intense stirring. Immediately before use 5 parts of solution A, 5 parts of solution B, and 1 part of solution C were mixed in the dark or dim light. The chromatograms then were dipped in the solution and when dark blue areas appeared on the paper they were washed for 30 seconds in tap water. The various compounds appeared as dark blue areas against a light blue background. Color stability reportedly is good for prolonged periods without fading. Since this stain is specific for the iodine moiety of the molecule it will stain iodide as well as the other organic iodinated compounds, thus those chromatograms which had iodide as carrier were always stained with this reagent.

C. Pauly reagent (13): 5.0 ml. of 1.0 percent sulfanilamide and 1.0 ml. of 5.0 percent $NaNO_3$ were mixed thoroughly in a 50 ml. graduate cylinder. N-butanol was added to the 50-ml. mark and the mixture shaken and allowed to stand for four minutes. The butanol was decanted off and the chromatograms were dipped in this solution. After drying they were dipped in 50 percent saturated Na_2CO_3 and dried. This reagent also is stable for prolonged periods with negligible amounts of fading.

CHAPTER IV

RESULTS AND DISCUSSION

Binding of T-4 and T-3 to Plasma Proteins

In most mammals the higher potency of T-3 as compared to T-4 is accompanied by a more rapid action which can be attributed to a faster rate of diffusion from blood to tissues than that of T-4. In birds, it has been reported that the half-lives of the two compounds as measured by whole-body disappearance rates, as well as their affinities for the plasma proteins are identical (109). This suggests that if the foregoing generalizations are correct, T-4 and T-3 should have similar potencies. In view of the fact that Newcomer (75), Newcomer and Barrett (76) and Mellen and Wentworth (74) have demonstrated that T-4 is more potent than T-3, the phenomenon of protein-binding of the hormones was re-investigated.

Protein-bound iodine determinations were made on plasma at various times following the intravenous injection of equimolar amounts of labelled T-4 or T-3. As can be seen from Figure 4-a, approximately twice as much T-4 was bound to protein as T-3; apparently binding was maximal within one hour and remained relatively constant throughout the time intervals studied. Since the administered dose was within the physiological range there was little chance of saturation of protein binding sites, which potentially could result in erroneous values. To demonstrate that the experimental procedure was measuring "bound" and not "free" hormone,

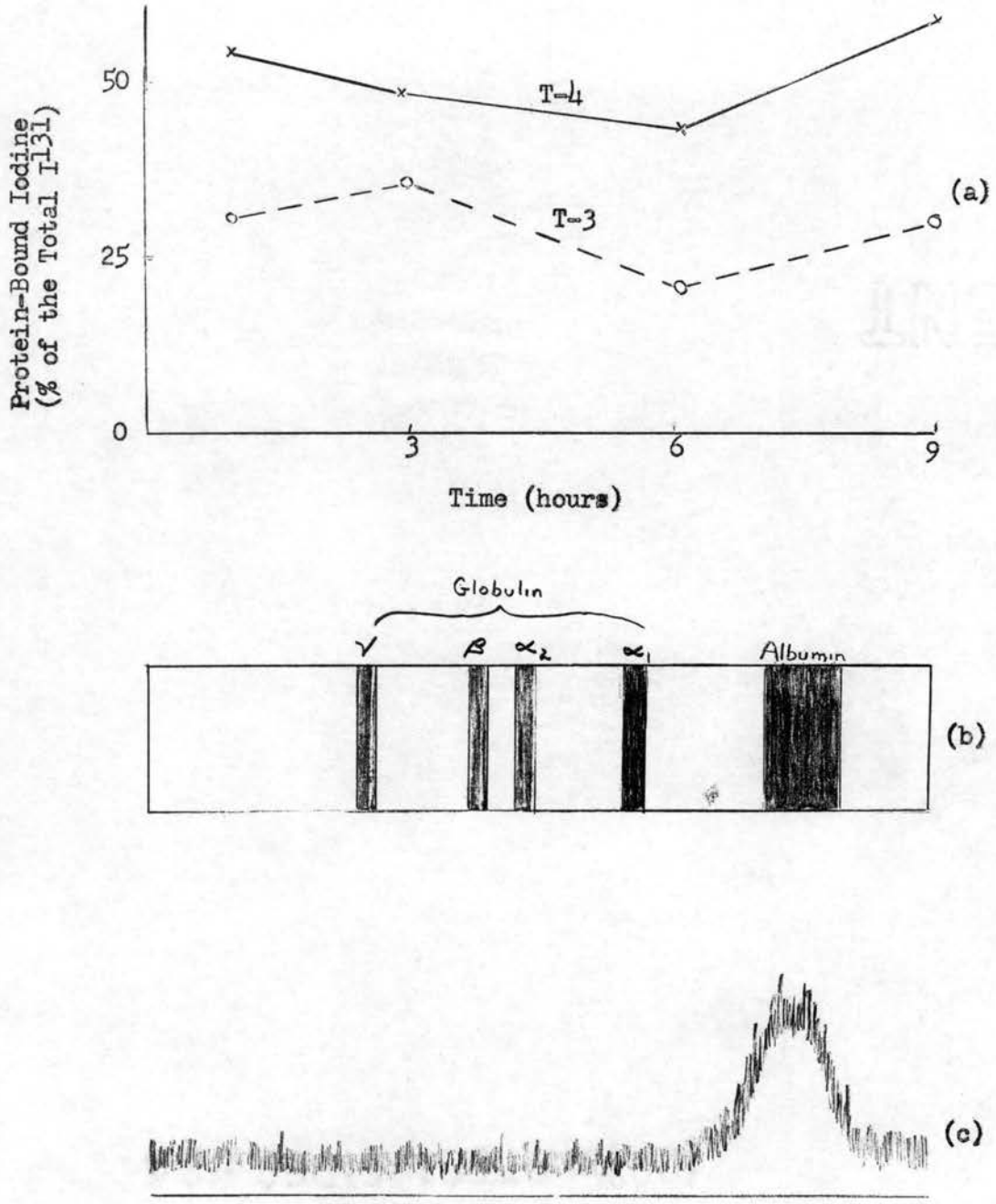

Figure 4. (a) Protein-Bound I^{131}-Labelled T-4 or T-3 in Chicken Plasma at Various Times Following Intravenous Injection of the Hormones, (b) Electrophoretic analysis of Chicken Plasma one Hour Following the Intravenous Injection of I^{131}-Labelled T-4 or T-3, (c) Scan of (b) with an Automatic Chromatogram Scanner after the Plasma had been Run through the Ioresin Column. (See Appendix, p. 90)

pure T-4 or T-3 in buffer solution was added to the column; 98-99 percent of the material remained on the column through several washes with saline. In addition, electrophoretic analyses were made on the plasma effluent from the "Ioresin" column. Figure 4-b and 4-c shows the results obtained in this study. It is evident that, in veronal buffer, all the radioactivity migrated with the albumin fraction, which according to various authors, is the primary site of binding of thyroid hormones to avian plasma under such circumstances (109, 6). These results are difficult to explain in the light of the data presented by Tata and Shellabarger (109) except that the techniques employed by these authors were based on entirely different premises than those employed in the present experiments.

To substantiate the foregoing observation of differences in protein binding of thyroid hormones in chicken plasma, experiments were performed on the in vitro uptake of T-4 and T-3 by chicken red blood cells. It has been observed that this phenomenon reflects the affinity of thyroid hormones for plasma proteins, that compound having the weakest affinity (or bound less firmly) would have the greatest in vitro uptake by the erythrocytes (20, 18, 33). Results of these experiments are summarized in Figure 5. During all the time intervals in the four hour incubation period, uptake of both compounds steadily increased. T-3 uptake increased from a mean uptake of 16.2 percent at 30 minutes to 45.2 percent at four hours; values for T-4 uptake were 12.7 percent at 30 minutes to 21.1 percent after four hours incubation. These values are considerably higher than those reported for human red cells (32). From the values of this experiment it is obvious that T-3 is taken up by the cells to a greater extent and at a faster rate than T-4. Maximal uptake was not achieved at the one hour time interval although Friis (32) reported a maximum

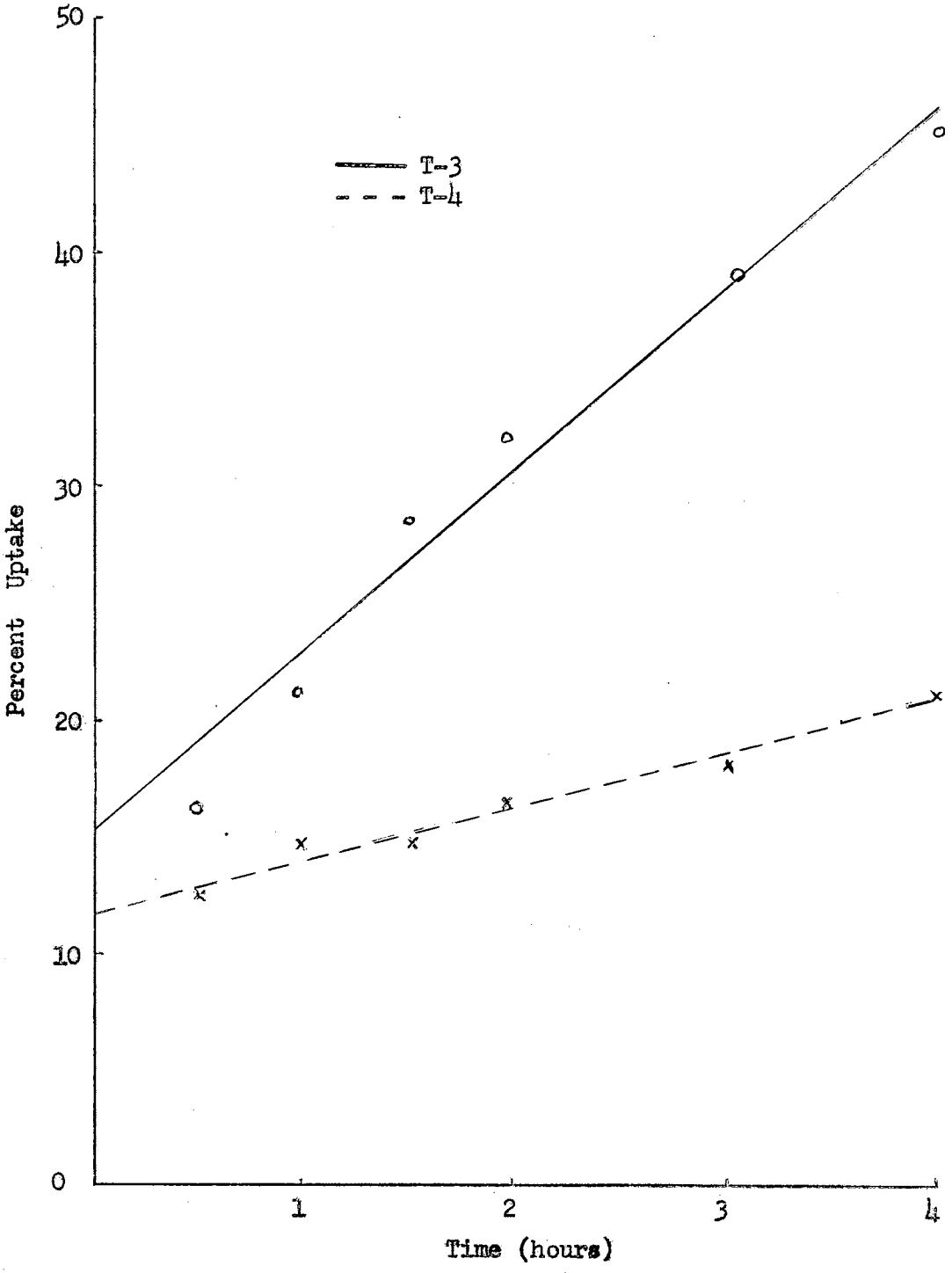

Figure 5. Average Uptake of I^{131}-Labelled T-4 and T-3 by Red Blood Cells from Six-Week Old Cockerels(see Appendix p. 91).

uptake after one hour incubation; Crispell, et al. (19) found maximal uptake after an incubation time of 10-20 minutes. The reason for this discrepancy is not apparent.

If protein binding is the main factor regulating the in vitro uptake of the hormones by red blood cells, dilution of the plasma proteins should result in an increase in uptake. Figure 6 shows the effects of various plasma-saline dilutions on red blood cell uptake during an incubation period of four hours. Quantitative values are lower than those seen in Figure 5 due to the modified technique used in this latter study. As the plasma was diluted the uptake of both hormones increased, the relative percent increase being greater for T-4 than T-3. This latter finding would be expected if more T-4 than T-3 was bound to the proteins. When only saline was used as the red cell suspending medium, uptake by the cells was identical for both hormones. These findings agree very well with those reported by Friis (33) and Crispell, et al. (20) in mammals.

Since the possibility arose that chicken red cell uptake may be affected by the presence of the nucleus, non-nucleated cells (dog) were incubated with chicken plasma to see if any qualitative difference in response would occur. As one would expect, extensive hemolysis occurred which yielded lower uptake values than normal, nevertheless, in the one experiment that was conducted the dog red blood cells took up the T-3 to a greater extent and at a faster rate than they did the T-4 (Appendix p.93).

Projecting these protein-bound iodine determinations and red cell uptake values in terms of affinities of T-4 and T-3 for chicken plasma proteins, T-3 has a markedly lower affinity for the plasma proteins than T-4. In support of this conclusion is the work of Balfour and Tunnicliffe

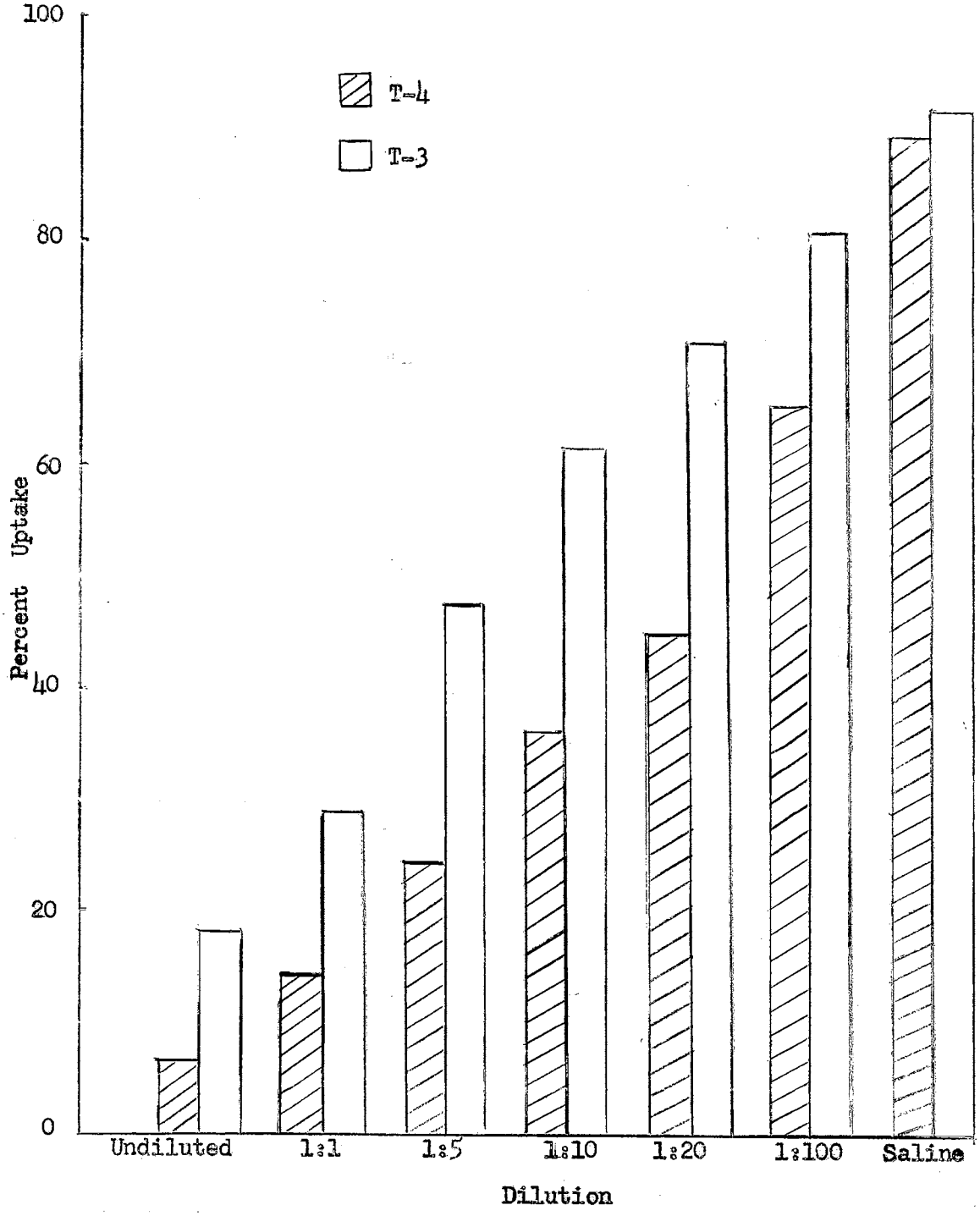

Figure 6. The Effect of Diluted Plasma on Chicken Red Blood Cell Uptake of I^{131}-Labelled T-4 and T-3 (see Appendix, p.92).

(6) who found that electrophoresis of duck serum on cellulose columns with borate-phosphate buffer, revealed the presence of a pre-albumin protein fraction which binds T-4 to a considerable extent. Moreover, as Ingbar (52) has found, pre-albumin possesses essentially no binding power for T-3, the net effect being a lower affinity of T-3 for avian plasma proteins than T-4.

In light of the present findings it is difficult to believe that, (a) T-4 and T-3 are bound to chicken plasma with equal intensities and (b) that protein binding is an adequate explanation for the differences in response of birds and mammals to thyroid hormones.

Biological Half-Life of I^{131}-Labelled T-4 and T-3 in Plasma and Cardiac Tissue

Biological decay rates of thyroid hormones in blood have been used extensively as an index of peripheral metabolism and turnover of the hormones (99, 100, 23, 1, 82). Consistently, T-3 disappears from the plasma of mammals at a rate approximately twice that of T-4; in avian species no data are available on disappearance rates from plasma. Characteristic of biological decay rates of thyroid hormones in blood of animals is the rapidity with which they disappear; Figure 7 graphically illustrates this phenomenon in the chicken. When the data are plotted on semilogarithm paper (Figures 8, 9) they form a straight line, illustrating the exponential nature of the decay rate. The deviations from linearity seen in Figures 8 and 9 are discussed below.

Table II summarizes half-life determinations of T-4 and T-3 in the plasma of sixteen-week old cockerels during various time intervals folfowing intravenous injection of each hormone. Time interval A is

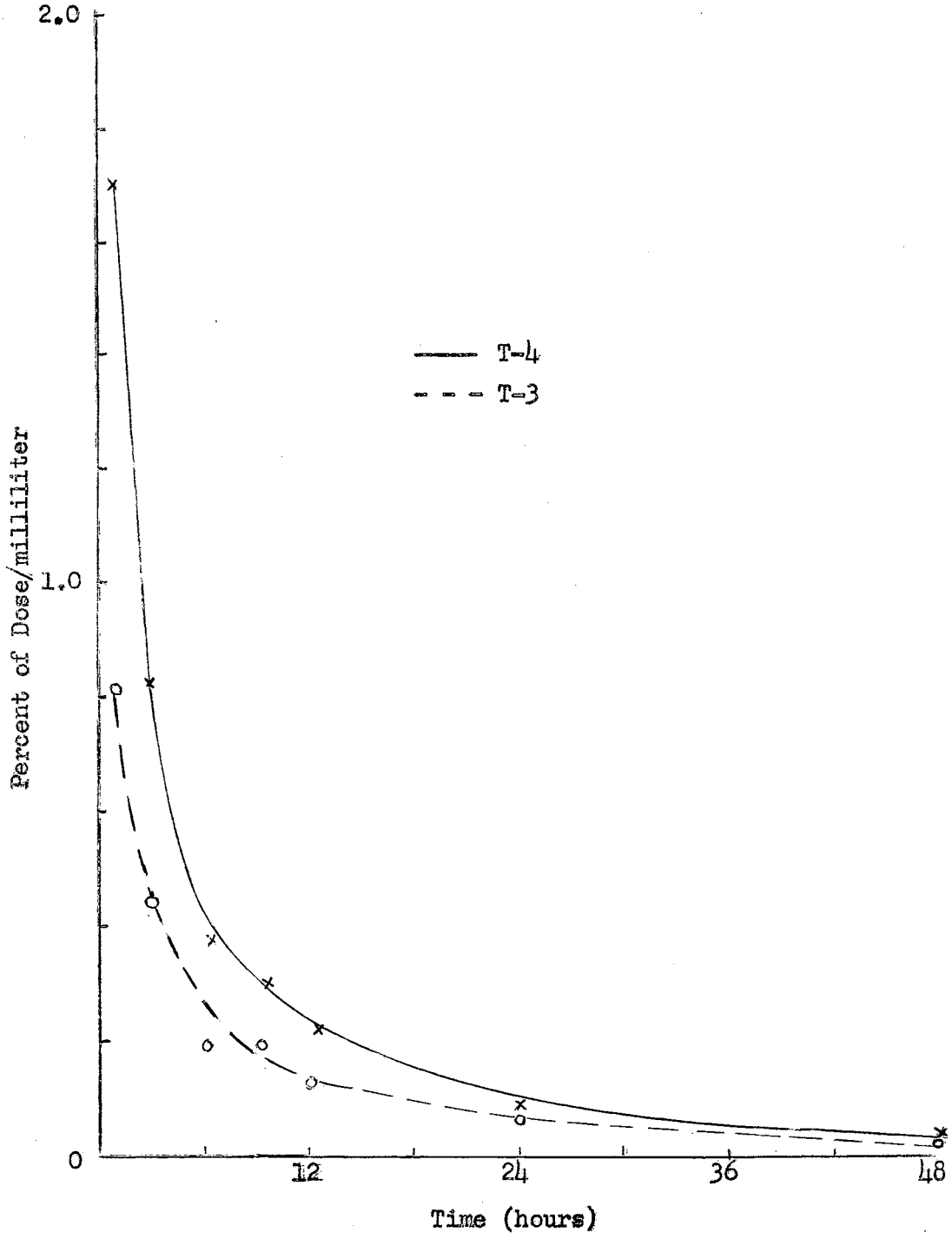

Figure 7. Typical Disappearance Curves of I^{131}-Labelled T-4 and T-3 from the Plasma of Six-Week Old Cockerels during the First Forty-eight Hours Following Injection.

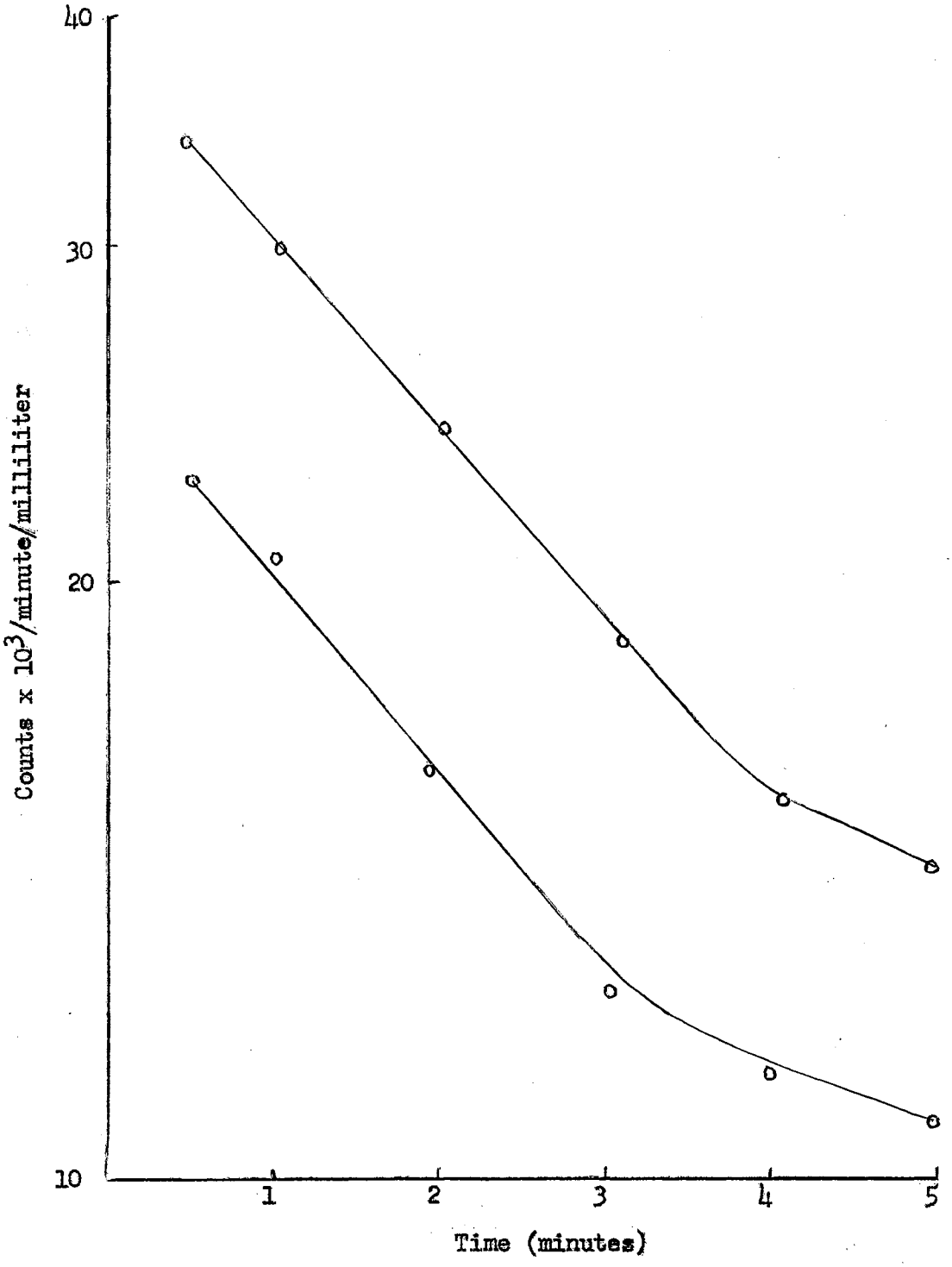

Figure 8. Semilogarithmic Plot of the Disappearance of I^{131} Labelled T-4 from the Plasma of Two Sixteen-Week Old Cockerels during the first Five Minutes Following Injection.

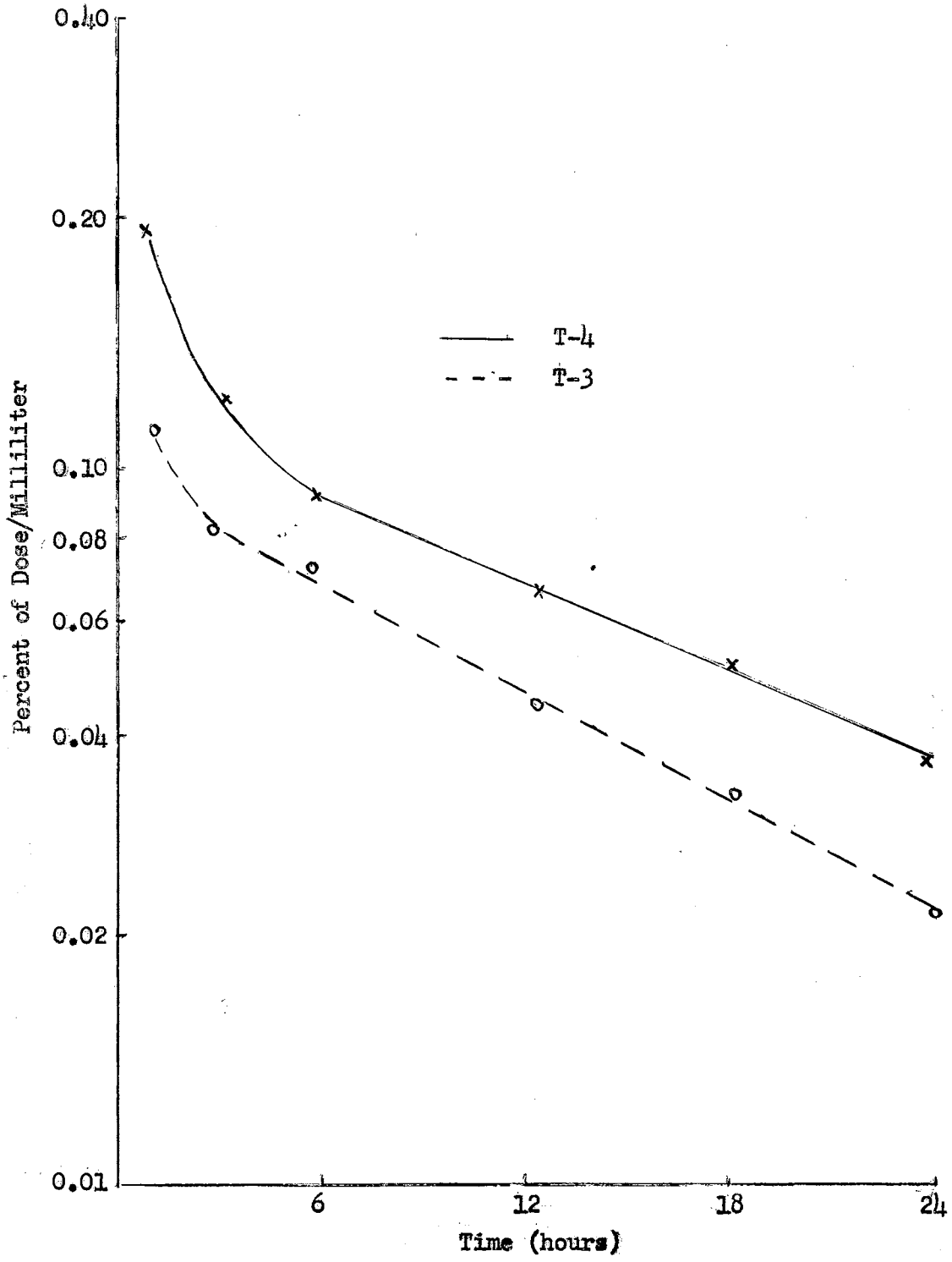

Figure 9. Semilogarithmic Plot of the Disappearance of I^{131}-Labelled T-4 and T-3 from the Plasma of Sixteen-Week Old Cockerels during the first Twenty-four Hours Following Injection.

characterized by a half-life of approximately 3.0 minutes for both hormones; time intervals B and C exhibit decay rates characterized by a half-life of approximately 29.0 minutes for both hormones; time interval D has a characteristic half-life of 7.2 hours for T-3 and 8.3 hours for T-4 ($p < 0.6$); time interval E is characterized by a half-life of approximately 16.4 hours for both hormones.

TABLE II

BIOLOGICAL HALF-LIFE OF I^{131}-LABELLED T-4 AND T-3 IN THE PLASMA OF SIXTEEN-WEEK OLD COCKERELS DURING VARIOUS TIME INTERVALS

	Time Interval			
	From	To	T-3	T-4
A	1.0	4.0 min.	3.4 min. (2)	3.0 min. (4)
B	5.0	20.0 min.	29.3 min. (4)	28.0 min. (5)
C	20.0	50.0 min.	27.3 min. (5)	31.1 min. (5)
D	1.0	24.0 hrs.	7.2 hrs. (8)	8.3 hrs. 9
E	1.0	4.0 days	16.3 hrs. (5)	16.5 hrs. (8)

( ) number of determinations

Clearly, at least four rate constants are involved in the disappearance of T-4 and T-3 from chicken plasma up to four days post-injection, three of which probably represent biologically important phenomena. Initially, the rate of disappearance of the hormones is very rapid, which undoubtedly represents the dilution of the hormones within the vascular compartment. Figure 8 shows a representative dis-

appearance curve of T-4 in two chickens during this initial time period. Although data on T-3 disappearance are not shown, the curves would be very similar. From the graph it is evident that the disappearance rate does not give a linear plot which possibly indicated two rate constants are involved during this period. Presumably, the initial "fast" component (up to 3 to 4 minutes) represents distribution throughout the large vascular pools, and the "slow"component represents equilibration of the hormones within the capillary beds; alternatively, the "slow" component may represent the rate constant characteristic of time intervals B and C. Time intervals B and C are characterized by identical half-lives for both hormones thus indicating identical rate constants are involved and the same events are occurring in the animal. Albert and Keating (1) have presented evidence to indicate that this time interval represents a distribution volume which is contained almost entirely within the gastrointestinal tract and does not represent general extravascular distribution. Engbring, et al. (23) report the 20 minute to 50 minute time interval represents rates of distribution of thyroid hormones into the body's extravascular iodine pools. Roche and coworkers (88) have given evidence to show that disappearance of T-4 or T-3 from the circulation during the first hour following injection represents extravascular distribution rates. Using indirect methods they have shown that 65 percent of the administered radioactive T-3 or T-4 appears in the interstitial fluid within one hour after injection. From Figure 9 it would appear that the rate constant characteristic of the first hour after administration actually may extend up to 3 to 6 hours. During the first 24 hours following injection (time interval D), T-3 was shown to have a slightly shorter half-life than T-4; a difference that was not statistically

significant. Albert and Keating (1) have suggested that this rate constant is indicative of the disappearance of thyroid hormone from an initial volume of distribution into the excreta and tissues. This is in general agreement with the conclusion of others (99, 100, 82). When plasma concentrations of T-4 and T-3 were measured once daily for four days, there was no difference in the rate of disappearance between T-4 and T-3. Half-life determinations during this period have been taken to represent metabolic degradation of the hormones within the cell (99, 100, 82) or T-4 leaving the blood by some route other than the urine and feces into some larger equilibrium volume in the body (1). In most instances a flattening of the curve occurred about the fourth day after injection; this was attributed to reutilization of the released I^{131} as the hormones were degraded. Sterling and Chodos (99) reported similar results.

In summary, it can be said that initially the hormones are distributed throughout the vascular compartment after which time they enter the extravascular space at approximately equal rates. As equilibrium is established between the vascular and extravascular compartment, they enter the cell and are metabolized at equal rates.

In the previous section, evidence was presented to indicate that T-3 has a lower affinity for chicken plasma proteins than does T-4. Accordingly, T-3 should have a shorter half-life in plasma if, indeed, protein binding is the main factor regulating the disappearance of the hormones from plasma. Roche, et al. (88) have postulated that the thyroxine-binding protein crosses directly from the capillary wall to the interstitial fluid and thus disappearance of the hormones from the blood may be independent of protein binding. Engbring and associates (23) have

stated that under various circumstances, prolonged half-life of T-4 (as in myxedema) may be independent of protein-bound iodine values. The fact that chicken blood lacks the specific thyroxine-binding protein of mammals may or may not allow the hypothesis of Roche, et al. (88) to apply to chickens.

Since there appears to be no difference in the half-lives of the two hormones in chicken plasma, factors other than protein binding must account for the disappearance rates from the circulation.

The half-lives of T-4 and T-3 in cardiac tissue are graphically represented in Figure 10. T-3 had a mean half-life of 3.9 hours; T-4, 4.9 hours. An analysis of covariance indicated a statistical difference between these two values ($p < 0.05$). This finding has an important functional interpretation. The fact that T-3 has a shorter half-life in cardiac tissue than T-4 may be one factor which could explain why T-4 is more potent in stimulating oxygen consumption in this organ (76). If T-4 remains in the tissue for a longer period than T-3, presumably it could stimulate metabolic processes for a longer period with the net result of a more potent action.

Intracellular Localization of I^{131}-Labelled T-4 and T-3

Implicit in every endocrinological experiment is the concept of a specific site of hormone action. This concept may be expressed with various degrees of precision and defined in either physiological or anatomical terms. Hormone action must ultimately be referrable to the chemistry of the cell. When viewed in this manner the following questions assume fundamental importance: (a) do hormones penetrate the cell membrane and enter the cytoplasm, and (b) if so, is the intracellular

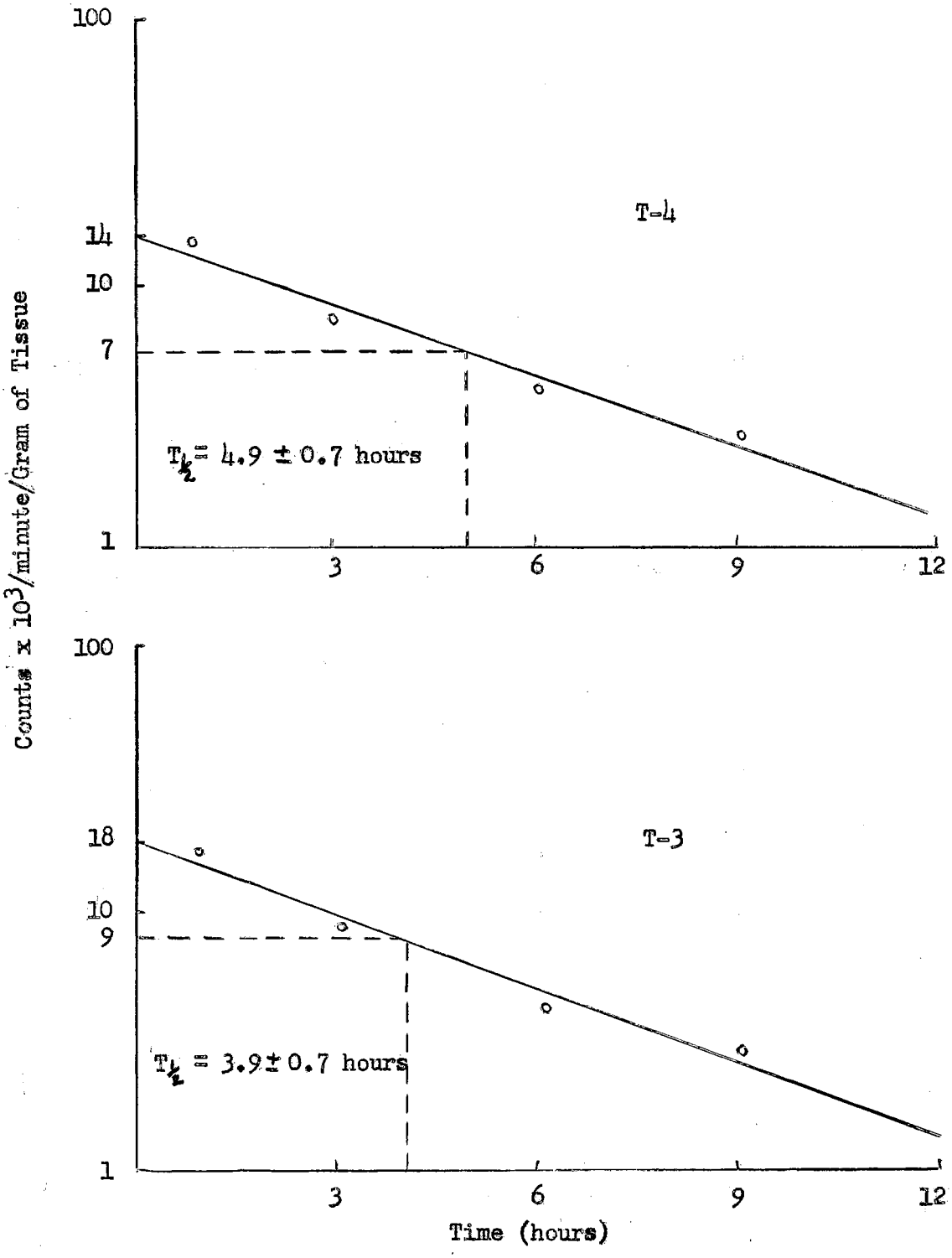

Figure 10. Graphical Representation of the Mean Half-Lives ($T_{\frac{1}{2}}$) of I^{131}-Labelled T-4 and T-3 in Chicken Cardiac Tissue. (see Appendix, p. 95)

existence associated with one or more of the various cytological structures?

Figure 11 shows the distribution of T-4 and T-3 in subcellular fractions of cardiac tissue at 1, 3, 6, 9 and 12 hours following the intravenous injection of each of the hormones. At first it would appear that the nuclear and microsome + supernatant (soluble) fractions concentrated the labelled hormones to a greater extent than did the mitochondria; in reality none of the fractions exhibited a marked ability to concentrate either hormone since comparative dry weights of the three fractions were determined on cardiac tissue from other chickens and the nuclear, mitochondrial and soluble fractions comprised 66.9, 8.9 and 24.2 percent of the dry weight respectively, giving a ratio between dry weight and radioactivity concentrations at one hour post-injection of near unity for each fraction.

From the relative changes in concentrations of the two hormones with time in the three fractions it is evident that the soluble fraction retains the hormones to a slight degree as is evidenced from a concentration at one hour of 24-28 percent to 40-42 percent at 12 hours. Although a statistical analysis of concentration of the two hormones revealed no difference in their concentration by this subcellular fraction, there was a statistical difference ($p < 0.05$) in the concentration at one hour as compared with that at 12 hours.

Concomitant with this increase in hormone concentration in the soluble fraction with time was the decrease in concentration in the nuclei; the percentage at one hour was statistically higher ($p < 0.05$) than the percentage at 12 hours. Again, no difference was observed between the two hormones.

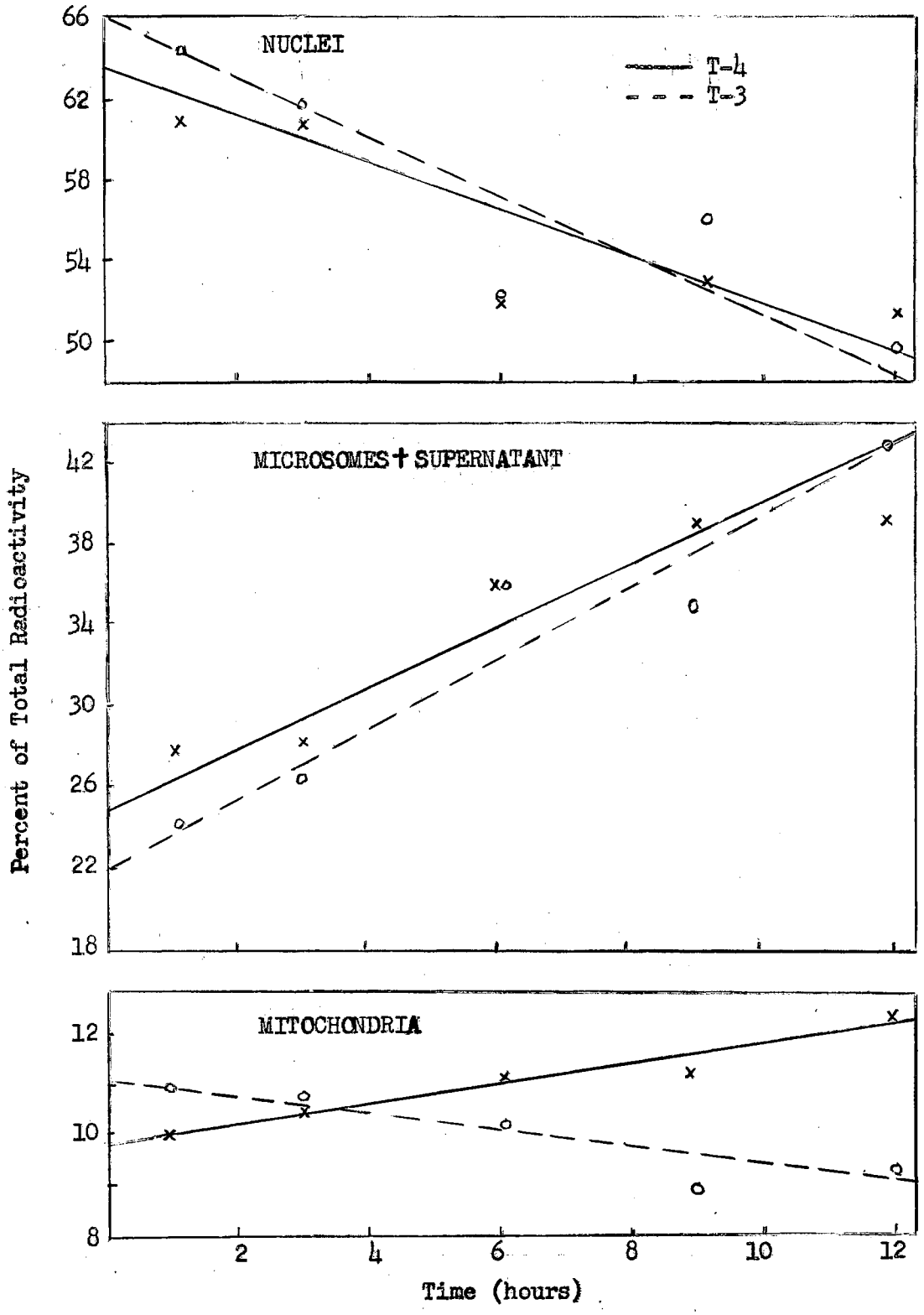

Figure 11. Distribution of Radioactivity in the Subcellular Fractions of Chicken Cardiac Tissue at Various Times Following the Intravenous Injection of I^{131}-Labelled T-4 or T-3 (see Appendix, p. 96-99).

Hormone concentration in the mitochondria showed a different picture. Relative percentages of the two hormones remained the same up to 12 hours as determined statistically but the concentration of T-4 at 12 hours was higher than the concentration of T-3 at this same time interval, the probability of significance being slightly greater than 0.06.

It could be argued that hormone concentration in subcellular fractions prepared by differential centrifugation does not necessarily reveal the concentrations that exist in the intact cell. Indirect evidence for such an idea has been presented by Lipner, et al. (68). They injected rats with radioactive T-4, isolated the "labelled" mitochondria from the liver and added them to a liver homogenate from a non-injected rat. The results showed that only 18.5 percent of the initial radioactivity remained in the "labelled" mitochondria, and the greatest radioactivity was found in the supernatant phase, showing T-4 from one cellular fraction can redistribute itself among other cell constituents. The extent of any redistribution that may have occurred during fractionation of the tissues cannot be ascertained from the present work, but the relative concentrations of the hormones in the fractions as a function of time would be valid inasmuch as identical fractionation procedures were followed for all time intervals studied.

Does the intracellular distribution of T-4 and T-3 represent an artifact produced by disruption of the cell, with a consequent redistribution of hormone from the cell surface into the cell? This does not seem to be the case for several reasons. Taurog, et al. (112) observed T-4 in the bile as a glucuronide conjugate, a product of an intracellular phenomenon. Secondly, Lipner et al. (69) administered C^{14}-carboxyl labelled T-4 and detected $C^{14}O_2$ in the expired air. Since decarboxylation

is an intracellular phenomenon they concluded that the T-4 and T-3 are most readily correlated with the protein content of the fractions and not with surface area. If the latter were true there would be a preponderance of T-4 and T-3 in the soluble fraction where the surface area is greatest and the smallest amount in the nuclear fraction, a condition that was not observed in the present experiments or in the work of others (68).

It is concluded, therefore, that since the relative concentration of hormone contained in the various fractions changed with time, there must be an active process associated with the distribution of T-4 and T-3 in subcellular fractions, a conclusion that has received some support (68, 16, 67).

The utility of the concentration of thyroid hormones by the soluble fraction is not readily apparent, although it may be associated with the higher rate of deiodination by this subcellular component as will be discussed in a later section.

Of interest is the condition that prevails in the mitochondria. It is a well established fact that the mitochondria are concerned with the oxidative processes that occur in the cell, and that a latent period occurs before the maximal effects of the thyroid hormones are produced (60, 48). Newcomer and Barrett (76) in studying the effect of T-4 and T-3 on oxygen uptake by surviving cardiac tissue observed this latent period to be 9-15 hours. In addition it was noted that the increased oxygen uptake following the injection of T-4 did not return to the control level until 36 hours post-injection; that of T-3 returned by 18 hours. This more prolonged response of T-4 could be explained, in part, by the fact that the T-4 was retained in the mitochondria for a longer period

than T-3 as indicated in the present experiments. This may be one factor that contributes to the difference in potencies of the two hormones.

The results obtained in this study on the subcellular localization of T-4 in cardiac tissue are contrary to the reports that have appeared on the distribution of T-4 in rat liver particulates; however, comparative studies with T-3 have not been done (68, 16, 67). In those reports that have appeared only one group of investigators (67) has shown a selective concentration of T-4 in subcellular fractions and this was observed in the mitochondria from five minutes to ten minutes post-injection, after which time the concentrations remained constant up to two hours. This discrepancy could be explained on the basis of the time intervals studied. In the current experiments ten minute time intervals were not studied nor were 12 hour time intervals studied by Lee and Williams (67). Another explanation may involve the type of tissue studied. All tissues are not equally responsive to thyroid hormone stimulation and one might speculate that differences in responsiveness are due to the extent, both qualitative and quantitative, to which the subcellular fractions of the various tissues concentrate the hormones.

When mitochondria from cardiac tissue were incubated in vitro with labelled T-4 and T-3 a response occurred that differed quite markedly from that observed when the hormones were administered to the intact animal (Figure 12). At a time not later than fifteen minutes after adding the hormones to the mitochondrial suspension the uptake by the mitochondria was maximal and a greater amount of T-3 was taken up than occurred for T-4. Two alternatives could account for the observed differences; first, the cell membrane may have limited the amount of hormone that entered the cell in vivo, but if this were the case the mitochondria still

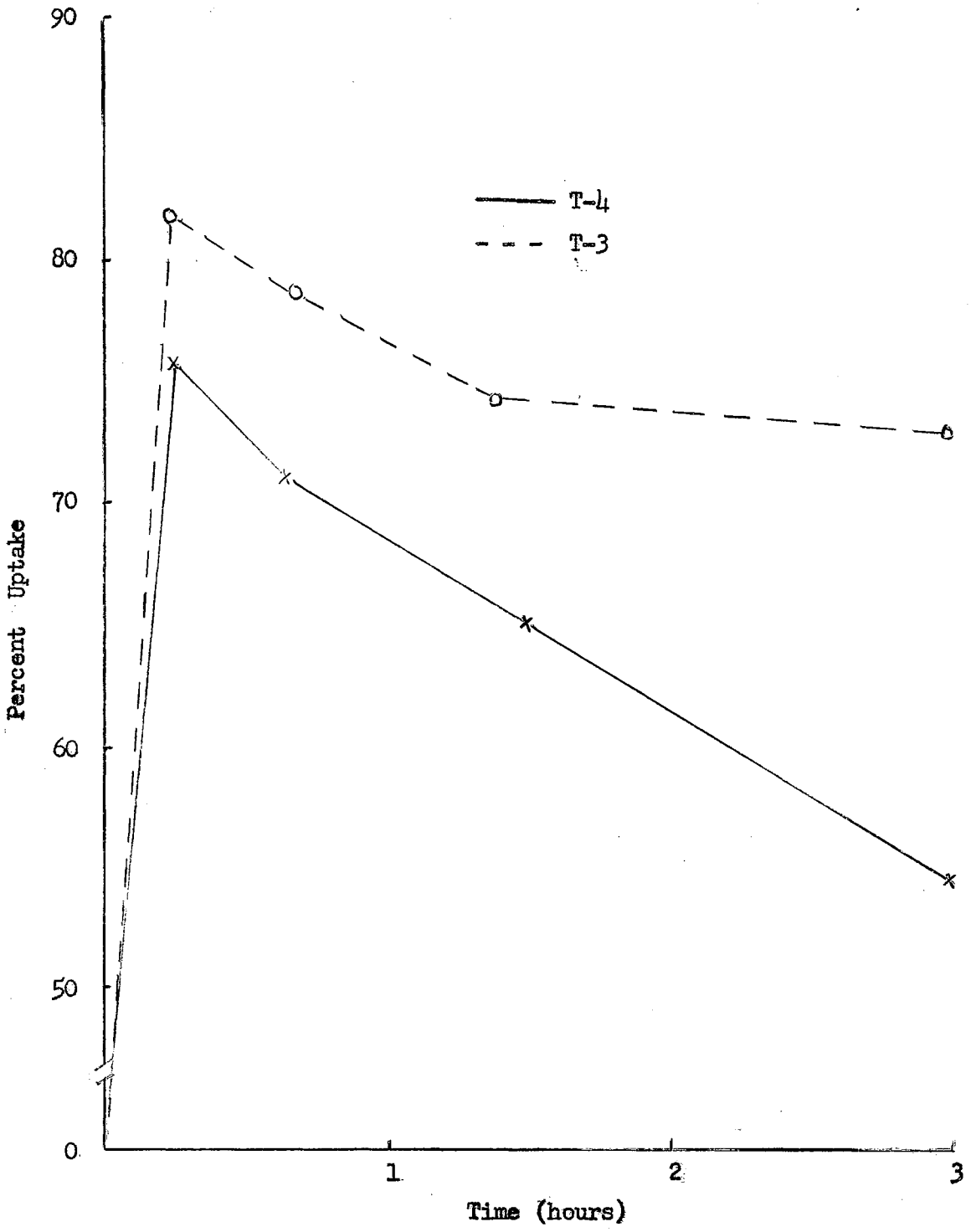

Figure 12. In Vitro Uptake of I^{131}-Labelled T-4 and T-3 by Chicken Heart Mitochondria (see Appendix, p. 100).

would have concentrated a considerable amount of the hormone that did enter the cell. This was not observed. Secondly, the difference in uptake by the mitochondria in vivo as compared to in vitro may be due to simple differences in affinity of the hormones for mitochondrial protein when incubated in vitro and not really associated with any active uptake. This appears to be the most tenable explanation in view of the data reported by others (61, 49). When mitochondria which had taken up T-4 or T-3 from a solution were suspended in a medium containing serum, the T-4 or T-3 moved from the mitochondria into the serum. Mitochondria denatured by heat took up over twice as much T-4 or T-3 as did the intact mitochondria. At an incubation temperature of 0°C., the uptake was maximal in 40 minutes; similar results were obtained at 20°C. incubation temperature. The uptake of these iodo compounds by mitochondria has features in common with the binding of dyes by proteins. Thus the greater uptake of iodo compound by heat-treated mitochondria recalls the increased binding of dyes by denatured proteins. It was suggested that the uptake of thyroid hormones represents a distribution between serum and mitochondrial protein, the greater uptake of T-3 than T-4 reflecting the different relative affinities of these compounds for serum and mitochondrial proteins. Since the mitochondria took up T-4 and T-3 at a very rapid rate (61) it seemed unlikely that the time required for uptake contributed significantly to the latent period observed before the iodo compounds uncouple oxidative phosphorylation (60, 48). Furthermore, since T-3 was taken up by mitochondria to a greater extent than T-4 the lower uncoupling activity of T-3 cannot be explained in terms of uptake. Hoch and Lipmann (49) studied uptake by rat and hamster liver mitochondria and reported 80-90 percent of the T-4 and T-3 taken up by the mitochondria but also found

no correlation between uptake and the metabolic effect of the hormones.

As will be noted from Figure 12 there was a decrease in concentration of T-4 and T-3 by the mitochondria as the time of incubation was increased. This phenomenon is difficult to explain if we accept the findings heretofore mentioned whereby uptake is related only to binding affinity of the hormones to mitochondrial protein. More logically, this could be explained if the mitochondria transform the hormones into compounds which are inactive metabolically and either loosely bound or not bound at all to protein, and thus be free to diffuse out of the mitochondria. However, no significant amounts of any intermediates were identified chromatographically when mitochondria from chick cardiac tissue were incubated for four hours with T-4 or T-3. To compare results obtained from in vivo studies with those conducted in vitro presupposes the same mechanisms are in operation, a supposition that may not be valid. Thus inferences made under such circumstances must be made with a certain degree of hesitancy.

Metabolism of I^{131}-Labelled T-4 and T-3 by Cardiac Tissue

In Vivo Studies

When I^{131}-labelled T-4 and T-3 were injected intravenously into six to sixteen-week old cockerels, iodide was the only radioactive metabolite detected. Figure 13 graphically represents the percentage deiodination of T-4 and T-3 at various times up to nine hours following injection of the hormones. At the end of nine hours approximately 50 percent of the I^{131} label had been removed from the T-3 molecules, while at the same time only approximately 27 percent of the T-4 was deiodinated.

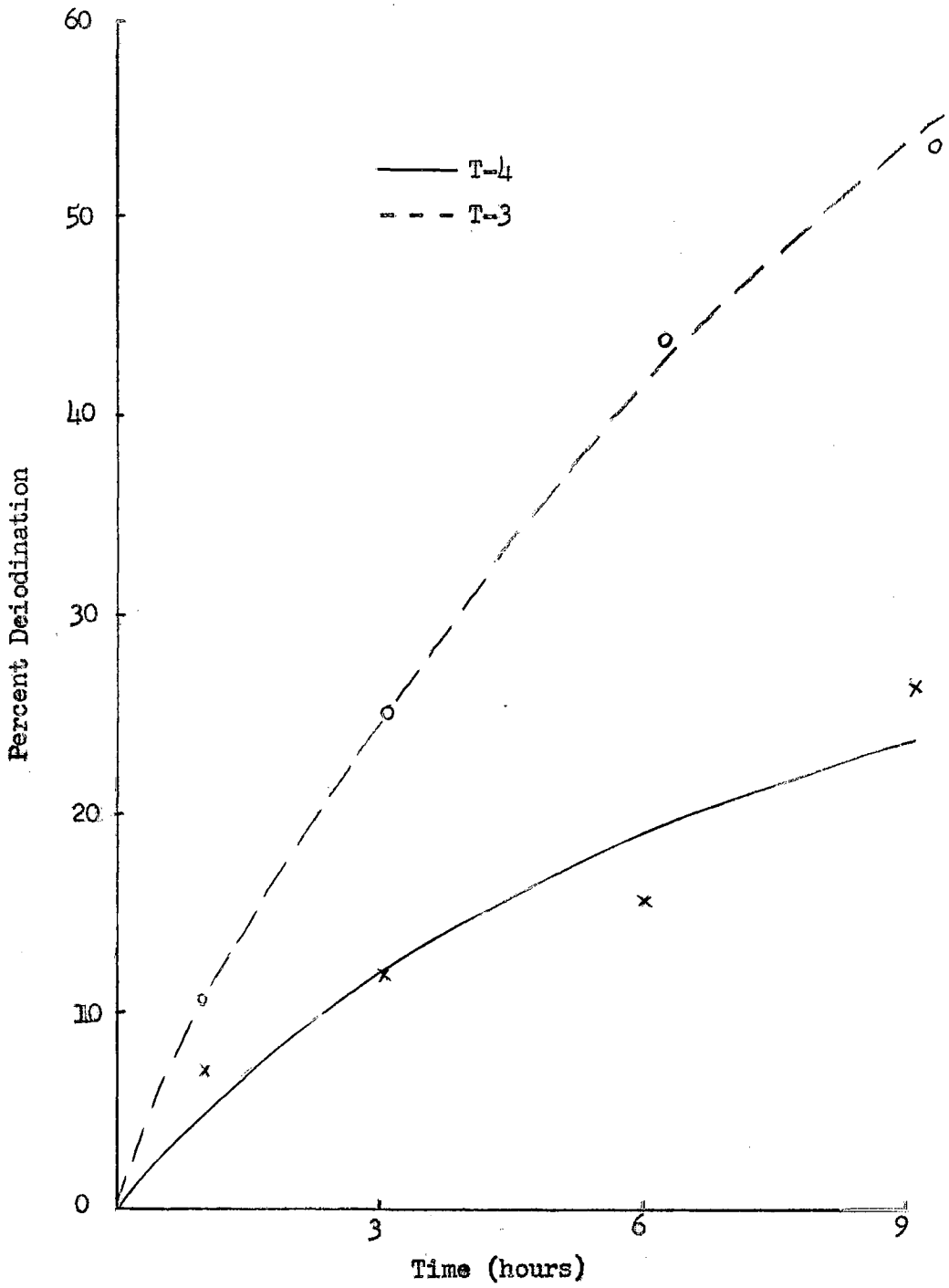

Figure 13. In Vivo Deiodination of I^{131}-Labelled T-4 and T-3 by Chicken Cardiac Tissue at Various Times Following Intravenous Injection into Six-Week Old Cockerels (see Appendix, p. 101).

This difference was statistically significant ($p < 0.005$). A statistical analysis of the differences at one and three hours post-injection indicated both hormones were deiodinated to the same extent.

Although it has been reported by numerous workers that T-3 is produced by various tissues following parenteral administration of T-4 into various laboratory animals (43, 79, 50, 58, 56, 30), others have failed to identify this component as a metabolite (89, 29, 66). When T-3 was injected, the primary metabolite was T-3A, and a small amount of T-2 in kidney and liver from rats (86, 90).

In the few reports that have appeared in the litarature on the in vivo metabolism of thyroid hormones in cardiac tissue, disagreement is encountered on the nature of the metabolites produced. Kallman and Starr (58) injected T-4 into rats and showed that T-3 comprised nine percent of the total redioactivity at five hours post-injection. These authors were unable to separate chromatographically T-4 from inorganic iodide using a tertiary amyl alcohol system, therefore any iodide that may have been liberated would have gone undetected. Moreover, since T-3 and T-4A have similar chromatographic mobilities in this system, the activity they attributed to T-3 may have been T-4A. Ford, et al. (20) prepared ethanol-ammonia extracts of guinea pig cardiac tissue two hours following injection of labelled T-4 or T-3. In the studies with T-4 a majority of the radioactivity was present as iodide but no T-3 was identified. With T-3 only a small amount of iodide was detected and no other radioactive component was visible.

Tata (108) has suggested that the reason for T-3 not being a consistent deiodination product of T-4 incubation in vitro is due to the fact that the deiodinase catalyses the simultaneous removal of the two

iodine atoms in the ortho positions to the hydroxyl group. Stanbury (97) has inferred that rupture of the phenolic ring accompanies deiodination. If either of these reactions occur in the intact animal, this could explain why no T-3 was identified following T-4 administration in the present experiments, and why no T-2 was demonstrated following T-3 administration. Unfortunately, no one has demonstrated whether or not either of the aforementioned reactions occur when T-4 or T-3 are administered to the intact animal.

The fact that T-3 is deiodinated to a greater extent than T-4 in cardiac tissue from cockerels could be another factor to help explain why T-4 is more potent in this species. This same response has been observed in mammalian tissues and has been explained on the basis of a more avid binding of T-4 to plasma proteins, resulting in more T-3 entering the cell and being subjected to the intracellular "deiodinase." If this explanation is true, then the results of the present experiments lend support to the earlier conclusion that T-3 is bound less firmly to chicken plasma proteins than is T-4.

Homogenate Metabolism

Figure 14 shows the results of incubating whole heart homogenates with I^{131}-labelled T-3 and T-4 for various periods up to eight hours. When homogenates were heated to 100°C. for 10 minutes for purposes of control, it was observed that in about 75 percent of the cases the rate at which deiodination of T-4 and T-3 occurred was sharply accelerated as compared to unheated samples. Within four hours deiodination was almost maximal; at the end of eight hours 94.9 percent and 68.5 percent of the T-3 and T-4 respectively was deiodinated. In the unheated samples

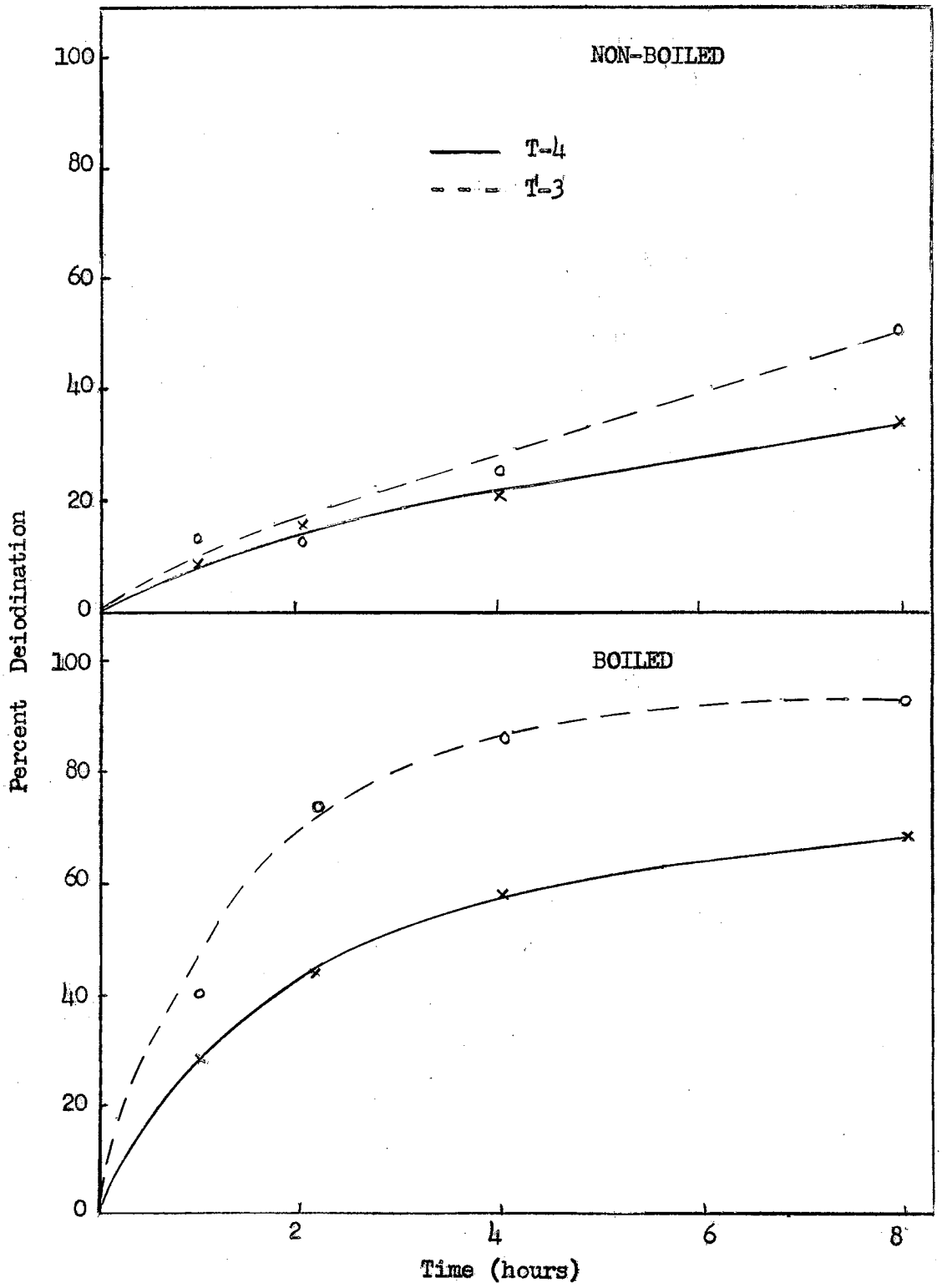

Figure 14. Deiodination of I^{131}-Labelled T-4 and T-3 by Homogenates of Chicken Cardiac Tissue (see Appendix, p.102).

iodide was shown to be the only metabolite in 80 to 90 percent of the determinations. Quantitatively, this amounted to an average value of 30.5 percent for T-4 and 46.5 percent for T-3 at the end of eight hours incubation. In about 10 percent of the determinations, five to ten percent of the radioactivity was found in a component having a chromatographic mobility corresponding to the acetic acid analogues of T-4 or T-3.

Thermal stability of a T-4 deiodinating system previously has been reported. Etling and Barker (25) found that heat-killed liver slices incubated for three days with T-4 deiodinated about as well as unheated samples. Lissitzky, et al. (70) found deiodinating activity in homogenates that had been heated for as long as two hours. Stanbury (97) and Stanbury, et al. (98) observed that microsomes exhibited far greater deiodinating ability when they were pre-heated to 100°C. for several minutes than did the non-heated preparations. Sprott and Maclagan (96) also have observed an augmentation of deiodination of T-4 by heating liver homogenates. In the present experiments it was found that boiling the homogenates for thirty minutes instead of ten minutes resulted in approximately a fifty percent decrease in deiodination; one hour boiling completely inhibited all deiodination. No deaminated products were found in the boiled homogenates indicating the heat lability of this enzyme system.

Mitochondria Metabolism

Mitochondria from chicken cardiac tissue seem to have a very limited ability to deiodinate thyroid hormones (Figure 15). Both hormones are deiodinated at the same rate and reach a value of 7.3 percent of the total

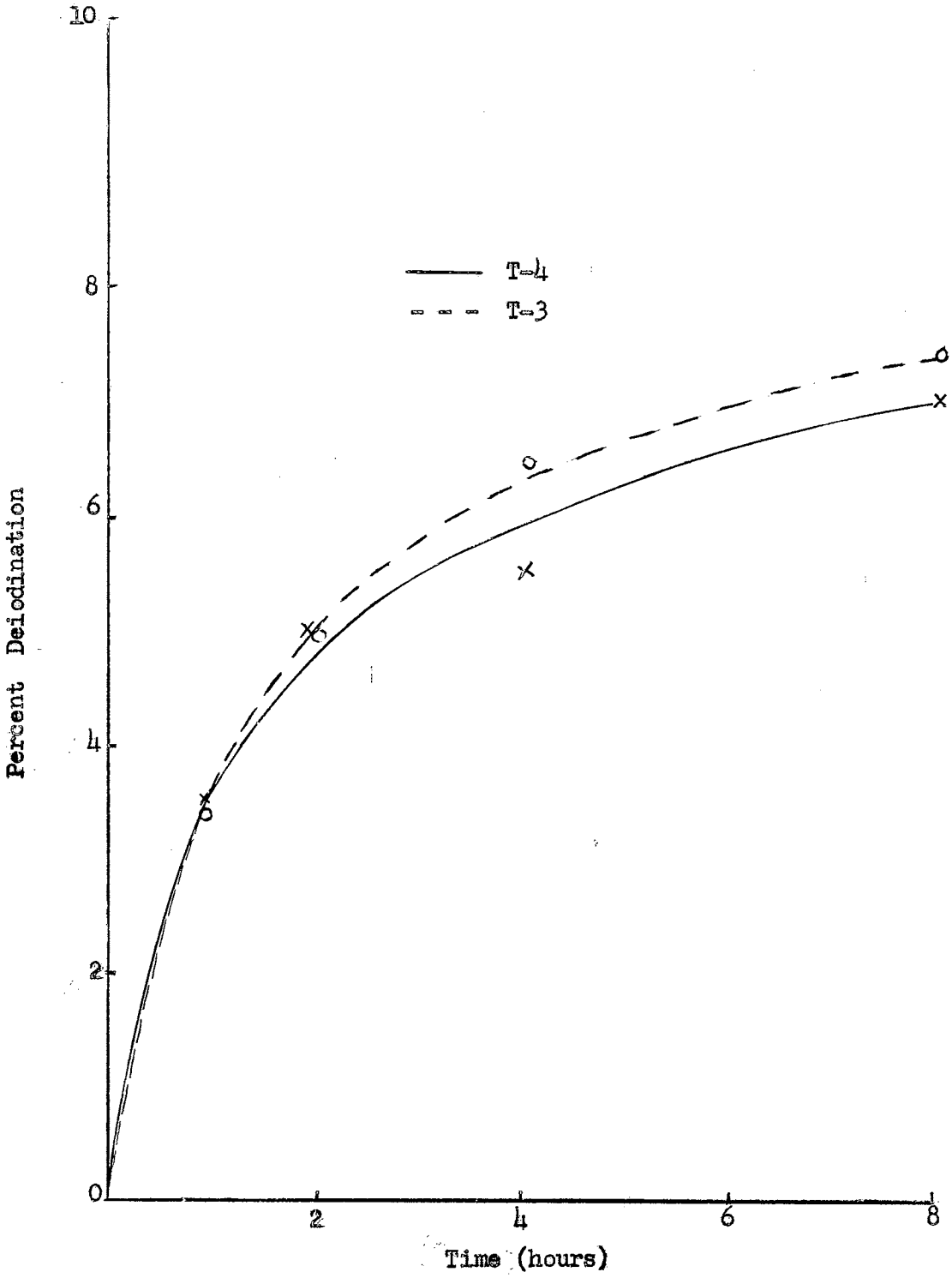

Figure 15. Deiodination of I^{131}-Labelled T-4 and T-3 by Mitochondria from Chicken Cardiac Tissue (see Appendix, p. 103).

radioactivity at the end of eight hours incubation. Tata (104), and Hogness, et al. (50) have shown that mitochondria are the least effective subcellular component in deiodinating capacity, while Yamazaki and Slingerland (120) reported that liver mitochondria deiodinate thyroid hormones as well as any of the subcellular fractions. The values reported here support the two former views.

A noteworthy difference in metabolism was observed in the mitochondria as compared to the other fractions in that in about 75 percent of the studies a metabolite appeared that corresponded to the acetic acid analogues of T-4 and T-3 (Figure 16). Its appearance was not observed until a minimum of four hours incubation time, and in most instances, was not detected in appreciable amounts until after eight hours incubation. In the few samples that were incubated for 12 hours the concentration of this compound continued to increase. The amount of the T-4A or T-3A produced after eight hours incubation ranged from 5.2 to 10.3 percent of the total radioactivity, giving a mean concentration of 7.6 percent. Although no differences were seen in the amount of the analogue produced when either T-4 or T-3 was used as substrate, the analogue of T-3 occurred more frequently than did the T-4 analogue when the parent thyronines were incubated. Whether or not we can interpret this quantitatively to mean that T-3A is produced to a greater extent than T-4A is questionable. When dealing with metabolites appearing in such low concentrations as encountered with the acetic acid analogues, caution is necessary in assigning a physiological role to any slight differences that might show up. Albright, et al. (2) have shown that solubilizing the mitochondria results in an increase in the deaminating activity. Conceivably, this could offer an approach to the question of whether or

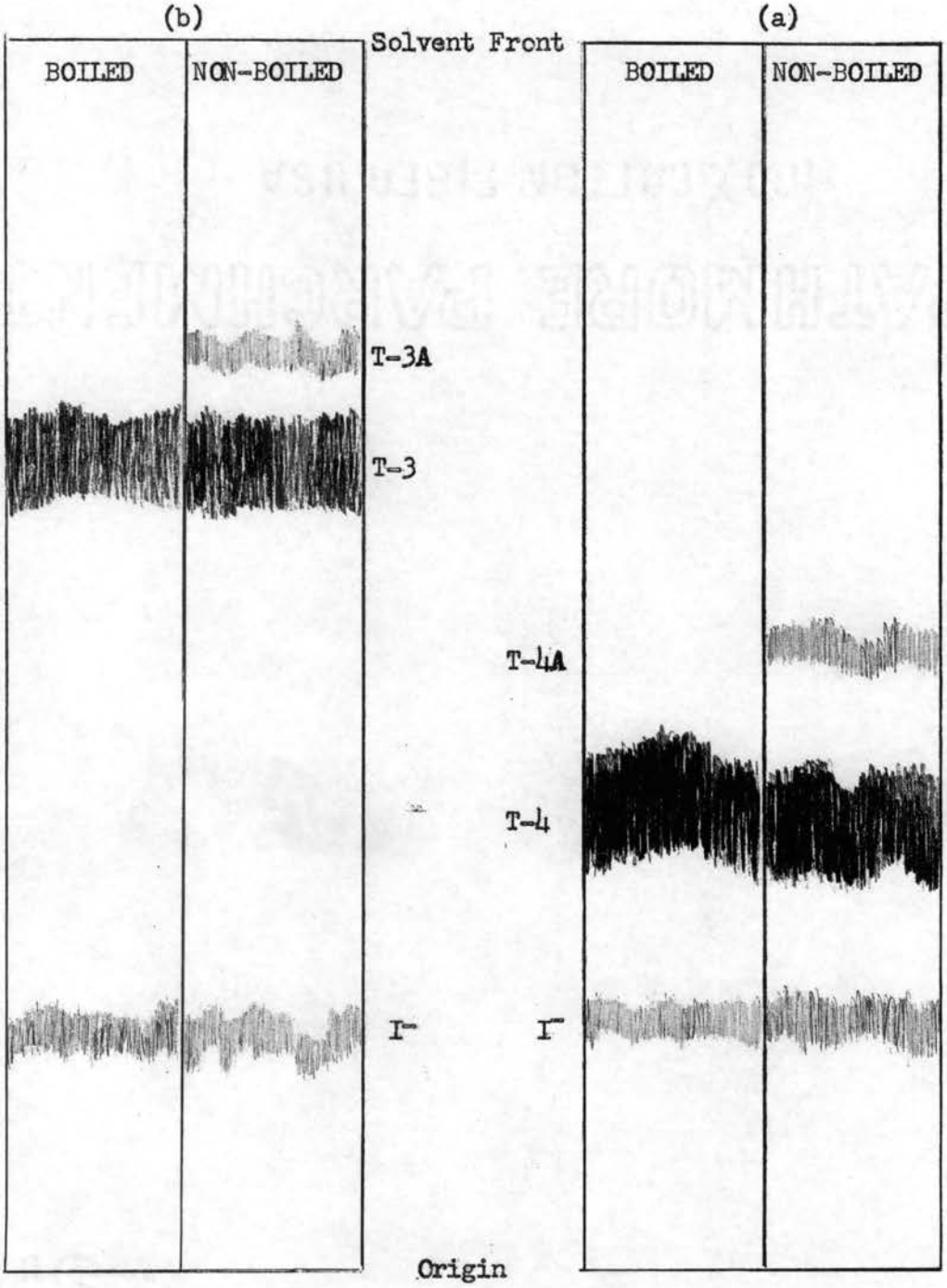

Figure 16. Radioautographs of Chromatograms of Chicken Heart Mitochondria Incubated for Eight Hours with I^{131}-Labelled T-3 (a) or T-4 (b). Solvent: tertiary amyl alcohol saturated with 2N ammonium hydroxide. (See Appendix, p. 104)

not the "deaminase" has a greater affinity for T-3 than T-4 by the fact that increased activity of this enzyme would produce enough of the analogues to enable quantitative comparisons to be made.

The identity of the deaminated products as being the acetic acid analogues is not altogether certain. Both the acetic and propionic acid analogues have almost identical R_f values in the solvents used in this study. An ideal method for identification would be co-crystallization of the radioactive derivative with the known analogues, but this was not possible due to the feeble activity of the compounds on the chromatograms. Two-dimensional chromatography of the mitochondria incubated for eight hours was done to attempt to verify that the components, T-3A or T-4A (Fig.17) were not T-2 or T-3, and to see if the propionic acid derivatives could be separated from the acetic acid derivatives. Figure 17 shows the radioautographs prepared from the chromatograms. The carrier T-3A and T-4A coincided exactly with the spots on the radioautograph; carrier T-4P (tetraiodothyropropionic acid) and T-3P (triiodothyropropionic acid) showed slight separation from the corresponding acetic acid derivatives and were not superimposable on the darkened areas on the film. Tomita (114) has demonstrated the conversion of C-14-labelled 3:5-diiodothyropyruvic acid to 3:5-diiodothyroacetic acid and ". . . it is likely on general grounds that this reaction should occur rather than deamination to a propionic acid, which has no biological parallel." Thus, tentatively, we will assume that the deaminated derivatives are the acetic acid analogues of the parent compounds.

Gross (40) has stated that T-3A can be produced from T-3 during chromatography in a tertiary amyl alcohol system. The danger of attributing any biological significance to such a reaction needs no further

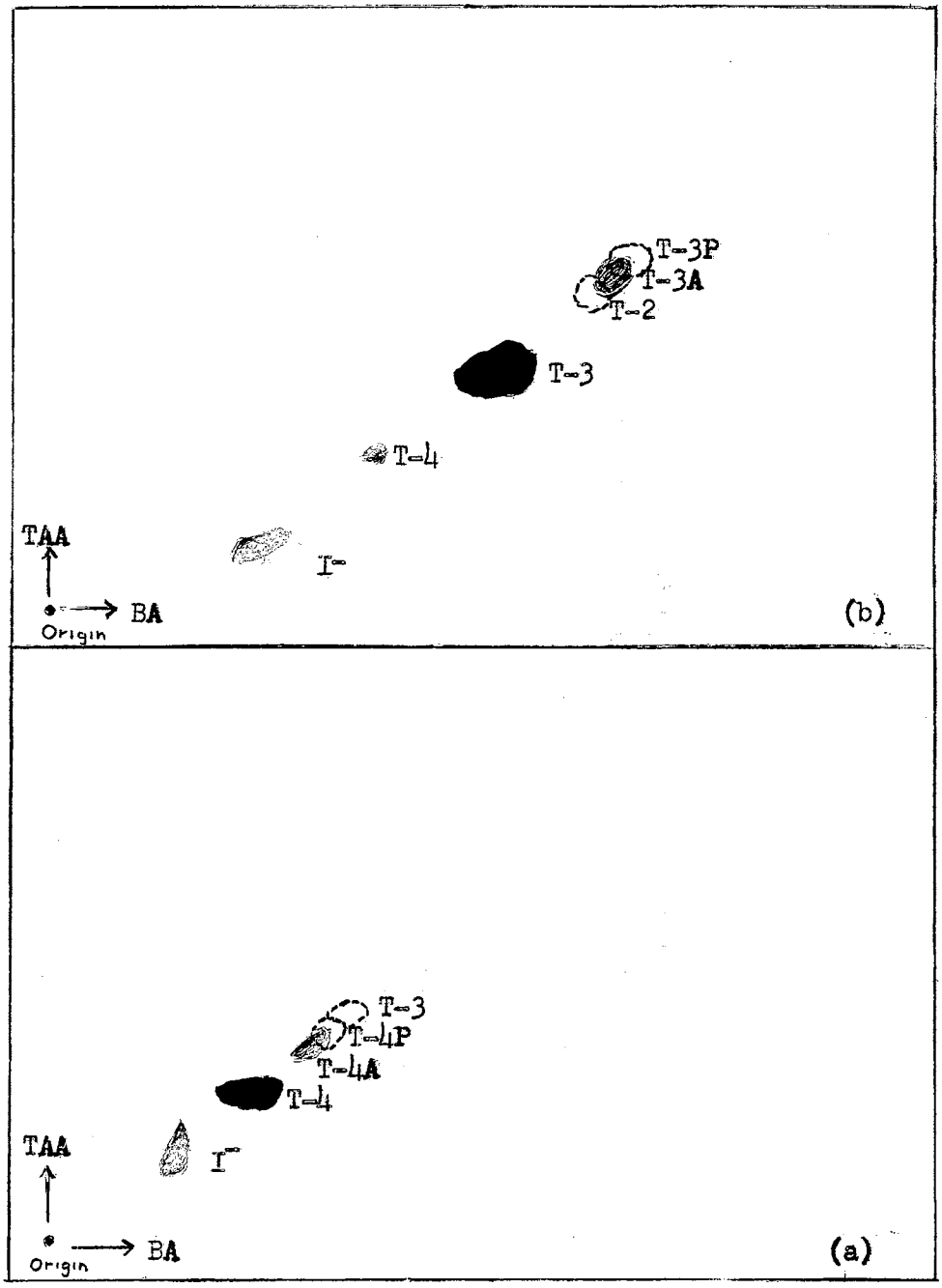

Figure 17. Radioautographs of Two-Dimensional Paper Chromatograms from Chicken Heart Mitochondria Following Eight Hours Incubation with I^{131}-Labelled T-4 (a) or T-3 (b). Solvents: tertiary amyl alcohol saturated with 2N ammonium hydroxide (TAA) and butanol saturated with 2N ammonium hydroxide (BA); ascending, 24 hours.

comment. In the present experiments the deaminated analogues were presumed to be true metabolites since they were not observed in the boiled samples in most instances. Occasionally, small amounts occurred in the boiled samples but there was always less than occurred in the unboiled preparations.

In contrast to the results reported here, Tata, et al. (110) have observed the deaminated analogues of T-4 and T-3 (either the acetic or propionic analogues) produced by brain homogenates to be in greatest concentration after one hour incubation and decreasing with time. They concluded that deamination must occur prior to deiodination. When the homogenates were fractionated into the subcellular components, deamination was found primarily in the mitochondria.

Teleologically, a role for the deaminated analogues may be that of "biological antagonism." In the process of exerting their metabolic effects, one method for inactivating the hormones, in addition to deiodination, may be the production of these analogues which actively inhibit the action of their parent thyronines. Barker, et al. (8) have reported that 3,3'-diiodothyronine and 3,3',5'-triiodothyronine, as well as their propionic acid derivatives returned thyroxine-maintained metabolic rates of thyroidectomized rats to the hypothyroid level.

Microsome and Supernatant Metabolism

Figure 18 depicts the results of incubating I^{131}-labelled T-4 and T-3 with the microsomal (and in some instances microsomes + supernatant) and supernatant fractions from chicken cardiac tissue. The non-particulate fraction had almost no ability to deiodinate either hormone as evidenced by a two to three percent deiodination at the end of eight hours

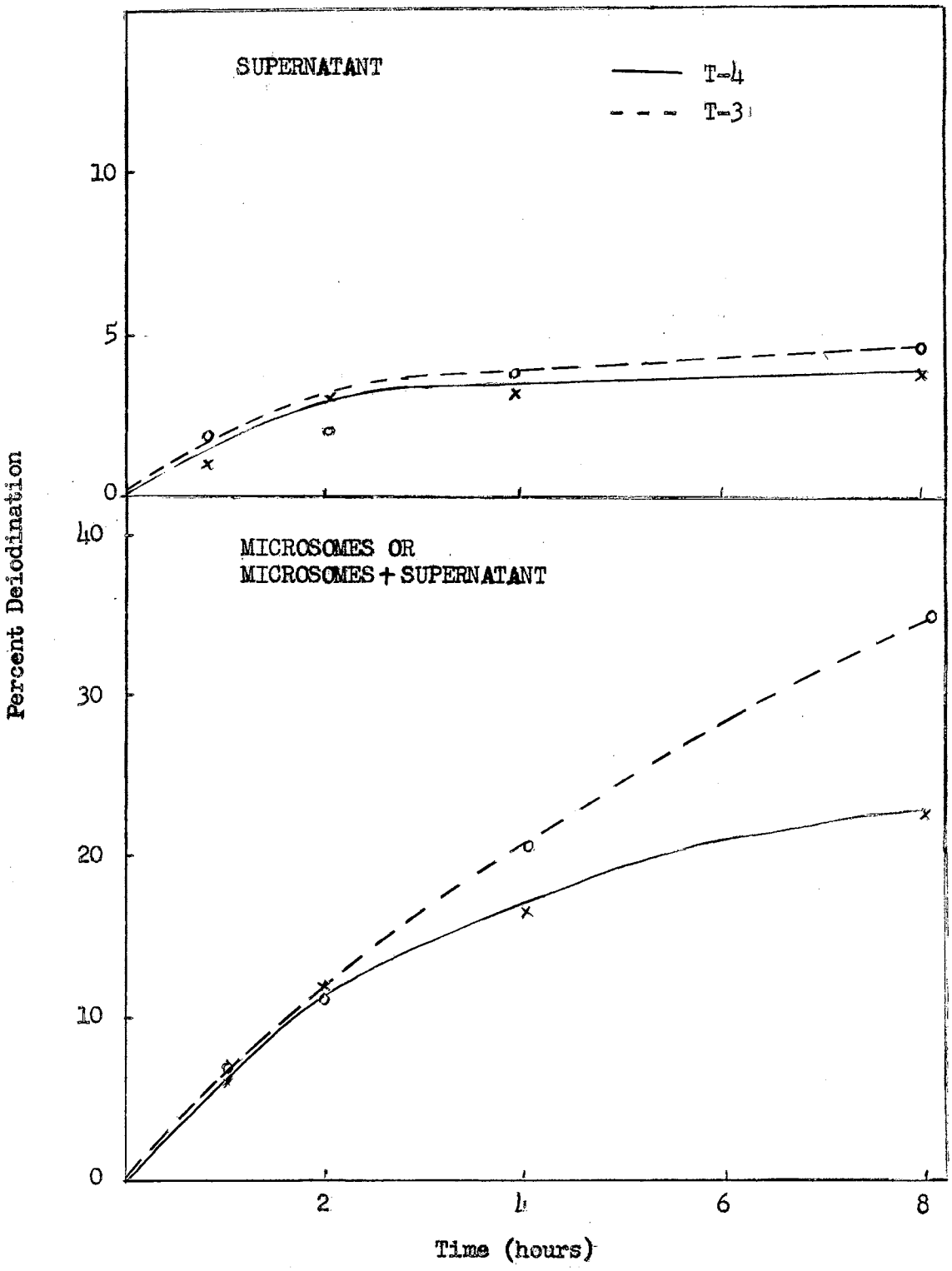

Figure 18. Deiodination of I^{131}-Labelled T-4 and T-3 by the Microsome and/or Supernatant Fractions of Chicken Cardiac Tissue (see Appendix, pp. 105-106).

incubation. No other radioactive component was evidenced in the radioautograms.

Considerable deiodination of the hormones occurred in the presence of the microsomal or microsomal+ supernatant fraction. At the end of eight hours incubation, 22.4 percent of the T-4 radioactivity appeared in the iodide region of the chromatograms, whereas 34.9 percent occurred in this locale when T-3 was used as substrate. No statistical difference in the deiodination of T-4 and T-3 was revealed in the time intervals shorter than eight hours. At the eight hour interval the percent deiodination of T-3 was statistically higher than T-4. Again, no other radioactive component was identified chromatographically. Maclagan and Reid (72) observed that the microsomal fraction of rat liver was the most active fraction in deiodinating T-4. Similar results have been reported by Tata (104) and Stanbury (97).

A correlation is seen in the results of microsomal deiodination reported here and the results of the intracellular distribution of the hormones in cardiac tissue. As was seen, T-3 and T-4 were concentrated in the microsomal + supernatant fraction to a much greater extent than occurred in the mitochondria; deiodination rate also was greatest in the microsomal fraction. Somewhat puzzling is the fact that no differences were observed in the concentrationof T-3 and T-4 by the microsomes, and yet, T-3 was deiodinated to a greater extent than T-4. An adequate explanation for this is not apparent; it may be that the two phenomena are unrelated, i.e., in the time intervals studied, both hormones may be retained in the microsomes at equal concentrations, yet still have different affinities for the "deiodinase."

It was noted that deiodination was augmented when whole homogenates

were boiled for 10 minutes; fractionation of the tissues followed by heating resulted in a loss of this ability. Similar results were reported by Stanbury (97) who noted extensive deiodination of T-4 by preheated liver homogenates. Virtually no deiodination occurred in the heated soluble or microsomal fraction; addition of preheated microsomes to unheated supernatant gave excellent deiodination and became maximal when both fractions were preheated. No explanation was presented to account for this phenomenon.

General Considerations

T-3 is deiodinated to a greater extent than T-4 by chicken cardiac tissue in vivo; this same relationship holds true when homogenates or microsomes are incubated with the hormones. In mammals, T-3 also is deiodinated to a greater extent than T-4 in vivo; the reverse is true when deiodination is studied in isolated tissues. Pitt-Rivers and Tata (80) suggest this difference reflects relative binding abilities of the hormones to plasma proteins. If their explanation is correct, and if T-3 and T-4 have equal affinities for the plasma proteins as has been suggested (109), then both T-4 and T-3 should have identical deiodination rates in vivo. Since, in this study, T-3 was deiodinated to a greater extent than T-4 in vivo, this would reflect a lesser binding ability of T-3 to the plasma proteins.

The observation that in vitro deiodination rates in mammals and chickens appear to be different suggests that chicken "deiodinase" and mammalian "deiodinase" differ. The demonstration of this suggestion must await further purification and characterization of the enzyme(s) involved in this reaction.

CHAPTER V

SUMMARY AND CONCLUSIONS

Six to sixteen-week old cockerels were injected with equimolar amounts of I^{131}-labelled T-4 or T-3 (0.75 - 1.0 ug.). Blood and/or cardiac tissue samples were taken at various time intervals and analysed in order to study the various parameters that may be involved in determining why T-4 has a greater biological potency than T-3 in this species. To extend these studies, cardiac tissue subcellular fractions were incubated with I^{131}-labelled T-4 or T-3 and chromatographed on paper in order to see if any difference exists in the metabolism of T-4 as compared to T-3 in these fractions.

Following the injection of T-4 or T-3, 51.2 and 27.4 percent of the T-4 and T-3 respectively were measured as being protein-bound. These values remained relatively constant up to nine hours post-injection. Confirmation that the hormones were protein-bound was obtained by locating the radioactivity in the albumin fraction of the plasma proteins after they had been separated by paper electrophoresis in veronal buffer. Red blood cell uptake of the hormones revealed T-3 was taken up to a greater extent and at a faster rate than T-4. T-3 uptake increased from a mean uptake of 16.2 percent after 30 minutes incubation, to 45.2 percent after four hours; values for T-4 were 12.7 and 21.1 percent after 30 minutes and four hours incubation time respectively. Dilution of the plasma with saline resulted in an increased uptake of both hormones

by the red cells; the increase was greater for T-3 than T-4. When plasma was replaced by saline, uptake reached a maximum value of approximately 90 percent for both hormones. By replacing the nucleated chicken red cells with non-nucleated dog cells and incubating them with chicken plasma containing the labelled T-4 or T-3, uptake in the case of T-3 was still greater than T-4.

The biological half-lives of T-4 and T-3 in plasma revealed similar values for all time intervals studied. In the time intervals four minutes, one hour, 24 hours, and four days following injection of the hormones the half-lives were 3.2 minutes, 28.6 minutes, 7.9 hours, and 16.4 hours respectively. The half-lives of the hormones in cardiac tissue were 3.9 hours for T-3 and 4.9 hours for T-4.

When cardiac tissue was fractionated at 1, 3, 6, 9 and 12 hours after the intravenous injection of T-4 or T-3, no striking selective concentration was found in the nuclear, mitochondrial or soluble fractions, although the concentration of both compounds in the soluble fraction increased from an average concentration of 25 percent of the total radioactivity at one hour post-injection, to a concentration of approximately 41 percent at 12 hours. The concentration of T-4 in the mitochondria increased from a mean value of 9.9 percent at one hour to 12.5 percent at 12 hours; T-3 concentration decreased from a mean value at one hour of 11.0 percent to 9.4 percent at 12 hours.

Metabolism studies of T-4 and T-3 by cardiac tissue in vivo showed inorganic iodide as the only radioactive metabolite. The amount of iodide produced by this organ nine hours following injection of T-3 or T-4 was 53.5 percent and 27.6 percent respectively. Metabolic studies of T-4 and T-3 by subcellular fractions of cardiac tissue during incubation

times of 1, 2, 4, and 8 hours showed inorganic iodide to be the major metabolite. Boiled homogenates deiodinated 94.9 percent of the T-3 in eight hours, whereas only 68.5 percent of the T-4 was deiodinated. In the unboiled preparations, 46.5 percent and 30.5 percent of the T-3 and T-4 respectively were deiodinated. No significant deiodination was observed in the mitochondrial and supernatant preparations, although an average of 7.6 percent of the total redioactivity was identified as the acetic acid analogues of T-4 and T-3 in approximately 80 percent of the mitochondrial preparations. Microsomal preparations had the greatest ability of all the subcellular fractions to deiodinate the two hormones; 34.9 percent of the T-3 and 22.4 percent of the T-4 were deiodinated in eight hours. Fractionation of the tissue resulted in a loss of deiodinating ability of the hormones by the boiled preparations.

The results of these experiments indicate that (1) thyroid hormones are not bound to chicken plasma proteins with equal intensities, and thus protein binding is not an adequate explanation for the difference in potency of T-4 and T-3 in avian species; (2) the disappearance rates of T-4 and T-3 from the circulation depend on factors not necessarily associated with protein binding; (3) retention of T-4 in the mitochondria for a longer period than T-3, in addition to the shorter half-life of T-3 in cardiac tissue as compared to T-4 may be a factor contributing to the difference in potency of the hormones in this organ; and (4) different rates of deiodination by cardiac tissue in vivo and in vitro could affect the extent to which T-4 and T-3 exert their metabolic effects in this organ.

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A P P E N D I X

R_f VALUES OF VARIOUS IODINATED DERIVATIVES IN TERTIARY AMYL ALCOHOL SATURATED WITH 2N AMMONIUM HYDROXIDE

	T-4	T-4A	T-3	T-3A	I^-
	0.28	0.43	0.47	0.61	0.22
	0.30	0.43	0.43	0.65	0.19
	0.28	0.43	0.49	0.54	0.18
	0.29	0.43	0.45	0.63	0.17
	0.31	0.44	0.43	0.61	0.14
	0.30	0.45	0.44	0.63	0.22
	0.27	0.45	0.47	0.62	0.22
	0.28	0.47	0.45	0.59	0.23
	0.29	0.44	0.46	0.50	0.21
	0.29	0.43	0.42	0.52	0.20
	0.26	0.46	0.45	0.80	0.23
	0.29	0.39	0.59	0.61	0.16
	0.29	0.43	0.55	0.68	0.15
	0.29	0.39	0.48	0.67	0.20
	0.30	0.45	0.36	0.57	
	0.31	0.38	0.65	0.69	
	0.29	0.35	0.52	0.57	
	0.32	0.49	0.47	0.64	
	0.31	0.32	0.51	0.52	
	0.32	0.36	0.58	0.59	
	0.27	0.40	0.54		
	0.26	0.40	0.43		
	0.29	0.38	0.52		
	0.35	0.48	0.49		
	0.25	0.51	0.51		
	0.21		0.49		
	0.21		0.52		
	0.37		0.50		
	0.35		0.50		
	0.25		0.55		
	0.26		0.47		
	0.18		0.53		
	0.19		0.46		
	0.19		0.46		
	0.23		0.50		
	0.33				
Mean	0.28	0.42	0.48	0.61	0.19
S.D.	0.14	0.14	0.18	0.22	0.03

PROTEIN-BOUND IODINE-I^{131}
(Percent of the total I^{131}
in 2.0 ml. plasma)

Time After Injection (Minutes)		T-4	T-3
1		52.8	29.2
		47.0	33.7
		59.7	34.5
		55.2	26.1
		56.8	28.7
		55.8	27.6
		53.6	30.8
		54.4	30.1
	Mean	54.4	30.1
3		43.0	30.8
		46.1	30.0
		49.6	42.2
		48.4	38.2
		49.8	36.7
		50.2	39.2
		50.1	35.8
	Mean	48.1	36.1
6		33.2	8.6
		53.6	10.9
		49.7	28.4
		38.2	28.6
		42.4	27.2
		41.6	8.4
			9.4
	Mean	43.1	17.3
9		57.7	28.4
		62.5	25.3
		64.3	31.6
		59.3	21.6
		56.4	25.1
		53.2	24.1
		60.2	26.8
	Mean	59.1	26.1
Blank (free hormone)		2.3	0.8

IN VITRO UPTAKE OF I^{131}-LABELLED T-4 BY CHICKEN RED BLOOD CELLS
(Percent of the added I^{131})

Determination No.	Incubation Time (hours) 0.5	1.0	1.5	2.0	3.0	4.0
1	7.7*	12.8	11.0	12.0	10.9	16.3
2	11.3	12.3	14.6	17.3	19.0	17.3
3	12.3	13.9	15.2	15.8	19.7	24.5
4	13.4	15.7	15.9	17.2	18.7	22.8
5	18.8	20.8	18.1	20.0	21.0	24.2
Mean	12.7	15.1	15.0	16.5	17.9	21.1
S.D.	4.0	3.2	2.5	2.9	4.0	3.8

IN VITRO UPTAKE OF I^{131}-LABELLED T-3 BY CHICKEN RED BLOOD CELLS
(Percent of the added I^{131})

Determination No.	Incubation Time (hours) 0.5	1.0	1.5	2.0	3.0	4.0
1	15.6*	20.9	25.4	28.4	30.4	34.5
2	18.2	22.5	26.8	31.8	40.4	--
3	19.4	25.4	30.3	35.3	39.7	45.0
4	17.4	19.7	29.1	31.2	40.2	51.1
5	12.8	20.6	30.5	33.2	41.5	59.2
6	14.0	21.3	28.9	33.1	41.8	46.0
Mean	16.2	21.7	28.5	32.1	39.0	45.2
S.D.	6.5	2.0	2.0	2.3	4.3	6.4

*Average of duplicate samples

THE EFFECT OF VARIOUS PLASMA DILUTIONS ON THE UPTAKE OF I^{131}-LABELLED T-4 AND T-3 BY CHICKEN RED BLOOD CELLS

Dilution	T-4		T-3	
	% Uptake	Mean	% Uptake	Mean
None	5.8		19.6	
	7.6	6.7	16.8	18.2
1:1	13.4		27.6	
	13.8	13.6	27.4	27.5
1:5	22.5		49.2	
	24.7	23.8	46.4	47.8
1:10	37.2		63.1	
	35.0	36.1	60.5	61.8
1:20	46.2		72.6	
	42.0	44.1	69.4	71.0
1:100	66.0		79.4	
	65.6	65.8	81.8	80.6
Saline only	87.3		93.4	
	91.3	89.3	92.0	92.7

UPTAKE OF I^{131}-LABELLED T-4 AND T-3 BY DOG RED BLOOD CELLS SUSPENDED IN CHICKEN PLASMA

(Percent of the Total Radioactivity Added)

Substrate	Incubation Time (Hrs.)	Percent Uptake	Mean
T-4	0.5	9.1 7.4	8.2
	1.0	4.9 4.6	4.7
	2.0	5.6 4.8	5.2
	3.0	5.5 5.4	5.4
	4.0	8.3 8.0	8.2
T-3	0.5	5.4 5.8	5.6
	1.0	5.8 5.9	5.9
	2.0	8.7 9.9	9.4
	3.0	11.2 12.2	11.7
	4.0	12.8 18.9	15.9

BIOLOGICAL HALF-LIVES OF I^{131}-LABELLED T-4 AND T-3 IN THE PLASMA OF SIXTEEN-WEEK OLD COCKERELS DURING VARIOUS TIME INTERVALS

Time Interval			Half-Life	
From	To		T-3	T-4
1.0	4.0 min.		1.4	2.7
			5.4	2.3
				3.5
				3.5
4.0		Mean	3.4 min.	3.0 min.
	20.0 min.		23.6	21.5
			28.0	21.4
			36.5	35.0
				28.7
				37.7
		Mean	29.3 min.	28.9 min.
20.0	50.0 min.		27.0	31.5
			29.5	34.0
			25.0	25.0
			30.0	35.0
			25.0	29.5
		Mean	27.3 min.	31.1 min.
1.0	24.0 hrs.		10.8	10.4
			8.1	9.6
			7.7	8.2
			6.0	6.4
			5.6	7.0
			5.0	8.0
			8.0	6.0
			6.2	9.0
				10.2
		Mean	7.2 hrs.	8.3 hrs.
1.0	4.0 days		10.0	20.0
			15.6	19.2
			26.4	14.6
			16.3	15.0
			13.2	16.5
				15.0
				17.4
				14.4
				16.5
		Mean	16.3 hrs.	16.5 hrs.

HALF-LIVES OF I^{131}-LABELLED T-4 AND T-3
IN CHICKEN CARDIAC TISSUE

Determination No.		Half-Life (Hours) T-4	T-3
1		4.8	3.8
2		4.8	4.4
3		6.0	5.0
4		4.4	3.4
5		4.5	3.0
	Mean	4.9	3.9
	S.D.	0.7	0.7

DISTRIBUTION OF I^{131}-LABELLED T-4 IN CHICKEN CARDIAC TISSUE SUBCELLULAR FRACTIONS

(Percent of the Total Radioactivity)

Hours Post-Injection		Subcellular Fraction		
		Nuclei	Mitochondria	Microsomes + Supernatant
1		63.2	11.8	32.8
		69.3	9.1	28.6
		60.3	10.1	28.0
		60.2	11.1	24.3
		66.0	10.5	28.6
		61.3	10.9	25.1
		63.8	10.7	27.3
		57.7	7.0	23.7
		53.7	8.9	30.8
		57.5		
	Mean	61.3	9.9	27.7
	S.D.	4.5	2.1	2.2
3		63.8	8.7	27.0
		60.8	8.8	32.7
		51.3	9.9	29.4
		61.9	7.8	24.9
		66.3	12.6	29.9
		60.2	10.6	25.1
		67.1	10.0	26.1
		64.2	11.6	27.6
		60.4	15.1	34.2
		56.7		
	Mean	61.2	10.6	28.5
	S.D.	5.6	2.1	3.1

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Hours Post-Injection		Subcellular Fraction		
		Nuclei	Mitochondria	Microsomes + Supernatant
6		62.1	8.3	45.6
		36.1	14.3	46.5
		52.9	7.9	45.2
		59.2	8.7	35.7
		56.1	12.4	32.5
		65.2	11.9	39.6
		46.1	9.7	26.9
		51.9	14.4	28.1
		43.1	13.3	29.5
				33.7
	Mean	52.5	11.3	36.3
	S.D.	9.4	3.0	7.6
9		40.5	7.0	40.0
		62.4	9.6	49.5
		42.6	12.0	39.8
		56.6	12.4	46.0
		63.5	11.6	34.7
		61.4	15.0	31.3
		62.0	12.8	30.0
		63.0	11.6	33.5
		49.8	11.8	43.2
		45.2	11.9	44.9
				44.0
	Mean	54.7	11.5	39.7
	S.D.	9.2	3.6	6.4
12		41.4	9.2	42.5
		50.9	12.3	44.9
		56.0	14.3	30.7
		60.1	11.6	30.9
		56.8	10.3	50.9
			16.5	38.7
	Mean	53.0	12.4	39.7
	S.D.	7.6	2.6	8.2

DISTRIBUTION OF I^{131}-LABELLED T-3 IN CHICKEN CARDIAC TISSUE SUBCELLULAR FRACTIONS

(Percent of the Total Radioactivity)

Hours Post-Injection		Subcellular Fraction		
		Nuclei	Mitochondria	Microsomes + Supernatant
1		62.7	10.5	22.1
		58.3	13.2	30.4
		74.4	9.2	17.5
		70.1	10.9	20.7
		62.9	10.1	26.8
		63.2	13.8	27.2
		69.3	12.6	22.3
		63.2	9.9	21.9
		60.3	8.5	24.6
	Mean	64.9	11.0	23.7
	S.D.	5.8	1.6	3.9
3		55.4	10.7	33.1
		53.7	11.8	34.1
		68.9	10.0	23.1
		68.6	9.5	23.9
		67.3	10.5	22.7
		67.9	12.4	22.7
		63.8	11.2	20.0
		60.8	10.2	28.0
		51.3	10.9	29.3
	Mean	62.0	10.8	26.3
	S.D.	6.6	1.7	4.9

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Hours Post-Injection		Subcellular Fraction: Nuclei	Mitochondria	Microsomes + Supernatant
6		59.0	11.9	30.1
		56.5	11.8	37.5
		62.9	12.1	27.8
		42.9	5.9	32.4
		52.0	7.1	31.1
		56.0	10.6	52.2
		62.1	9.6	43.4
		36.1	11.8	38.3
		52.9	10.5	34.7
	Mean	53.3	10.2	36.3
	S.D.	9.1	3.4	6.6
9		46.2	12.7	46.5
		60.1	11.5	36.1
		65.5	8.1	46.5
		62.3	4.2	30.9
		60.7	10.6	28.2
		67.2	2.4	31.7
		40.5	7.6	34.2
		62.4	8.4	29.4
		42.6	10.2	26.9
			9.9	31.2
				35.2
	Mean	56.4	9.1	34.3
	S.D.	9.9	3.2	6.6
12		41.4	9.0	44.2
		50.9	10.7	44.8
		58.4	8.4	32.6
		52.5	8.8	46.5
		51.5	6.9	48.0
		46.9	12.6	49.5
				30.4
	Mean	50.2	9.4	42.3
	S.D.	6.2	2.0	7.0

IN VITRO UPTAKE OF I^{131-} LABELLED T-4 AND T-3 BY MITOCHONDRIA FROM CHICKEN CARDIAC TISSUE
(Percent of the added I^{131})

Incubation Time (minutes)		T-4	T-3
15		78.7	84.4
		78.6	85.0
		72.9	79.5
		73.9	80.7
	Mean	76.1	82.4
45		75.0	83.1
		76.0	83.3
		66.3	76.5
		70.8	76.7
	Mean	72.0	79.9
90		71.6	82.1
		72.8	79.5
		65.4	73.6
		57.5	72.3
	Mean	66.8	76.9
180		63.6	77.2
		65.4	77.4
		43.4	72.0
		51.9	73.7
	Mean	55.9	75.1

IN VIVO DEIODINATION OF I^{131}-LABELLED T-4 AND T-3 AT VARIOUS TIMES FOLLOWING INTRAVENOUS INJECTION OF THE HORMONES

(Percent of the Total Radioactivity Found as Iodide-I^{131})

Hours Post-Injection		Substrate T-4	Substrate T-3
1		5.0	8.3
		10.3	11.2
			12.5
	Mean	7.6	10.7
3		4.2	29.8
		14.3	20.3
		10.5	22.3
		17.5	
	Mean	11.6	24.1
6		7.5	48.6
		18.4	39.0
		22.4	41.8
	Mean	16.1	43.1
9		28.7	59.2
		29.5	49.4
		25.3	52.0
		27.1	
	Mean	27.6	53.5

IN VITRO DEIODINATION OF I^{131}-LABELLED T-4 AND T-3 BY HOMOGENATES OF CHICKEN CARDIAC TISSUE

(Percent of the Total Radioactivity Found as Iodide-I^{131})

Incubation Time (Hrs.)		Boiled Homogenate		Non-Boiled Homogenate	
		T-4	T-3	T-4	T-3
1		31.9	44.1	12.0	10.5
		35.6	27.4	5.5	6.4
		24.9	14.0	0.7	13.1
		28.3	49.6	9.6	2.8
		17.1	70.0	5.2	17.3
		37.6		13.3	12.6
	Mean	29.2	40.2	7.7	10.4
2		56.3	84.9	9.4	8.7
		40.0	63.0	18.6	15.9
		29.4	64.1	21.2	6.0
		50.8	83.3		25.8
			87.5		
	Mean	44.1	76.6	16.4	14.1
4		75.0	93.8	27.2	17.9
		63.6	89.3	12.9	10.9
		63.7	85.0	4.7	25.7
		45.4	91.2	28.2	22.8
		66.1		30.7	48.0
		44.2		27.8	20.0
	Mean	59.7	89.8	21.9	24.2
8		76.4	95.5	34.2	40.2
		75.6	92.1	22.3	24.2
		55.9	93.4	13.3	47.1
		70.2	94.8	36.4	45.9
		52.3	99.0	38.3	54.6
		80.8		38.8	67.1
	Mean	68.5	94.9	30.5	46.5

IN VITRO DEIODINATION OF I^{131}-LABELLED T-4 AND T-3 BY MITOCHONDRIA FROM CHICKEN CARDIAC TISSUE

(Percent of the Total Radioactivity Found as Iodide-I^{131})

Incubation Time (Hrs.)		Boiled Mitochondria		Non-Boiled Mitochondria		Net Deiodination	
		T-4	T-3	T-4	T-3	T-4	T-3
1		1.6	1.4	2.0	5.7	0.4	4.3
		2.0	1.2	4.8	5.8	2.8	4.3
		1.4	2.3	6.2	4.9	4.8	2.6
		1.2	2.1	4.0	5.0	2.8	2.9
		2.1		8.5		6.4	
		1.6		6.5		4.9	
	Mean	1.6	1.8	5.3	5.3	3.7	3.5
2		4.2	3.5	9.6	10.5	5.4	7.0
		3.1	4.8	9.6	7.8	6.5	3.0
		2.7	4.1	3.5	9.1	0.8	5.0
		5.1		14.9		9.8	
		4.7		7.5		2.8	
	Mean	4.0	4.1	9.0	9.1	5.1	5.0
4		3.2	3.6	10.2	6.6	7.0	3.0
		2.7	4.7	11.1	12.0	8.4	8.3
		1.6	4.2	2.1	11.4	0.5	7.2
		4.7	2.2	9.8	10.3	5.1	8.1
		4.5		13.1		8.6	
		1.9		5.4		3.5	
	Mean	3.1	3.7	8.6	10.1	5.5	6.7
8		5.2	4.2	8.6	14.1	3.4	9.9
		4.4	5.6	11.0	15.9	6.6	10.3
		4.8	6.2	7.3	15.2	2.5	9.0
		5.6	5.8	16.7	16.0	11.1	10.2
		6.2	4.2	15.6	14.3	9.4	10.1
		3.2	4.8	10.7	14.2	7.5	9.4
		5.9		14.8		8.9	
	Mean	7.0	5.1	12.1	14.9	7.1	7.5

CONCENTRATION OF T-4A OR T-3A AFTER EIGHT HOURS INCUBATION OF I^{131}-LABELLED T-4 OR T-3 WITH MITOCHONDRIA FROM CHICKEN CARDIAC TISSUE

(Percent of the Total Radioactivity on the Chromatograms)

Determination No.	Percent T-4A	Percent T-3A
1	--*	7.2
2	5.8	9.0
3	7.1	7.3
4	10.3	5.6
5	--	5.2
6	8.0	9.6
7	--	--
Mean	7.8	7.3

*No analogue observed

IN VITRO DEIODINATION OF I^{131}-LABELLED T-4 AND T-3
BY MICROSOMES OR MICROSOMES + SUPERNATANT
FROM CHICKEN CARDIAC TISSUE

(Percent of the Total Radioactivity
Found as Iodide-I^{131})

Incubation Time (Hrs.)		Boiled		Non-Boiled		Net Deiodination	
		T-4	T-3	T-4	T-3	T-4	T-3
1		1.8	3.3	8.0	14.4	6.2	11.1
			1.2		5.3		4.1
	Mean	1.8	2.3	8.0	9.9	6.2	7.6
2		4.2	3.6	14.3	17.0	10.1	13.4
		4.8	4.2	17.2	15.8	12.4	11.6
		3.8	5.2	18.8	15.2	15.0	10.0
	Mean	4.3	4.3	16.7	16.0	12.5	11.6
4		5.3	4.6	18.8	26.4	13.5	21.8
		3.7	4.5	17.3	30.5	13.6	26.0
		6.1	4.8	30.4	20.3	24.3	15.5
			5.6		24.4		18.8
	Mean	5.0	4.9	22.2	25.4	17.1	20.5
8		6.4	5.4	31.8	40.1	25.4	34.7
		5.7	5.1	26.2	42.4	20.5	37.3
		4.8	6.1	26.0	39.5	21.2	33.4
			6.2		40.4		34.2
	Mean	5.6	5.7	28.0	40.6	22.4	34.9

IN VITRO DEIODINATION OF I^{131}-LABELLED T-4 AND T-3
BY THE SUPERNATANT FRACTION FROM
CHICKEN CARDIAC TISSUE
(Percent of the Total Radioactivity
Found as Iodide-I^{131})

Incubation Time (Hrs.)		Boiled		Non-Boiled		Net Deiodination	
		T-4	T-3	T-4	T-3	T-4	T-3
1		1.6	2.5	2.2	3.8	0.6	1.3
		3.2	2.1	4.2	4.8	1.0	2.7
		0.4	1.9	2.7	3.5	2.3	1.6
	Mean	1.7	2.2	3.0	4.0	1.3	1.9
2		3.5	1.7	7.0	4.3	3.5	2.6
		4.2	4.1	7.3	5.8	3.1	1.7
		2.8	1.9	3.9	3.2	1.1	1.3
	Mean	3.5	2.6	6.1	4.3	2.8	1.9
4		4.2	5.3	7.0	9.5	2.8	4.2
		1.6	4.2	4.4	7.0	2.8	2.8
		6.6	3.9	10.4	7.0	3.6	3.1
	Mean	4.1	4.4	7.3	7.8	3.1	3.4
8		5.6	3.9	8.8	8.8	3.2	4.9
		4.6	6.2	7.2	8.8	2.6	2.6
		4.0	5.1	8.1	8.5	4.1	3.4
	Mean	4.7	5.1	8.0	8.7	3.3	3.6

VITA

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