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EFFECT OF IODOCOMPOUNDS, SULFHYDRYL REAGENTS, PROPYLTHIOURACIL AND DISULFIDE HORMONES ON MITOCHONDRIAL VOLUME

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Pharmacology and Therapeutics, Faculty of Medicine of the University of Manitoba

by

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TO JEAN

OUR CHILDREN

AND

FRANCIS A. LEESON

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ABBREVIATIONS

ADP ..... adenosine-5'-diphosphate

AMP ..... adenosine-5'-monophosphate

ATP ..... adenosine-5'-triphosphate

C-factor ..... contraction factor

cyclic-AMP ..... adenosine-3', 5'-cyclic monophosphate

DNP ..... dinitrophenol

EDTA ..... ethylenediamine tetraacetate

FMA ..... fluorescein mercuric acetate

GSH ..... reduced glutathione

GSSG ..... oxidized glutathione

I^+ ..... iodinium ion

IA ..... iodoacetamide

ICN ..... cyanogen iodide

I_2 ..... iodine

NAD ..... nicotinamide adenine dinucleotide

NADH ..... reduced nicotinamide adenine dinucleotide

NEM ..... N-ethylmaleimide

p-HMB ..... p-hydroxymercuribenzoate

PO_4 ..... inorganic phosphate

PTU ..... propylthiouracil

T2 ..... diiodothyronine

T3 ..... triiodothyronine

T4 ..... thyroxine

Tris ..... tris(hydroxymethyl)aminomethane

TSH ..... thyroid stimulating hormone

U-factor ..... uncoupling factor

ABSTRACT

Studies on mitochondrial swelling demonstrated that thyroxine (T4)-induced swelling could be modified by certain compounds. A swelling threshold concentration of N-ethylmaleimide (NEM), 10^{-5}M, inactive by itself in promoting mitochondrial volume changes or on spontaneous mitochondrial volume changes, inhibited T4- but not cyanogen iodide (ICN)-induced mitochondrial swelling. Preincubation of NEM with mitochondria enhanced the NEM inhibition of T4-induced swelling. Conversely, increasing the T4 concentration resulted in a reduction in the magnitude of the inhibition by NEM. NEM, 10^{-5}M, also inhibited Ca^{++}- and inorganic phosphate (PO_4)-induced swelling. In a swelling threshold concentration, $HgCl_2$, 5 x 10^{-8}M, inhibited the T4-induced swelling but not fluorescein mercuric acetate (FMA), iodoacetamide (IA), and p-hydroxymercuribenzoate (p-HMB). NEM may be inhibiting T4-induced swelling as a result of its reaction with sulfhydryl groups and it is possible that the sulfhydryl groups of T4 deiodinase might be a site of action.

FMA was examined for its effect on mitochondrial volume as well as for the effects of iodocompounds on FMA's action. FMA-induced mitochondrial swelling displayed characteristics similar to that induced by sulfhydryl reagents. In the presence of mitochondria, the quenching of FMA's fluorescence precedes the mitochondrial volume changes. T4 enhanced and ICN inhibited the quenching of FMA by mitochondria. CN^-, 5 x 10^{-6}M, was able to inhibit the quenching of FMA's fluorescence and a 10^{-3}M concentration inhibited both the quenching of FMA's fluorescence by mitochondria and FMA-induced swelling. The ICN molecule in the presence of mitochondria is split and CN^- may prevent the fluorescence quenching of FMA. Iodine (I_2), ICN, I^- and T4 enhanced FMA-induced swelling in that order of potency. At 0°C, FMA-induced swelling was reduced and I_2, ICN and I^-, but not T4, retained their ability to enhance FMA's action. NEM-induced swelling was also enhanced by I_2 and ICN, but T4 and I^- were ineffective.

The mitochondrial swelling induced by FMA and the quenching of the fluorescence of FMA is probably a result of its reaction with mitochondrial sulfhydryl groups. Enhancement of FMA-induced mitochondrial swelling may be due to an increased reactivity of sulfhydryl groups in the presence of iodocompounds.

Propylthiouracil (PTU), by itself, promoted mitochondrial volume changes which were inhibited by CN^- and dinitrophenol (DNP). Adenosine-5'-triphosphate (ATP) reversed the swelling effects of PTU. PTU, *in vitro*, was unable to inhibit the T4-induced swelling. However, mitochondria isolated from PTU treated rats displayed a reduced swelling response to T4 compared to that of normal, but not to ICN. The *in vivo* administration of PTU, acutely or chronically, reduced the T4-induced swelling response. Since T4-induced mitochondrial swelling was reduced and ICN-induced was not reduced, it may be that PTU is interfering with T4 deiodinase activity.

Furthermore, mitochondria isolated from a thyroidectomized rat also displayed a reduced sensitivity to T4, but the effect of ICN was not reduced. This may also be due to a reduction of T4 deiodinase activity known to occur in thyroidectomized animals.

The disulfide hormones, vasopressin, oxytocin and insulin, modified the T4-induced swelling response. The highest concentration of vasopressin did not modify the T4-induced swelling, whereas the lower concentrations employed enhanced it. On the other hand, oxytocin and insulin in the higher concentration enhanced the T4 response and the lower concentrations were not as effective. In the presence of insulin or vasopressin, 10^{-6}M, T4 concentrations of 2.5×10^{-6} and 5×10^{-6}M displayed the greatest enhancement of their swelling curves. It may be that this interaction of the disulfide hormones with T4 may be occurring at the level of the sulfhydryl groups of the mitochondrial membrane.

The T4-induced swelling by rat liver mitochondria was reduced by

(a) NEM, 10^{-5}M, (b) PTU administration and (c) thyroidectomy, whereas the mitochondrial swelling in response to ICN was not modified. These results indicate that T4-induced swelling may be associated with T4 deiodinase activity and are consistent with the concept that the iodinium ion (I^+) is responsible for the action of T4. The T4-induced swelling response is able to be modified by compounds which can enter into reactions with sulfhydryl groups such as NEM, $HgCl_2$ and the disulfide hormones. In addition, the FMA- and NEM-induced swelling is enhanced by iodocompounds. Therefore, the mitochondrial sulfhydryl groups may be the level at which the action occurs.

STATEMENT OF PROBLEM

Vasopressin and oxytocin, hormones of the posterior pituitary gland, are octapeptides containing a disulfide group. In this laboratory, it has been shown that both vasopressin and oxytocin release the thyroid stimulating hormone (TSH) from the anterior pituitary gland *in vivo* and *in vitro* (LaBella, 1964a). Furthermore, thyroid hormone, T4, in low concentrations releases TSH and, in addition, modifies its release by vasopressin and oxytocin (LaBella, 1964b). In recent years, evidence has accumulated which indicates that the disulfide hormones, vasopressin, oxytocin and insulin (Ussing and Zerahan, 1951; Levine and Goldstein, 1955; Rasmussen *et al.* 1960), as well as T4 (Lehninger, 1962a), act on membranes to modify their properties. In order to obtain some knowledge of the manner by which these hormones exert their effects and interact to modify membrane properties, one might study a model membrane system. Lehninger and Neubert (1961) had suggested that the mitochondrion might be a useful model membrane system in the study of drugs and hormones acting on membranes.

A number of reasons prompted the selection of the mitochondrion. Mitochondria are readily obtained by the utilization of cell fractionation techniques and undergo volume changes in response to tonicity changes in the suspending medium, as well as in response to specific compounds. These volume changes can be monitored by light absorption techniques. Moreover, it is known that T4 (Klemperer, 1955; Tapley, 1956; Lehninger *et al.* 1959), as well as vasopressin and oxytocin (Lehninger and Neubert, 1961), cause marked increases in mitochondrial volume. Other factors which support such use of the mitochondrial membrane are the similarities that membranes of cells have in common. Cell membranes appear to have the same general composition, consisting of approximately 40% protein and 60% lipid, and similar permeability characteristics. The penetration of these membranes by solutes in a given series is proportional to their oil-water partition coefficients (Davson and Danielli, 1952). This permeability

relationship has been demonstrated for the mitochondrion also (Watanabe and Williams, 1953; Tedeschi and Harris, 1955).

The thyroid hormones have many biological effects, and, in the course of exerting certain of these effects, T4 deiodination might be required (Galton and Ingbar, 1962). Furthermore, it has been suggested that the formation of the I^+ might be responsible for hormonal effects of T4 (Galton and Ingbar, 1962; Roche et al. 1962). To test these theories, the effects on mitochondrial swelling of a number of experimental conditions and specific compounds, such as ICN which dissociates to a I^+, was compared to T4.

Since sulfhydryl groups were suggested to be involved in T4-induced swelling (Lehninger and Schneider, 1959), the effects of compounds which show a marked specificity for reacting with sulfhydryl groups (sulfhydryl reagents) were investigated. FMA was utilized because it is a sulfhydryl reagent, whose native fluorescence is quenched by sulfhydryl compounds.

PTU was examined for possible modification of the mitochondrial swelling response of T4, as well as for its effect on mitochondria, because the thiouracil derivatives have been shown to interfere with the peripheral deiodination of thyroid hormones (Greer et al. 1964).

In addition, observations in this laboratory have indicated that T4 and the disulfide hormones interact to modify the release of TSH (LaBella, 1964b). Experiments were performed to study the possibility of such an interaction on the mitochondrial membrane, in vitro.

INTRODUCTION

MITOCHONDRIAL CHARACTERISTICS

Mitochondria are cytoplasmic organelles of animals, plants, protozoans, and have also been reported in bacteria, but the latter reports have been questioned (see Novikoff, 1961, for discussion of mitochondria in bacteria). Numerous names have been assigned to these organelles (Cowdry, 1918); however, the term mitochondria (Gr. mitos, thread; chondros, granule or grain) is now generally accepted.

These organelles are characterized by their distinctive biochemical and morphological features. Mitochondria contain the associated enzyme systems of electron transport and oxidative phosphorylation, as well as specific enzymes, such as succinic dehydrogenase. Among the important biochemical properties of mitochondria are their tinctorial reactions with compounds which permit their identification with the light microscope. Crystal violet, acid fuchsin and iron hematoxylin have a long history of use as mitochondrial stains, and three additional stains whose chemical reactions with mitochondrial constituents have been intensively studied and elaborated are the G-Nadi staining reaction, Janus Green B and the tetrazolium salts. The G-Nadi reaction probably demonstrates the presence of cytochrome oxidase through a reaction in which dimethylphenylenediamine is oxidized to produce indophenol blue in the presence of a-naphthol (Novikoff, 1961). Janus Green B was introduced by Michaelis (1900) and its mechanism of staining has been intensively investigated by Lazarow and Cooperstein (1953). It reacts with flavoprotein enzymes which reduce the dye to a leuco-form. Flavoprotein enzymes are also located in other subcellular structures but it is in mitochondria, which contain a large amount of cytochromes, that the leuco-form of Janus Green B is oxidized to a blue derivative. The tetrazolium salts are also reduced by flavoprotein dehydrogenases and the reduced form is blue (Novikoff, 1961). Morphological detail of mitochondria can be viewed in electron micrographs, and Novikoff (1961)

presents a strict morphological definition of mitochondria as cellular organelles, which exhibit, by electron microscopy, a smooth outer membrane and an infolded inner membrane when fixed in osmium tetroxide.

Mitochondrial shape is variable *in situ*. Generally, it is either filamentous or granular. It may be vesicular with a central clear zone, and in certain cells shows enlargement at one end to resemble a club. The enlarged end of this club-like mitochondrion may exhibit a central clear zone (De Robertis *et al*. 1963). Cycles of variation in mitochondrial shape have been reported (Noël, 1923).

Rat liver mitochondria are generally 0.3 μ in width and 0.5 to 1.0 μ in length. Mitochondrial counts of rat liver cells give a mean of 800 per cell with a range of 500 to 2500 per cell and comprise 18.6% of the cell's volume and 15-20% of the cell's nitrogen (Lehninger, 1964).

IMPORTANT EVENTS OF EARLY MITOCHONDRIAL RESEARCH

Cytologists between 1850 and the beginning of this century described many cytoplasmic inclusions. During this period, a variety of histological fixative procedures were introduced which did not yield reproducible results. Owing to these inadequate techniques, many artifacts no doubt received proper names. Kölliker, an important pioneer in cytology, began his research on cytoplasmic particles around 1850 and probably performed the first volume-change experiments with mitochondria (Kölliker, 1888). He observed that particles of insect muscle sarcoplasm increased in volume in a hypotonic environment. Retzius (1890) named these particles sarcosomes (muscle mitochondria). Benda (1897-1898) introduced the term mitochondria to describe the "fadenkornern", i.e. thread granules, observed in cells during spermatogenesis.

An important event in mitochondrial research was the introduction of the supravital stain, Janus Green B, by Michaelis (1900). Not only did

this compound generate interest in the chemistry of mitochondria, but it was instrumental in negating the opinion that mitochondria were fixative artifacts. Later, their chemical nature began to be elaborated when Regaud (1908) identified phospholipid and protein in mitochondria. Furthermore, Warburg (1913) isolated cellular particles and demonstrated their involvement in oxygen uptake. Apparently, he was first in the application of centrifugation to segregate cell components and to study the biochemical activity of cellular particles. This represents an early study of cellular respiration, presumably involving a mitochondrial fraction.

Mitochondrial research received a major impetus with the introduction of improved techniques to isolate cellular components. In 1934, Bensley and Hoerr isolated mitochondria from homogenized guinea pig liver by differential centrifugation of brei. Later, Claude (1943) used refined differential centrifugation techniques and obtained cell fractions which he called large and small granules. Both Bensley and Hoerr and Claude employed saline media in their isolation procedures. The small granules of Claude were sub-microscopic and he named them microsomes, which today are regarded as derivatives of the endoplasmic reticulum. Claude's large granule fraction contained secretory granules and mitochondria (Claude, 1943, 1946). Hogeboom et al. (1948) utilized sucrose as a medium for isolating subcellular components and were able to segregate nuclei, microsomes and mitochondria from each other by differential centrifugation. Furthermore, liver mitochondria isolated in 0.88 M sucrose retained their characteristic elongated form as seen in intact cells. Moreover, these isolated mitochondria displayed the same reactivity to the specific stains, e.g. Janus Green, as they do in the intact cell.

Finally, the mitochondrial ultrastructure was first described in the electron microscopy studies of Palade (1952) and S'jostrand and Rhodin

(1953).

EARLY THEORIES OF THE PHYSIOLOGICAL ROLE OF MITOCHONDRIA

A number of physiological roles have been assigned to mitochondria in the early period of research. These were discarded and replaced by the modern concept that mitochondria are the sites of respiration and oxidative phosphorylation. It will suffice to mention briefly the early theories without giving detailed accounts of the experimental evidence cited in formulating or refuting them.

An early negative concept concerning mitochondria held these organelles were artifactual. This represented an extension of the concept that cytoplasmic structures seen by light microscopy were mere artifacts of preparation. Altmann (1890) described cytoplasmic granules, which were identified with mitochondria, as being micro-organisms imbedded in a ground substance. This idea apparently received little support. The theory concerning the physiological role of mitochondria which dominated early mitochondrial research was that advanced by Benda (1902) in his conclusion that mitochondria were permanent cell organelles. He suggested that they were important in heredity and during histogenesis differentiated into other cellular structures.

Subsequent theories were concerned mainly with biochemical functions of mitochondria. According to the Electosome theory of Regaud (1909), mitochondria can take material from the surrounding cytoplasm and transform it into diverse products. Furthermore, Kingsbury (1912) suggested that mitochondria were cell structures associated with reducing substances concerned in cell respiration. Another theory which indicated a biochemical function was "the surface film theory" (Cowdry, 1926). These theories were not supported by experimental facts and either were discarded or incorporated into modern concepts, i.e. that of Kingsbury.

DESCRIPTION OF MITOCHONDRIAL ULTRASTRUCTURE

The introduction of improved tissue preparation techniques, such as fixation, embedding and sectioning, in electron microscopy permitted the demonstration of the ultrastructure of mitochondria. Palade (1952) and Sjostrand and Rhodin (1953) first described the ultrastructure of the mitochondrion. The dimensions of the ultrastructures are below the resolution of the light microscope. Mitochondria are surrounded by a double membrane system of which the outer one is smooth and approximately 60 Å in width and an infolded inner membrane which also approximates 60 Å in width. The infoldings of the inner membrane are called cristae mitochondriales or cristae (Palade, 1952). These two membranes are separated by an electron transparent space of 60-80 Å. Consequently, two chambers exist within mitochondria, one between the outer and inner membranes and the other within the limits of the internal membrane. The inner chamber is continuous, at least in liver mitochondria, with the cristae jutting into it. Filling this internal chamber is a material showing considerable fluidity, the mitochondrial matrix.

MITOCHONDRIAL MEMBRANE

Indirect evidence for the existence of a mitochondrial membrane has been gathered in a number of ways. Initially, osmolarity studies indicated the presence of a semipermeable membrane. This type of study was probably first performed by Kölliker (1888) who observed that sarcosomes of insect muscle had swollen when placed in water. In 1914, Lewis and Lewis demonstrated that mitochondria of intact tissue culture cells were responsive to osmotic pressure changes, and Harris (1943) also observed that cytoplasmic particles behaved as osmometers in the intact cell. Later, Claude (1946) demonstrated that isolated mitochondria took up water in response to hypotonic conditions. Hogeboom et al. (1948) reported

similar observations. Furthermore, a change in the environmental conditions of mitochondria caused marked changes in their permeability, as demonstrated by Lehninger (1951). He showed that there was an increase in mitochondrial permeability to reduced nicotinamide adenine nucleotide (NADH) after a brief exposure to water. Comparing the concentration of substances within the mitochondria with that of the surrounding cytoplasm, and following their release from mitochondria exposed to adverse conditions, is another line of evidence in favor of the existence of a mitochondrial membrane. Mitochondria contain a high content of soluble enzymes (Kielly and Kielly, 1951), and ions were reported to be in greater concentration within mitochondria than in the surrounding fluid (MacFarlane and Spencer, 1953; Bartley and Davis, 1954). In addition, disruption of mitochondria caused a loss of small molecules (Schneider, 1953), and sonic vibrations resulting in mitochondrial disintegration released 60% of the original nitrogen in soluble form, most of it as protein nitrogen (Hogeboom and Schneider, 1950). Moreover, studies on the differential permability of mitochondria with molecules of different sizes supported this concept of the mitochondrial membrane's existence. Werkheiser and Bartley (1957) found that Na^+ and K^+ penetrated mitochondria, whereas high molecular weight compounds, such as nucleotides or polyglucose, would not. Finally, the quantitative demonstration that mitochondria behaved as osmometers was strong proof for the evidence of a membrane. Tedeschi and Harris (1955) demonstrated that mitochondria followed the Boyle-van't Hoff law and behaved as osmometers if a dead space of 40% was assumed. Thus, indirect experimental evidence and the direct graphic evidence of electron micrographs of mitochondria by Palade (1952) and Sjostrand and Rhodin (1953), demonstrated, conclusively, the existence of a mitochondrial membrane.

It seems to be a common assumption among cytologists that the

membrane systems of subcellular particles are similar to the cell membrane. This assumption has some experimental basis since a number of similarities between the cell and mitochondrial membranes can be cited. The chemical composition of both systems is similar, as well as the lipid-protein arrangement, and comparable physical dimensions are shared, in common. Electron micrographs of both membranes reveal that they exist as two electron dense lines between which is a less electron dense central zone (Palade, 1952). Furthermore, electron micrographs of high resolution have revealed that an individual membrane of the mitochondrion displays two electron dense outer lines and a less dense middle line. Its construction is in harmony with the "unit membrane" concept proposed by Robertson (1959). The "unit membrane" is considered as the fundamental structure of all membranes and possesses a total width of 75 Å and in an electron micrograph is composed of two outer electron dense lines of 25 Å and a lighter middle of 25 Å. Cell membranes of various tissues, as well as the membranes of subcellular structures, such as nuclei and mitochondria, exhibit this construction pattern. Electron micrographs of a "unit membrane" may reflect the lipid-protein bimolecular layers according to the membrane structure proposed by Danielli (in Davson and Danielli, 1952).

FUNCTION OF MITOCHONDRIA

Within the preceding two decades, a voluminous literature has accumulated indicating that each of the subcellular particles, such as nuclei, mitochondria, and lysosomes, is endowed with a special chemical composition and function. Subsequent to the introduction of cell fractionation techniques, interest in the function of mitochondria intensified and an important mitochondrial function was established, i.e. they are the site of the chain of enzymes concerned in electron transport and oxidative phosphorylation. According to Lehninger (1961), the outstanding

properties of mitochondria are catalysis of respiration and oxidative phosphorylation, the occurrence of reversible mitochondrial swelling and contraction associated with respiration, and the transport of ions both into and out of mitochondria related to respiration. Moreover, Lindberg and Ernster (1954) point out the enzymatic processes of importance in mitochondrial function and subdivides them into the energy-generating oxidations of the Krebs cycle and the mechanisms through which energy is conserved in the form of high energy bonds, energy transferring processes which result in ATP formation, and enzymes concerned in energy utilization processes.

Cellular respiration is concerned with the oxidative metabolism of fuel molecules associated with energy release through enzymatic reactions and transference of electrons to oxygen. The enzyme system catalyzing the transfer of electrons from substrates to oxygen is called the respiratory chain. At 3 points of the chain, adenosine-5'-diphosphate (ADP) is enzymatically phosphorylated to ATP, thus conserving the liberated energy and this process is termed oxidative phosphorylation. It is expressed in the experimental situation as the P:O ratio, which is the atoms of PO_4 esterified to the number of oxygen utilized. Depending on the point of entry of substrate electrons into the respiratory chain, the ratio number will ideally be 3, 2 or 1. A substance which lowers this ratio is classified as an uncoupler of oxidative phosphorylation. The rate of respiration is dependent on the concentration of substrates, oxygen, ADP and inorganic PO_4, and ATP. The influence of ADP on respiratory rates was first studied by Lardy and Wellman (1952). They introduced the concept of respiratory control and demonstrated that maximum respiratory rates depend on the presence of a PO_4 acceptor, such as ADP. Chance and Williams (1956) made a detailed study of the factors influencing the respiratory rate and agree on the critical importance of ADP. Experimentally, the

respiratory control index is expressed as the ratio of the respiratory rate in the presence of ADP to that in its absence. Mitochondria with good respiratory control are called tightly coupled and have a high numerical value (can be in the forties). Conversely, mitochondria are loosely coupled when they are not dependent on the ADP concentration and the numerical value may be as low as 1.

MITOCHONDRIAL VOLUME CHANGE - HISTORICAL

Volume changes in mitochondria were probably first observed by Kölliker (1888). Other workers observed changes in volume of mitochondria and other cytoplasmic particles in intact cells in response to anisotonic conditions (Lewis and Lewis, 1914; Harris, 1943). Isolated mitochondria also displayed this sensitivity to osmolarity changes (Claude, 1946; Hogeboom *et al.* 1948). These studies were qualitative and were performed with direct visual examination or gravimetric methods (Claude, 1946). However, the introduction of light scattering techniques to the study of mitochondrial volume changes (Cleland, 1952; Raaflaub, 1952a, b, 1953) permitted easy and rapid monitoring of these changes. The first quantitative treatment of these volume changes was that of Tedeschi and Harris (1955), who demonstrated that mitochondria follow the osmotic law if a dead space of 40% is assumed. Finally, the report that T4 stimulated water uptake by mitochondria (Klemperer, 1955) and the detailed study of Tapley (1956) generated interest in this area of research.

IN SITU CHANGES IN MITOCHONDRIAL SHAPE AND VOLUME

Mitochondria, *in situ*, undergo changes in shape and volume which can be influenced by the physiological state of the cell, the functional state through which it is passing, and by the physical and chemical environment. Cells grown in tissue culture or leaf epidermis cells of plants have been employed extensively in studies on the changes in shape and volume of

mitochondria, since these preparations permit direct light microscopic observation. Intensive early studies on mitochondrial changes demonstrated the plasticity of mitochondria (Lewis and Lewis, 1914). These authors produced a large number of drawings which illustrated features of mitochondrial change: (a) mitochondria fusing together, (b) undergoing division, (c) changing shape repeatedly and (d) decreasing in size independent of fusion or divison processes. The overall conclusion drawn from their work was that mitochondria are extremely plastic bodies and often react more rapidly to environmental changes than other cell structures.

Although Lewis and Lewis had demonstrated adequately that mitochondria undergo morphological changes, phase contrast cinemaphotomicrographs, which showed these changes, illustrated the point definitively (in Novikoff, 1961). The mitochondrial changes which occurred upon the administration of exogenous chemicals to the media in which the cells were bathed may not necessarily reflect a direct action on mitochondria. They may merely represent changes which occur through a modification of the total cellular function or functions.

In addition to plasticity, mitochondria may exhibit mobility. In cells of liver, mitochondria may be free to move in the cytoplasm or be moved as a result of cytoplasmic movement and in others, such as those of cardiac muscle or the kidney tubule cells, the mitochondria are probably anchored. Palade (1956) suggested that those mitochondria which move, may do so to acquire needed substrates.

<u>in vivo</u> mitochondrial volume changes have been observed under pathological conditions, but in general, this swelling has not been characterized, since one does not know what effects the preparative procedures might produce.

CLASSIFICATION OF MITOCHONDRIAL VOLUME CHANGES

The volume changes which mitochondria undergo *in vivo* and *in vitro* are classified as passive or active. Passive volume changes do not involve the expenditure of energy, e.g. those occurring following tonicity changes in the media. Active volume changes, such as T4-induced mitochondrial swelling, are associated with energy expenditure.

Furthermore, mitochondrial volume changes of the active type can be characterized by the degree of change in light scattering (Packer, 1961; Lehninger, 1962a) and are classified as small or large volume changes. Small light scattering changes are referred to as low amplitude swelling and correspond to a volume change of only 1 to 2% of the mitochondrial volume, whereas large light scattering or optical density changes which are referred to as large amplitude swelling may involve volume changes of 200 to 300%, as in the case of liver mitochondria (Lehninger, 1962a). Packer (1961), in order to distinguish the types of swelling observed in mitochondria, classified them as phase 1 or phase 2. Phase 1 refers to small volume changes which are fully reversible in tightly-coupled mitochondria, and phase 2 or large volume changes are associated with irreversible changes, such as uncoupling of oxidative phosphorylation. Phase 1 swelling is equivalent to low amplitude swelling and phase 2 to large amplitude swelling.

In a medium containing respiratory substrates, ADP, Mg^{++}, and PO_4, it was demonstrated (Harman and Feigelson, 1952; MacFarlane and Spencer, 1953) that mitochondria carrying out phosphorylating respiration maintained a low water content. If respiratory substrates or ADP were absent, the mitochondria would swell. Therefore, low amplitude swelling may be studied in a medium containing respiratory substrates but not ADP. Swelling of mitochondria, therefore, occurs in a respiratory substrate deficient medium, and the addition of ADP causes a contraction. This

sequence is referred to as a swelling-contraction cycle. Low amplitude swelling is measured at a neutral wavelength, 410 mμ, isosbestic for the carriers of the respiratory chain and a decrease in optical density is presumed to represent mitochondrial swelling, and an increase in optical density, contraction. While addition of ADP to mitochondria in the low amplitude swelling stage results in contraction of the mitochondria (Holten, 1957; Chance and Packer, 1958; Beechey and Holten, 1959; Packer, 1960, 1961), the addition of adenosine-5'-monophosphate (AMP) or ATP is not effective (Packer, 1960). Moreover, Packer (1960) also reported low amplitude swelling-contraction can occur in concentrations of sucrose as high as 0.88 M, and that agents which uncouple oxidative phosphorylation also produce mitochondrial contraction. Lehninger (1962a) pointed out that low amplitude changes, as measured by light scattering techniques, have not been established definitively as reflecting changes in volume, unlike large amplitude changes which have been verified by gravimetric procedures. Consequently, Lehninger feels there may not be sufficient data to conclude that these light scattering changes reflect changes in mitochondrial volume. He proposes that low amplitude studies may be measuring mitochondrial structural changes which may be quite different from volume changes and suggests they may reflect volume changes in mitochondrial compartments, changes in the membrane, as well as a rearrangement of internal components which could lead to a redistribution of light refracting compounds.

Large amplitude swelling of the active type, which is associated with respiratory chain activity, is slow compared to large amplitude swelling induced by hypotonicity. Active swelling proceeds to an apparent equilibrium volume. ATP reverses T4-induced swelling and ATP + Mg^{++} + bovine serum albumin reverses swelling induced by most agonists. Mitochondrial swelling promoted by reduced glutathione (GSH) is reversed by the addition

of ATP + Mg^{++} + bovine serum albumin + contraction factor (C-factor). Large amplitude swelling is sensitive to inhibitors of the respiratory chain or uncouplers of oxidative phosphorylation. Harman and Feigelson (1952) and Raaflaub (1953) observed that respiration might be necessary for the occurrence of active mitochondrial swelling and evidence for this has been reported by Hunter and Ford (1955), Hunter et al. (1956), Lehninger and Ray (1957), and Lehninger et al. (1959). These studies demonstrated that active swelling is blocked by inhibitors of the respiratory chain and by anaerobic conditions. Additional evidence for dependence of active swelling on respiratory chain activity is provided by the reported observations that DNP blocks active swelling (Tapley, 1956) as does dicumarol (Tapley, 1956), which are inhibitors of oxidative phosphorylation.

Although a substance induces active swelling of the large amplitude type, it does not necessarily mean that a common mechanism is operating to promote swelling. Two substances may induce swelling but the associated events may markedly differ. T4, for example, promotes swelling which is associated with uncoupling factor (U-factor) formation, whereas thiol-induced swelling is not, but is accompanied by peroxide formation, thereby giving rise to different hypothesis concerning their mechanism of action. The mitochondrial swelling curves induced by T4 or thiols are also characteristically different.

Of the many agonists of mitochondrial swelling, most are not as potent as T4. The thiols require concentrations of 10^{-4} to 10^{-3}M to promote mitochondrial swelling (Lehninger and Schneider, 1959). T4 promotes swelling in concentrations as low as 10^{-8}M and 10^{-5}M produces maximal swelling in a few minutes (Lehninger et al. 1959). However, a few compounds exhibit greater potency than T4. Greenbaum and Dicker (1963) reported a vasopressin preparation induced swelling in a concentration of

10^{-13}M, but Lehninger and Neubert (1961) had observed that 2 x 10^{-5}M of vasopressin, oxytocin and insulin was extremely effective in promoting rapid and extensive swelling. Furthermore, gramicidin, 10^{-11}M (Neubert and Lehninger, 1962a), and fatty acids in concentrations less than 10^{-6}M induced swelling (Lehninger, 1962a).

THYROXINE-INDUCED SWELLING

Swelling of mitochondria in response to T4 had been described first by Klemperer (1955) in his monograph on the binding of iodothyronines by mitochondria, and detailed studies of this swelling phenomena were subsequently reported by Tapley et al. (1955), Tapley (1956), and Lehninger (1959a). It has been confirmed repeatedly (Beyer et al. 1956; Dickens and Salmoney, 1956; Emmelot and Bos, 1957; Shaw et al. 1959). The mitochondrial swelling curve, in response to T4, exhibits a lag phase (markedly demonstrable at low concentration and low temperature) which is followed by a rising phase to a plateau. Increasing the concentration of T4 increases the rate of swelling of mitochondria (Tapley, 1956) and as little as 10^{-8}M T4 is effective in promoting swelling (Lehninger et al. 1959). In studies on mitochondrial swelling, two types of media mainly have been employed, one utilizing sucrose, 0.3 M sucrose-0.02 M tris (hydroxymethyl) aminomethane (Tris)-HCl buffer, pH 7.4, and the other KCl, 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Mitochondria are more sensitive to the effects of T4 in the latter medium. The replacement of sucrose by other solutes, such as glucose, raffinose, KCl or NaCl, does not qualitatively influence the T4-induced swelling response. Isolated mitochondria of various rat tissues do not exhibit the same degree of responsiveness to T4. Liver and kidney mitochondria exhibit the greatest volume changes, while those of diaphragm, heart, spleen, brain or testes respond slightly (Tapley and Cooper, 1956). In the measurement of

mitochondrial volume changes by optical methods, the decrease in optical density reflects an increase in mitochondrial volume, and Lehninger (1959a) has presented evidence that the optical density decrease occurring after exposure of mitochondrial suspensions to T4 is associated with an increase in wet weight of mitochondria as determined by gravimetric methods, thus indicating an increased uptake of water. Temperature affects mitochondrial swelling markedly (Tapley, 1956), swelling being extremely rapid at 37°C. Due to the high temperature coefficient of swelling, most studies are performed at 20°C to retard the process. In addition, T4-induced swelling is affected by the pH of the medium. Swelling is maximum at pH 7.3 or pH 7.4, an increase or a decrease in the pH resulting in a decrease in swelling and beyond the pH limits of 6.5 and 8.5 it is absent. The lag phase of the T4-induced swelling curve is marked at low temperatures and increases in temperature reduce the lag phase (Lehninger et al. 1959). Increases in T4 concentration caused a corresponding decrease in the lag interval.

Other studies (Tapley, 1956; Hunter and Ford, 1955; Lehninger et al. 1959) have demonstrated inhibition of T4-induced swelling by a variety of agents. Sucrose in high concentration inhibits T4 swelling (Tapley, 1956; Lehninger et al. 1959). It was postulated that sucrose exerts its inhibitory effect by two mechanisms, one as a result of the increase in osmolarity at high concentrations and the other by its ability to inhibit enzymatic reactions at lower concentrations. High concentrations of high molecular weight substances, such as serum albumin, gamma globulin and polyvinylpyrrolidone, which increase the osmolarity of the medium, also inhibit T4-induced swelling. Serum albumin inhibits at 10^{-5}M, a concentration which does not produce a substantial increase in the osmotic pressure of the medium (see section on U-factor). Inhibitors of the respiratory chain, such as sodium amytal, antimycin A and CN^-, block the

swelling response of mitochondria to T4 (Tapley, 1956; Lehninger and Ray, 1957; Lehninger et al. 1959). DNP, pentachlorophenol and gramicidin, which are inhibitors of oxidative phosphorylation, prevent the effects of T4 on mitochondria (Tapley, 1956), as does ATP, ethylenediamine tetra-acetate (EDTA), Hg^{++}, and a variety of ions (Tapley, 1956). The addition of sucrose to the medium prior to the advent of swelling induced by T4 caused inhibition, but sucrose added after swelling has begun is without effect. Apparently there is an increase in mitochondrial permeability to sucrose in the presence of T4 (Lehninger et al. 1959). Lehninger et al. believe that a small amount of T4-induced swelling may occur before permeability changes occur, since it would be expected that T4 could cause swelling even in the presence of high concentrations of sucrose. They believe that the lag period before swelling commences supports this explanation. ATP is of special interest since it will inhibit the effects of T4 when added to the medium before swelling occurs and will reverse it when added later (see section on mitochondrial contraction). Ethyl alcohol, 0.25 M--1.5 M (Karler et al. 1965) blocks the swelling response to T4, although the mechanism by which this occurs has not been established.

Aging of stock suspensions of mitochondria for 6 hr or longer at 0°C reduces the sensitivity of mitochondrial swelling response to T4. This loss of sensitivity is correlated with a loss of nicotinamide adenine dinucleotide (NAD). Lehninger et al. (1959) and Hunter and Ford (1955) have shown that PO_4-induced mitochondrial swelling is also accompanied by a loss of NAD. The level of NAD remained constant up to 9 hr periods during the aging process and then fell off sharply. On the basis of these and other observations, Lehninger et al. (1959) suggested that the bound form of mitochondrial NAD might be the site of T4 action in inducing mitochondrial swelling.

Aebi and Abelin (1953) reported that mitochondria isolated from

thyroid-fed rats swelled at a faster rate than normal. Tapley (1956) confirmed this finding and reported also that mitochondria isolated from hypothyroid rats showed a reduced swelling response. Moreover, Tata (1963) demonstrated that isolated liver mitochondria of thyroidectomized rats were less sensitive to PO_4, T4, oleate and hypo-osmolarity.

MITOCHONDRIAL CONTRACTION

Isolated mitochondria of liver or kidney, which have undergone active volume changes with an increase in their water content, can be contracted and water extruded by the addition of certain compounds.

Experimental results on the extrusion of water from mitochondria in the presence of phosphorylating respiration are vague, since most of these studies have been done in rather complex medium containing swelling promoting factors, as well as factors which have been reported to aid in the contraction of swollen mitochondria. Harman and Feigelson (1953) demonstrated that the shape of heart muscle mitochondria could be correlated with their oxidative activity. Following these observations, it was demonstrated that mitochondria, in the absence of a PO_4 acceptor, readily increased their water content and in the presence of a PO_4 acceptor the water content was maintained at a low level (MacFarlane and Spencer, 1953). Price et al. (1956) suggested that the contractile elements of mitochondria are associated with oxidative phosphorylation. They put forward this suggestion as a result of experiments in which they were able to cause contraction of mitochondria with MgCl and NAD added to their medium with AMP. Contraction with AMP followed after a lag period during which the AMP presumably became phosphorylated to ATP, because the addition of ATP to the medium caused an immediate contraction. Other workers have reported on mitochondrial contraction during phosphorylating respiration (Beyer et al. 1955; Hunter and Ford, 1955; Dianzani and Scuro, 1956,

Ulrich, 1959, 1960; Packer, 1960, 1961; Packer and Tappel, 1960).

Early studies on the contraction of mitochondria by ATP in the absence of added respiratory substrates were reported by Lehninger (1959a, b). It was found that 0.125 M KCl-0.02 M Tris-HCL buffer, pH 7.4, was the most effective medium with respect to both the rate and final extent of the contraction of T4-induced swollen mitochondria. Sucrose inhibited this contraction. This observation accounted for those of Cleland (1952), Raaflaub (1953) and Tapley (1956) who reported that ATP did not induce contraction of mitochondria. These authors used a medium which contained a relatively high sucrose concentration.

ATP contracts T4 swollen mitochondria. This process can take place under conditions in which the K^+ of the medium is replaced by other cations, such as Na^+, lithium, rubidium, ammonium, or Tris and Cl^- is replaced by bromide, I^-, fluoride, nitrate, chlorate, perchlorate, sulfate, or acetate (Lehninger, 1959a, Lehninger, 1961). Moreover, no nucleoside-5'-triphosphate other than ATP caused contraction. The rate of contraction decreased slightly as the K^+ concentration was increased. Furthermore, mitochondria swollen in distilled water are contracted by ATP in the presence of T4, while in the absence of T4, ATP + Mg^{++} + serum albumin are required for contraction but contraction is faster in the presence of T4 (Lehninger, 1959b). Lehninger (1961) demonstrated that ATP-induced contraction was independent of phosphorylating respiration and occurred in a highly "unphysiological media". He then concluded that an ATP driven contractile mechanism in mitochondria causes extrusion of water and probably solutes from swollen mitochondria independent of active transport of ions. This extrusion could be the result of a two dimensional contraction of one or both membranes of the mitochondria or a closure of cristae folds. He also postulated that an internal mitochondrial substance might cause a reduction in the "colloid" osmotic pressure with the resulting outward

movement of water (Lehninger, 1959a, b, 1960).

The concentration of ATP required to produce mitochondrial contraction was found to range between 0.0005 M and 0.05 M. These studies of contraction have utilized optical density changes as an index of both mitochondrial swelling and contraction. Lehninger (1959a) compared optical density measurements with gravimetric measurements and demonstrated that the optical density changes are qualitatively reliable for contraction and swelling. It was found that 60 µmoles of ATP would result in the extrusion of over 650 µmoles of water from mitochondria. ATP was shown to be a highly (but not absolutely) specific reversing agent for T4 swollen mitochondria. Although T4 swollen mitochondria are contracted by ATP, the addition of Mg^{++} may cause some inhibition of the ATP-induced contraction. Furthermore, the addition of serum albumin accelerates the ATP-induced contraction of T4 swollen mitochondria. Presumably, serum albumin combines with Lehninger's U-factor, thus removing an endogenous swelling-inducing agent. Moreover, the contractile system of mitochondria appears to be very stable (Lehninger, 1959a). Mitochondria in a swollen state for 3 hr undergo immediate reversal when ATP is added to the suspension.

If ATP is to exert its effect on the contractile mechanism of mitochondria which have been exposed to swelling-inducing agents other than T4, such as PO_4, Ca^{++}, fatty acids, or U-factor, the addition of other factors to the suspension is required, such as Mg^{++}, serum albumin and C-factor. C-factor is of special interest relative to the mitochondrial swelling induced by GSH and will be discussed under the sections dealing with the effects of thiols on mitochondrial volume changes. Mitochondria swollen under hypotonic conditions are contracted by the addition of ATP + Mg^{++} + serum albumin, or by ATP + Mn^{++}, while spontaneously swollen mitochondria in isotonic medium require ATP + Mn^{++} + serum albumin (Lehninger *et al.* 1959). ATP + Mg^{++} are required to produce contraction

of PO_4 swollen mitochondria. EDTA + serum albumin + ATP + Mg^{++} contracts Ca^{++} swollen mitochondria. Swelling induced by p-HMB and phloridzin is reversed by ATP + Mg^{++} or serum albumin + Mn^{++}. The swelling produced by carbon tetrachloride or digitonin is not reversed by any of these agents or combinations of them. Swelling by U-factor, as well as that by sodium oleate (Lehninger and Remmert, 1959), is reversed by a combination of ATP + Mg^{++} + serum albumin.

EFFECT OF IODINE COMPOUNDS ON SWELLING

I_2 (Rall et al. 1962a) and ICN (Rall et al. 1962b) promote mitochondrial swelling. In the presence of equimolar concentrations, 5 x 10^{-6}M, of T4, ICN and I_2, swelling is more rapid with I_2 than with ICN which, in turn, is faster than T4. As little as 10^{-6}M ICN or 10^{-7}M I_2 induces mitochondrial swelling. Amytal, antimycin-A, CN^- (Rall et al. 1962a), DNP (Rall et al. 1963), 0.1% serum albumin, 0.75 M sucrose, EDTA (Rall et al. 1962a), block the swelling induced by these agonists. The blockade by CN^-, antimycin A or amytal can be overcome with high concentrations of ICN (Rall et al. 1962b), but cannot be overcome by I_2. Chloroacetophenone, monoiodoacetate and NEM, which are reagents that react with sulfhydryl groups, were reported to be ineffective in blocking swelling by T4, I_2 or ICN (Rall et al. 1962a; Rall et al. 1963).

ATP reversed I_2- or ICN-induced swelling (Rall et al. 1962a, 1962b). The ATP-induced mitochondrial contraction was able to be completely blocked by arsenate or p-HMB but not, or slightly blocked, with NEM. When gradually increasing concentrations of ATP were employed to prevent mitochondrial swelling induced by T4, I_2 or ICN, an order of susceptibility to ATP was apparent. With 2 x 10^{-5}M ATP, no inhibition of swelling was observed, whereas, with 6 x 10^{-5}M ATP, T4-induced swelling was inhibited but not that by I_2 or ICN. Swelling promoted by T4 and ICN could be prevented by

2×10^{-4}M ATP. Finally, 5×10^{-4}M ATP inhibited swelling by all three substances.

Michel et al. (1964) reported that the flow of electrons through the cytochrome b was necessary for the iodo-compounds, T4, I_2 and ICN to produce swelling. On the other hand, Scott and Hunter (1966) reported that the occurrence of electron transport at any one of the three energy conservation sites supported the T4-induced swelling.

MITOCHONDRIAL CHANGES ASSOCIATED WITH THYROXINE-INDUCED SWELLING AND POSSIBLE MECHANISM(S) OF ACTION

During T4-induced swelling, these events occur: (1) electron transport occurs, (2) U-factor is released, (3) NAD is lost, (4) respiratory control is lost, (5) oxidative phosphorylation does not proceed. Evidence that the respiratory chain activity is linked to T4-induced swelling is derived from the observations that anaerobic conditions and substances which inhibit respiratory chain activity and oxidative phosphorylation prevent T4-induced swelling (Tapley, 1956; Lehninger and Ray, 1957; Lehninger et al. 1959; Lehninger and Schneider, 1958; Chappell and Greville, 1959a; Hunter et al. 1959). Lehninger and Ray (1957) suggested that reduction of respiratory chain carriers prevents swelling. Subsequent studies with respiratory chain inhibitors (Lehninger and Schneider, 1958; Lehninger et al. 1959) prompted them to further suggest that some of the NAD must be oxidized to permit swelling to occur and that the oxidation-reduction state of the remaining respiratory chain carriers was not important. Moreover, Chappell and Greville (1958b) demonstrated that swelling required electron transport activity, since it would occur in either fresh or aged mitochondria (Hunter et al. 1959; Chappell and Greville, 1958b) with the addition of both the swelling agent and an oxidizable substrate. Moreover, ferricyanide, an electron acceptor, was

able to restore swelling of fresh or aged mitochondria which were inhibited by CN^- or anaerobic conditions in the presence of oxidizable substrate (Hunter et al. 1956; Chappell and Greville, 1960). Chappell and Greville (1960) concluded that electron transport chain activity supports swelling with succinate as substrate through a restricted portion of the respiratory portion of the respiratory chain, that is, cytochrome b to cytochrome c.

The discovery that T4-induced swelling is accompanied by the release of long chain fatty acids (Lehninger and Remmert, 1959; Wojtczak and Lehninger, 1961), and that these isolated fatty acids could promote mitochondrial swelling and uncouple oxidative phosphorylation, led to the suggestion that this might constitute the mechanism of action of T4 in promoting mitochondrial swelling (Lehninger and Remmert, 1959). The isolated mixture of these long chain fatty acids, which they had called U-factor, was extracted by isooctane from aged mitochondria or membrane fragments. T4-, Ca^{++}-, or phloridzin-induced mitochondrial swelling is associated with a parallel formation of U-factor (Lehninger and Remmert, (1959), while swelling induced by PO_4, GSH, or carbon tetrachloride is not. Wojtczak and Lehninger (1961) demonstrated that concentrations of serum albumin (0.2 mg/ml) inhibited swelling induced by T4, Ca^{++} or phloridzin but not by PO_4, GSH or carbon tetrachloride. The swelling induced by T4, Ca^{++}, or phloridzin is apparently mediated by a substance, presumably a fatty acid (U-factor), which can be trapped by serum albumin (Wojtczak and Lehninger, 1961; Lehninger, 1962a). Freshly prepared mitochondria contained little U-factor, whereas after spontaneous or T4-induced swelling there was a gradual increase in U-factor content which paralleled the swelling reaction.

T4 apparently releases U-factor, which increases electron transport and thus leads to swelling, or U-factor may affect the permeability of

mitochondria directly. These points have not been established conclusively. Serum albumin in relatively low concentrations (10^{-5}M) inhibits U-factor-induced swelling, and this inhibition is considered diagnostic for mitochondrial swelling associated with the release of U-factor. U-factor causes a loss of oxidative phosphorylating ability (Ernster and Lindberg, 1958), as does T4-induced swelling (Lehninger, 1960). However, Emmelot (1962) was not able to detect mitochondrial swelling in the presence of oleate or long-chain fatty acids isolated from the BY 448 rat liver hepatoma at a concentration of 0.6 to 20 µg/ml in a 0.3 M sucrose--0.2 M Tris (pH 7.4) medium. On the other hand, Avi-Dor (1960) reported swelling of mitochondria by fatty acids in a sucrose—Tris medium. The addition of ATP to mitochondria swollen spontaneously or with T4 caused contraction and a parallel decrease in U-factor (Wojtczak and Lehninger, 1961). When the fate of U-factor was followed by the use of C^{14}-oleate, it was found that C^{14} was incorporated into the phospholipid fraction of mitochondria at a rate paralleling the kinetics of contraction. In addition to uncoupling oxidative phosphorylation, U-factor inhibits the ATP-PO_4 exchange and stimulates adenosine triphosphatase activity.

Another consequence of T4-induced swelling is the loss of NAD (Ernster, 1956; Emmelot and Bos, 1958; Lehninger et al. 1959; Lester and Hatefi, 1958), and the sensitivity of mitochondria to swelling-inducing agents is related to their total NAD content. Lehninger and Ray (1957) suggested that the reduced state of the carriers, especially NAD, is an important determinant of swelling. Reaffirming this view, Lehninger (1962a) cited Kaufman and Kaplan (1960), who reported that loss of NAD from mitochondria involves an oxidation of the reduced form and a leakage of the oxidized form, which is then attacked by extramitochondrial NADase. Kaufman and Kaplan (1960) found that mitochondrial swelling accompanied the oxidation of the reduced form, specifically. However, Chappell and

Greville (1963) stated that the oxidation of NADH can hardly be a direct or even sufficient cause of the extensive swelling which follows, since (a) both oxidizable substrate and an agent is needed to cause swelling of aged mitochondria, and this can scarcely lead to the oxidation of NADH, (b) if respiratory chain inhibitors are added during the course of mitochondrial swelling, swelling is abruptly stopped (Chappell and Greville, 1959b), and (c) swelling of mitochondria inhibited by amytal in the presence of b-hydroxybutyrate, a condition under which extensive reduction of NAD occurs, is restored by succinate. Chappell and Greville (1963) suggest the loss of NAD may result from permeability changes which presage extensive swelling.

Lehninger (1962a) reported that early in the course of T4-induced swelling, mitochondria exhibit loose coupling. In addition, Lehninger et al. (1958) observed that mitochondria which remained intact and unswollen did not lose phosphorylating ability but did when swollen. Fragments prepared by sonic disruption of mitochondria, unlike those prepared by digitonin, were able to carry out oxidative phosphorylation.

THE ACTIVE FORM OF THYROXINE

Deiodination of thyroid hormones is recognized as one of their important metabolic pathways (Pitt-Rivers and Tata, 1959; Stanbury, 1960). The deiodinating enzymes remove I_2 from iodotyrosines and iodothyronines, and administration of I^{131} labeled iodotyrosines or iodothyronines results in the excretion of I^{131} as I^- and negligible amounts of the original material. Deiodinase distribution has been studied in different species and tissues, such as liver, kidney, muscle, brain (Stanbury, 1960). Mitochondria of rat liver and kidney (Albright et al. 1959; Lissitzky et al. 1961; Yamamato et al. 1964) exhibit deiodinase activity. Tata (1958) studied the subcellular localization of the enzyme in brain and skeletal

muscle and reported activity in the nuclear, microsomal, mitochondrial and supernatant fractions.

The deiodination of T4 is considered by some to be a necessary prerequisite for the activation of T4. This theory was invoked by Galton and Ingbar (1961) to explain their observation of the loss of deiodinating ability of frog and tadpole tissue homogenates coincidental with metamorphosis. On the other hand, Lissitzky (1960) proposed that T4 deiodinase serves to terminate the metabolic activity of T4. Furthermore, Tata (1961) implied the necessity of deiodinase activity for T4 to exert its action, when he considered that the deiodination of thyroid hormones is linked somehow to their calorigenic action. The ideas of Tata and Galton and Ingbar are somewhat similar in that they both suggest the requirement of deiodinase action on T4 for it to exert its hormonal action.

Disturbances of the deiodinating mechanism may be responsible for decreased growth and development (Stanbury et al. 1956), and genetically defective deiodination has been associated with a goitrous condition (Kusakabe and Miyake, 1963).

Since deiodination does occur, a logical question arises relating to the significance of this process. If we accept the role of deiodinase as proposed by Lissitzky (1960), then the intact T4 molecule is responsible for the observed hormonal activity. Evidence cited by Lissitzky (1960) as supporting his view is (a) that metabolic products with less I_2 than T4 or triiodothyronine (T3) are not detected in vivo or in vitro and (b) of the many analogues of T4 synthesized, none have a biologic activity greater than T4 except T3 and this may be due to the ease of the latter penetrating to its sites of action.

On the other hand, if deiodination is required for T4 to exert its metabolic effects. then some product of deiodination might be responsible for the hormonal activity. The possible products are T3, iodothyronines

containing less I_2 than T3, I^- and I^+. Tata (1961) detected I^- but not any T3 when T4 was incubated with tissue isolated from a thyroidectomized rat and thus believes that T4 is not converted to a more active form. The iodothyronine products which have less I_2 than triiodothyronine have weak hormone activity and have not been readily isolated, since most of the labeled I^{131} appears in the urine as I^-. There is evidence that I^- can mimic thyroid hormones. Evans et al. (1960) favor the theory that I^- has a T4-like action, especially in high concentrations. They found that the administration of 5 mg/day of I^- restored the growth curve of thyroidectomized rats to near normal with no detectable organification of the administered I^- (Evans et al. 1960). Furthermore, I^-, like T4, can uncouple oxidative phosphorylation (Middlebrook and Svent-Györgyi, 1955) in high concentrations.

Another theory of the hormonal effects of products of deiodination has been proposed. Galton and Ingbar (1961) had observed while studying the deiodination of T4 and its analogues by tissue breis, that I^- and an iodinated substance, which remains at the point of origin of paper chromatograms, was formed. The addition of I^- as substrate did not result in the formation of this iodinated material. This substance has not been characterized but it has been suggested to be a protein (Yamazaki and Slingerland, 1959; Tata, 1959). Therefore, Galton and Ingbar (1961) suggested that deiodination could lead to formation of the two products, I^- and a product which results in the formation of iodinated origin material, or a single product which can form either I^- or origin material. They further suggested that this product might be the hypoiodous acidium ion, or I^+, since aromatic iodinations require oxidation of I^-. Subsequently, Galton and Ingbar (1962) suggested that reduction of the oxidized form of I_2 into organic I^- could be associated with the hormonal action of T4. Roche et al. (1962), while studying the effects of a number of I_2

containing compounds on mitochondrial swelling, observed that T4, I_2 and ICN possessed similar properties with respect to the swelling response. On the basis of these similarities and, since these compounds could potentially give rise to I^+, they suggested that these compounds exert their effects as a result of a transient reversible formation of the I^+. This theory requires the removal of I_2 from the T4 molecule by way of an enzyme, T4 deiodinase, with the resultant formation of the I^+. However, Roche et al. (1963) and Rall et al. (1964) were unable to detect any I^- formation during the course of mitochondrial swelling.

Barker (1963) proposed that the action of T4 was exerted via a iodothyronine-protein complex. According to Barker's theory, T4 reacts with binding sites on protein and two processes may occur: (1) there may be non-specific deiodination or: (2) a specific protein - 3-5, 3' iodothyronine complex may be formed with a concomitant formation of free I^-. This complex then forms a specific protein-activated iodothyronine complex which exerts the hormonal activity and I^- and tyrosine are formed. This theory attempts to unify the knowledge relating to the physiological importance of T4 deiodinase and the form of T4 responsible for hormonal activity. It incorporates data suggesting binding of T4 as the first step (Roche et al. 1962), as well as the theories of Lissitzky (1960) and Galton and Ingbar (1962). In addition, Barker (1963) reported that analogues of T4 which were 3' substituted in the molecule were effective in stimulating the heart rate of thyroidectomized animals, whereas 5' substituted analogues displayed very low responses. These data were suggested to support the concepts that 5' deiodination of T4 is of importance (Barker, 1962).

The available evidence does not permit a final definition of the physiological significance of deiodination.

MITOCHONDRIAL SWELLING INDUCED BY THIOLS AND DISULFIDE COMPOUNDS

Mitochondrial swelling is induced by thiols (Lehninger and Schneider, 1959), such as GSH, cysteine, and reduced coenzyme A. Swelling induced by these agents differs markedly from that induced by T4 with respect to the kinetics, extent, inhibition, and especially its reversal. The mitochondrial swelling curve produced by GSH in a concentration of 0.01 M or higher, displays a 10-15 min lag period followed by a rapid drop in optical density. The optimum pH range for swelling induced by thiols is 7.5 to 8.0. Neubert and Lehninger (1962b) found that all thiols examined did not promote the same degree of swelling. Moreover, thiourea, thioacetamide and diethyldithiocarbamic acid induced swelling which was different from that produced by simple thiols (Neubert and Lehninger, 1962b).

Thiol-induced swelling also differs from T4 with respect to its inhibition. DNP, dicumarol, Mg^{++}, sucrose, serum albumin or polyvinylpyrrolidone do not inhibit GSH promoted mitochondrial swelling. ATP, inosine-5'-triphosphate or guanosine-5'-triphosphate prevented swelling due to GSH but uridine-5'-triphosphate did not. Mn^{++} inhibited swelling by GSH, but among the respiratory chain inhibitors CN^{-}, but not amytal and antimycin A, was inhibitory. Lehninger and Schneider (1959) suggested that thiols act on specific chemical receptors in mitochondria and promote changes in both the structure and physical properties of the mitochondria. These changes are characteristic for thiols but differ from those produced by T4. Furthermore, the complexity of mitochondrial structure, which includes the presence of two intramitochondrial compartments, could permit more than one kind of swelling. In addition, mitochondrial swelling induced by GSH and oxidized glutathione (GSSG) appears to depend on the associated activity of the respiratory chain, since it is inhibited by certain respiratory chain inhibitors and anaerobic conditions (Lehninger and Schneider, 1959; Neubert and Lehninger, 1962b).

The disulfide polypeptide hormones, vasopressin, oxytocin and insulin were reported to promote mitochondrial swelling (Lehninger and Neubert, 1961). These substances had approximately the same order of activity in inducing mitochondrial volume changes. Each produced marked swelling in a concentration of 2×10^{-5}M. In 0.125 M KCl-0.02 M Tris-HCl buffer, the optimal pH for their induction of swelling is 7.0 to 7.3. The addition of ATP to the mitochondrial suspension caused contraction of vasopressin, oxytocin or insulin induced mitochondrial swelling. Growth hormone of the pituitary gland also has been reported to promote mitochondrial swelling (Melhuish and Greenbaum, 1961). This compound contains four disulfide bridges and is more effective than T4 on a molar basis. The neurohypophyseal hormones, vasopressin and oxytocin, are 50 to 100 times more active than GSSG in inducing swelling.

Combinations of thiols and disulfide compounds promote mitochondrial swelling which was greater than that of each compound individually (Neubert and Lehninger, 1962b). Furthermore, concentrations of both thiol and disulfide just less than that required by either alone to induce swelling produced a marked mitochondrial swelling response. The extent of this swelling depends on the concentration of the thiol and disulfide and the ratio of the concentrations of the two compounds. This relationship has been reported for GSH and GSSG, for GSH plus other simple disulfide compounds, and for GSH plus the disulfide hormones (Lehninger and Neubert, 1961; Neubert and Lehninger, 1962b).

Another difference, which is quantitative, between the mitochondrial swelling actions of GSSG and GSH was evident in the presence of Fe^{++}. Swelling of mitochondria induced by GSSG was accompanied by little lipid peroxide formation, whereas that by GSH promoted lipid peroxide formation which was five times greater than that by Fe^{++} itself. GSH (5-10 mM), in the presence of Fe^{++}, promoted lipid peroxide formation, the rate of

formation paralleling the rate of swelling. The combination of GSH and GSSG in the presence of Fe^{++} may rupture structural links or promote peroxidation of lipids at sites which are not ordinarily reached by the Fe^{++} (Hunter et al. 1963).

A proposal has been put forward to explain how thiols and disulfides cause changes in the mitochondrial membrane which would lead to the uptake of water. Lehninger and Neubert (1961) and Neubert and Lehninger (1962b) suggested that thiols and disulfide react with some specific structure controlling sulfhydryl or disulfide groups in a critical portion of the mitochondrial membrane. As supporting evidence of this theory, they cited the swelling induced by reagents reacting with sulfhydryl groups, Hg^{++}, Cu^{++}, p-HMB, IA and NEM. Furthermore, they pointed out that, since combinations of thiols and disulfides are effective swelling-inducing agents at low concentrations, these compounds might exert a concerted attack on both the sulfhydryl and disulfide groups of the mitochondrial membrane. Thus, a sulfhydryl-disulfide reaction, could produce mixed disulfides, as outlined by Boyer (1959) and Jensen (1959). The exogenous sulfhydryl group could react with a membrane disulfide and the exogenous disulfide with a membrane sulfhydryl group, thus setting up mixed disulfide reactions and altering the membrane structure, perhaps leading to increased polymerization or depolymerization of proteins, as first postulated by Huggins et al. (1951). This sulfhydryl-disulfide interaction was invoked by Fong et al. (1960) to explain the binding of vasopressin to kidney proteins.

CONTRACTION FACTOR

Swelling promoted by GSH is not reversed by factors which reverse swelling by most other agents, e.g. ATP + Mg^{++} + serum albumin (Lehninger and Schneider, 1959; Lehninger, 1959b). It was found that a substance released from mitochondria was required for the contraction in the presence

of ATP + serum albumin to take place. The substance was named C-factor and it is soluble, non-dialyzable and heat labile (Lehninger and Gotterer, 1960). Lehninger and Gotterer (1960) and Lehninger (1962b) suggested that C-factor is a protein or is bound to a soluble protein carrier. Swelling promoted by GSH is not reversed in the relatively dilute suspensions which are used for optical density studies. However, reversal by ATP can occur without the addition of C-factor, if the concentration of mitochondria is greatly increased (Lehninger, 1962b). C-factor is released in both situations but at low concentrations of mitochondria is diluted below an effective level (Lehninger, 1962b).

GSH, compared to a number of thiols, was the most effective in causing the loss of C-factor. Furthermore, swelling promoted by GSSG or by the combination of GSH and GSSG at low concentrations is reversed by ATP, indicating slight or no leakage of C-factor from mitochondria.

The contraction which occurs in the presence of C-factor is sensitive to 0.3 M sucrose or azide, both of which are inhibitory (Lehninger, 1962b). These observations indicate possible involvement of parts of the phosphorylation-coupling mechanism in the contraction mechanism. Highly purified preparations of C-factor have been found to inhibit swelling. CN^- or DNP is ineffective against C-factor-induced contraction. In order to exert its effect, C-factor must be present before the addition of ATP. Although ATP inhibits T4-induced mitochondrial swelling, it has no effect, by itself, on swelling by GSH. In fact, ATP stimulates mitochondrial swelling promoted by GSH, as does ADP which is less effective. Lehninger (1962b) suggested that ATP displaces endogenous C-factor by competing for the binding site in the presence of GSH. Contraction by C-factor, which is optimal at pH 7.7, is more sensitive to the ionic components of the medium than is contraction of T4 swollen mitochondria by ATP (Neubert et al. 1962a).

An assay method for C-factor has been described (Neubert et al. 1962c), based on Lehninger's (1962b) observation that this agent influences the extent but not the rate of contraction. The source of C-factor used in the assay procedure was rat liver mitochondria which had been subjected to sonic oscillation.

Neubert et al. (1962b) studied the distribution of C-factor and found it in mitochondria of liver, kidney, heart, pancreas, skeletal muscle, brain and spleen. In addition, they reported C-factor activity in maize rootlets, several species of bacteria, and in the membranes of red blood cells.

Neubert et al. (1962c) purified C-factor of rat liver mitochondria and chromatographically separated 3 forms which were designated C-factor I, II, and III. C-factor I and II were found to be identical with glutathione peroxidase and catalase respectively and C-factor III was an unidentified lipid or lipoprotein. The role of catalase and glutathione peroxidase in swelling and contraction is unclear.

GSH and GSSG were reported to contain amounts of metal contaminents, which could account for their induction of mitochondrial swelling (Cash and Gardy, 1965). Similar observations were reported for 8-lysine vasopressin and oxytocin (Cash et al. 1964). However, Lehninger and Beck (1967) observed that the trace metal-induced swelling (Fe^{++}, Ca^{++}, or Cu^{++}) was reversed by ATP + Mg^{++} + bovine serum albumin, but that induced by the GSH preparation required ATP + Mg^{++} + bovine serum albumin + catalase (contraction factor II). They concluded that if the swelling induced by the contaminated GSH is due only to the trace metals, then it's course is so modified by GSH that catalase is required.

PROPOSED MECHANISM OF ACTION OF VASOPRESSIN

Vasopressin and oxytocin are cyclic octapeptides differing in two

amino acids in which a ring is formed via a disulfide bond between 2 cysteine residues (du Vigneaud, 1954). There are two vasopressins occurring naturally which are characterized by the amino acid in the 8 position, arginine-8-vasopressin and lysine-8-vasopressin (Sawyer, 1961). Insulin also possesses disulfide groups and is composed of 51 amino acids in two distinct peptide chains, A and B, joined together by two disulfide linkages; a third disulfide group is within the A chain of the molecule (Sanger, 1960).

There is not complete agreement on the mechanism by which vasopressin increases the permeability of membranes. Four possible mechanisms have been proposed. Vasopressin may act by increasing pore size (Koefoed-Johnsen and Ussing, 1953), by stimulating collecting duct cells to secrete a substance containing hyaluronidase, which depolymerizes intercellular mucopolysaccharide complexes, thus increasing permeability to water (Ginetzinsky, 1958), by increasing carbonic anhydrase activity (Kashiwagi, 1959), or by accelerating adenyl cyclase (Orloff and Handler, 1961, 1962).

Following the observations that vasopressin increased the permeability of amphibian membranes to water movement which was osmotic in nature, it was suggested that vasopressin increased membrane pore size (Ussing and Zerahan, 1951; Koefoed-Johnsen and Ussing, 1953). Leaf and colleagues have extended the pore theory and Leaf (1962) suggested that under vasopressin's influence the functional pore area is enlarged by the fusion of existing small channels and/or the formation of new pores.

In order to explain the manner in which vasopressin is bound to membranes and how it increases pore size, the sulfhydryl-disulfide interchange reaction has been suggested as the basis of both processes. Sulfhydryl-disulfide interchanges lead to the formation of mixed disulfides (Boyer, 1959; Jensen, 1959), and these interchanges could account for protein polymerization (Huggins et al. 1951). Fong et al. (1959, 1960) reported that

tritiated vasopressin was readily released by thiol compounds from kidney membranes isolated at peak antidiuretic activity and that the number of reactive sulfhydryl groups in the distal convoluted tubules was reduced after administering vasopressin. They suggested that in its binding and action a specific sulfhydryl-disulfide interchange was involved between the hormone's disulfide group and a membrane sulfhydryl group. A new disulfide is formed linking hormone to membrane and the free sulfhydryl group of the hormone is free to react with another membrane disulfide group, which triggers a series of membrane sulfhydryl-disulfide interactions. This wavelike interchange reaction has been invoked to explain increases in membrane permeability. Evidence not in agreement with the sulfhydryl-disulfide interchange concept exists. Vasopressin analogs without a disulfide group have been demonstrated to increase membrane permeability (Schwartz and Livingston, 1964).

It has been suggested (Ginetzinsky, 1959) that vasopressin effects on kidney function may be due to activation of hyaluronidase. Ginetzinsky reported that hyaluronidase in the urine of dogs disappeared during water diuresis and was present during osmotic diuresis. He also observed that the intercellular cement substances between collecting tubule cells reacted as hyaluronic acid when the rats were water loaded and as its depolymerization products during dehydration of the animal. Consequently, he suggested that vasopressin stimulates the production of hyaluronidase by the collecting tubule cells of the kidney, this, in turn, depolymerizes the intercellular mucopolysaccharide complex, thus increasing the water permeability. Dicker and Eggleton (1960a, b: 1961a) have confirmed Ginetzinsky's observations and have extended them to man. However, Berlyne (1960) was unable to confirm Ginetzinsky's findings and both Berlyne (1960) and Kaplan (1961) have raised objections to the hyaluronidase assay technique used by Ginetzinsky.

Carbonic anhydrase of renal tubular cells have been implicated as a mediator of vasopressin's action on the basis that the hormone increased the activity of a carbonic anhydrase preparation (Kashiwagi, 1959). However, this apparently is the only report.

Finally, Orloff and Handler (1961, 1962) proposed that adenosine-3', 5'-cyclic monophosphate (cyclic AMP) mediates vasopressin's action. They reported that cyclic AMP mimics vasopressin in the toad bladder. Theophylline, an inhibitor of the inactivating enzyme for adenosine 3', 5' monophosphate, also does. These authors suggested that vasopressin acts as a cofactor for adenyl cyclase, which converts ATP to cyclic AMP. Additional evidence supporting this theory are the reports that vasopressin increases cyclic AMP concentration in dog kidney (Brown et al. 1963) and toad bladder (Handler et al. 1964), as well as the observation of Davoren and Sutherland (1963) that adenyl cyclase is associated with all membranes.

PROPYLTHIOURACIL

Antithyroid compounds, such as the thioureas, were discovered in four independent laboratories (Trotter, 1964) and extensively studied by Astwood (1945). These compounds produce goiters as a result of a decreased formation of thyroid hormones. As a result of the low level of circulating thyroid hormone, the anterior pituitary gland is stimulated. This results in an increased release of thyroid stimulating hormone which stimulates thyroid gland growth. The thionamide derivatives are antithyroid agents and act by interfering with the biosynthesis of thyroid hormone (Greer et al. 1964). They act at several steps in the biosynthetic pathway and there is an apparent order of sensitivity. The reaction coupling two iodinated molecules of tyrosine to produce T4 or T3 is more sensitive to thionamide effects than the iodination of monoiodotyrosine which, in turn, is more sensitive than the iodination of tyrosine.

PTU, a thionamide derivative, also has extra-thyroidal effects (peripheral effects). The peripheral metabolism of T4 and T3 is reduced by the administration of PTU (Van Arsdel and Williams, 1956; Escobar del Rey and Morreale de Escobar, 1961; Jagiello and McKenzie, 1960; Jones and Van Middlesworth, 1960). An increase in the renal clearance of I^- was noted after PTU administration (Brown, 1956), as well as a water diuresis in rats (Fregly, 1961) apparently due to a direct effect.

METHODS

EXPERIMENTAL ANIMALS

Mitochondria were isolated from the livers of hooded rats, Quebec Breeding Farm strain, weighing 150 to 200 gm, which were fed *ad libitum* with Victor Fox Food cubes to initiation of experiments. For studies with thyroidectomized rats, surgical thyroidectomy was verified by comparing uptake of I^{131} in the neck region and body growth with normal animals. Animals were killed by a blow on the head.

GLASSWARE CLEANING PROCEDURES

Nalgene centrifuge tubes used in the preparation of isolated mitochondria were hand washed with Heikol® and rinsed six times in hot water and ten times in deionized-distilled water. This procedure was utilized also in the cleaning of test tubes serving as reaction vessels. Some difficulty was encountered with excessive spontaneous swelling, and tubes not adequately rinsed free of detergent promoted mitochondrial swelling. The use of chromate-sulfuric acid cleaning solution as a glassware cleansing agent promoted mitochondrial swelling, as seen in Table I. All other glassware was washed in a Heinecke Laboratory Glassware Washer and subsequently rinsed several times with deionized-distilled water.

PREPARATION OF RAT LIVER MITOCHONDRIA

Mitochondrial isolation was according to the method of Schneider (1948) and recommendations of Lehninger *et al.* (1959) with slight modifications. All solutions were made in distilled water deionized by an Illco Way research model ion exchanger. The liver was immediately removed and placed in 0.125 M KCl to remove excess blood. Throughout the mitochondrial preparation procedure, all solutions were maintained at 0°C and tissue was brought to this temperature before weighing. The liver was blotted and dried with filter paper, a 5 gm portion was weighed, cut into small pieces and added to 30.0 ml of a 0.25 M sucrose solution.

Table 1

EFFECT OF CLEANING GLASSWARE IN DICHROMATE-SULFURIC ACID CLEANING SOLUTION ON MITOCHONDRIAL VOLUME

Optical Density Change after 10 min	
without cleaning solution	with cleaning solution
0.047±0.003	0.210±0.006

Table 1. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Reaction vessels cleaned with dichromate-sulfuric acid solution were rinsed 10 times in deionized-distilled water. Reaction vessels without cleaning solution were cleaned with Heikol® and rinsed 6 times with hot water and 10 times with deionized-distilled water. Values are mean and SE of 3 determinations. Experiment is representative of 4.

Homogenization was carried out with a Potter-Elvehjem homogenizer in a cold room (temp 0-4°C). The homogenization procedure never lasted more than 10 sec. Centrifugation of the brei was carried out in a Servall refrigerated centrifuge at a running speed temperature of 0°C, at 700 g for 10 min. The supernatant was then centrifuged at 8,400 g for 10 min and the resulting supernatant discarded. The dark brown mitochondrial pellet obtained was resuspended in 12.0 ml of 0.25 M sucrose, transferred to a 15.0 ml plastic centrifuge tube and centrifuged at 8,400 g for 10 min. Once again, the pellet was resuspended in an additional 12.0 ml of the 0.25 M sucrose solution and centrifuged at 8,400 g for 10 min. The final mitochondrial pellet was resuspended in the sucrose solution. In this final suspension, 1 gm of liver is equivalent to 1.0 ml of the stock suspension of mitochondria.

SELECTION OF REACTION VESSELS

Kimax round bottom test tubes 11 mm x 110 mm served as vessels in which mitochondrial swelling was monitored. Each tube was selected free of scratches, filled with buffer and examined in a spectrophotometer at 520 mμ. If the optical density changed more than 0.01 units during the course of a full rotation of the tube, it was discarded.

MEASUREMENT OF MITOCHONDRIAL VOLUME CHANGES

Studies on mitochondrial volume changes were performed at 18-20°C. Volume changes were determined by following optical density changes at 520 mμ, as described by Cleland (1952), with a Bausch and Lomb Spectronic 20 spectrophotometer. For each optical density determination, the reaction tube was removed from the bath, wiped dry, read as rapidly as possible, and immediately returned to the water bath. The reaction mixture contained 4.9 ml of 0.125 M Tris-HCl buffer, pH 7.4, as the suspending medium for mitochondria, 0.04 to 0.06 ml of the mitochondrial stock suspension

depending on the mitochondrial preparation and the test substance in 0.1 ml. In all cases, a volume of mitochondrial stock suspension, which in a preliminary test with the reaction mixture had yielded an optical density of approximately 0.6, was used. The zero reading was recorded 10 sec after the addition of mitochondria and experimental time intervals are indicated on the abscissa of each figure. Mitochondrial protein was determined according to the method of Lowry *et al.* (1951). Statistical procedures as described by Burn *et al.* (1953) were utilized. For mitochondrial swelling experiments, test substances were added to the suspending media prior to the addition of mitochondria.

EXPERIMENTS USING PROPYLTHIOURACIL

In vivo experiments with PTU were of two types, acute and chronic. In the acute experiments, rats were injected with a single dose of PTU, 15 mg i.p. and liver mitochondria isolated at time intervals up to 24 hr. Four groups of rats were used in chronic experiments which were fed food and water *ad libitum*. These groups received (1) saline, (2) T4, 25 µg i.p, (3) PTU, 15 mg i.p. and (4) PTU and T4. T4 was dissolved in a small volume of 0.01 N NaOH and diluted to the required volume. PTU was dissolved in a small volume of 1.0 N NaOH and adjusted to pH 8.5. The compounds were injected daily up to 10 days in a total volume of 0.5 ml and no overt signs of peritoneal irritation was noted after the injections. Mitochondria were isolated 16 hr after the last drug administration in the chronic experiments. The isolated rat liver mitochondria of each of the above groups were exposed to T4 and the resulting mitochondrial swelling curve was monitored.

ASSAY FOR THYROXINE DEIODINASE

In the determination of T4 deiodinase activity, the experimental conditions were maintained as closely as possible to those of the swelling

studies. Consequently, two procedures were employed. In the first procedure, the reaction mixture was composed of 0.125 M-0.02 M Tris-HCl buffer, pH 7.4, 0.05 ml of mitochondrial stock solution and 0.1 ml of a T4 solution (final concentration 5×10^{-6}M). The reaction was initiated by the addition of mitochondria. In the second procedure, the method of Wynn et al. (1962) and the recommendations of Yamamoto (1964) were used with modifications. The composition of the reaction mixture was 1.5 ml of 0.5 M Tris HCl buffer, pH 7.4, 0.1 ml of T4 (final concentration 5×10^{-6}M), and the addition of 0.1 ml of the mitochondrial stock solution initiated the reaction. In both procedures, incubation was carried out at 20°C in the light with shaking in a Dubnoff metabolic shaker.

T4 was dissolved in a minimum amount of 0.1 M KOH and diluted to the required volume with distilled water. This solution also contained 2.0 µC of I^{125} labeled T4 in 5.0 ml.

After incubation, a 1.0 ml aliquot was removed and 0.2 ml of dog blood plasma added to absorb the T4. This mixture was cooled for 30 min and 0.3 ml of 50% trichloroacetic acid containing carrier I^- in a concentration of 2×10^{-3}M (with a trace of ascorbate). A 0.3 ml aliquot of the supernatant was added to 20 ml of scintillation fluid and radioactivity counted in a Packard Tri-Carb scintillation counter. Alternately, a 100 µl aliquot was applied to chromatographic paper and descending chromatography was performed in a solvent system consisting of n-butanol : acetic acid : water (78 : 5 : 17). After drying the paper strips were cut in 1 cm sections and placed in the scintillation fluid for counting.

ESTIMATION OF THE BINDING OF THYROXINE TO MITOCHONDRIA

T4 labeled with I^{125} was added to the cold T4, 0.225 mg/ml of L-T4 sodium pentahydrate, and the experimental procedure was as described previously. Each reaction vessel contained 0.02 µl of I^{125}-T4. After 8 min

of incubation, the mixture was placed in a Spinco Ultracentrifuge and centrifuged at 20,000 g's for 5 min. The supernatant was decanted and the mitochondrial pellet was washed with 5 ml of 0.125 M KCl-0.02 M Tris - HCl buffer, pH 7.4, and recentrifuged. The mitochondria were washed and recentrifuged at 20,000 g's 5 times. After the fourth washing, the mitochondria were transferred to another tube in order to remove radioactive T4 adhering to the tube. The mitochondrial pellet was then transferred to 10.0 ml of a scintillation mixture and counted in a Packard liquid scintillation spectrometer or dissolved in 1.0 N NaOH and then the radioactivity was counted.

FLUORESCEIN MERCURIC ACETATE

FMA was prepared according to the method described by Karush et al. (1964) and exhibited the appropriate fluorescence spectrum. Subsequently, a commercial source of FMA became available and the commercial material showed the same fluorescence and biological activity. In these FMA experiments, the fluorescence changes were monitored with an Aminco-Bowman spectrophotofluorometer. The experiments were performed at 0°C and 20°C. In order to measure both the mitochondrial volume changes and the fluorescence intensity changes, the spectrophotofluorometer and spectrophotometer were placed in close proximity to each other. After preparation of the mitochondrial suspension, an aliquot was removed for fluorescence studies and the remainder was utilized in swelling studies. Fluorescence intensity was only measured at 20°C. In the mitochondrial swelling experiments, the temperature was maintained at 0°C by keeping the tubes containing the mitochondrial suspension in a tub of melting ice. For each spectrophotometer reading, the reaction vessel was removed, wiped dry, read rapidly and immediately returned to the tub of melting ice.

REAGENTS, DRUGS AND HORMONES

The following chemical compounds have been utilized in this investigation. All compounds were dissolved in deionized-distilled water and any specific manipulations for certain compounds are described.

Acetic Acid Glacial	The British Drug Houses, Ltd.
Albumin (bovine)	Nutritional Biochemicals Corp.
1, 4-bis-2-(4-methyl-5-phenyloxazolyl) benzene, Dimethyl POPOP	Packard Instrument Co. Inc.
Butanol	The British Drug Houses, Ltd.
n-Butyl 3, 5-diiodo-4-hydroxybenzoate	Aldrich Chemical Co.
Chromic Chloride	The British Drug Houses, Ltd.
Cyanogen Iodide	Nutritional Biochemical Corp.
Diethyl Ether	The British Drug Houses, Ltd.
2-5-diphenyloxazole, PPO	Nuclear Enterprises, Ltd.
3-5-Diiodo-L-Thyronine	Mann Research Laboratories, Inc.
N-Ethyl Maleimide	Mann Research Laboratories, Inc. and Sigma Chemical Co.
Ethylene Glycol Monomethyl Ether	Fisher Scientific Co.
Fluorescein Mercuric Acetate	Nutritional Biochemical Corp.
Fluorescein Sodium	The British Drug Houses, Ltd.
Glutathione (oxidized)	Sigma Chemical Co.
p-Hydroxymercuribenzoate	Sigma Chemical Co.
Insulin	bovine pancreas, recrystallized, 24.5 u/mg, Mann Research Laboratories, Inc.
Iodide Potassium	Nutritional Biochemicals Corp.
Iodine	Merck & Co. Ltd.
Iodoacetamide	Sigma Chemical Co.

Lysine-8-vasopressin	synthetic, 260 U/mg, Sandoz Chemical Co. and purified, 260 U/mg, National Institutes of Health, U.S.A. were obtained in sealed vials. The vial contents were diluted to concentrations of 10 U/ml with saline and a 1 ml aliquot was placed in a plastic vial and stored at -20°C. Preparations were thawed immediately prior to utilization. When more concentrated solutions were desired, the original sealed vial was diluted to the proper concentration and 1 to 10 dilutions were carried out with distilled water.
Mercapto Acetic Acid (Sodium Thioglycolate)	Matheson, Coleman & Bell
Mercuric Acetate	The British Drug Houses, Ltd.
Mercuric Chloride	The British Drug Houses, Ltd.
Ninhydrin Spray	Sigma Chemical Co.
Nitric Acid	Fisher Scientific Co.
Oxytocin	synthetic, 410 U/mg, obtained in a sealed vial from Sandoz Chemical Co. Oxytocin dilutions were carried out according to procedures described under lysine-8-vasopressin.
Phenol Reagent, 2 N (Folin-Ciocalteau)	Fisher Scientific Co.
Potassium Chloride	The British Drug Houses, Ltd.
Potassium Cyanide	Merck & Co. Ltd.
Potassium Hydroxide	The British Drug Houses, Ltd.
Propylthiouracil	Nutritional Biochemical Corp.

Pyridoxal HCl	California Foundation for Biochemical Research and Nutritional Biochemical Corp.
Silver Nitrate	Merck & Co. Ltd.
Sodium Iodide I^{131}	Abbott Laboratories
Standard Buffer Solutions pH 7.0	Fisher Scientific Co.
Sucrose	The British Drug Houses, Ltd.
Tham, Tris (Hydroxymethyl) Aminomethane	Fisher Scientific Co.
L-Thyroxine I^{125}	Volk Chemical Co.
L-Thyroxine Sodium Pentahydrate	Mann Research Laboratories, Inc.
Toluene	The British Drug Houses, Ltd.
Trichloroacetic Acid	The British Drug Houses, Ltd.

RESULTS

EFFECT OF N-ETHYLMALEIMIDE ON THYROXINE-INDUCED MITOCHONDRIAL SWELLING

The effects of varying concentrations of T4, ICN and NEM on mitochondrial volume are illustrated in Fig 1. T4, 2.5×10^{-6}M, promoted measurable mitochondrial swelling and, with increases in T4 concentration, the rate of swelling was increased. T4, 5×10^{-6}M, promoted rapid swelling within 10 min and between 15 and 20 min the swelling curve began to plateau. The 10^{-5}M concentration of T4 induced near maximal mitochondrial volume changes in 4 min. In concentrations equimolar with T4, ICN promoted mitochondrial swelling which was more rapid and of greater magnitude than T4. Mitochondrial volume changes in the presence of ICN, 10^{-6}M, were slightly above the level of spontaneous mitochondrial swelling. The rate of mitochondrial swelling induced by ICN was proportional to the concentration. On the other hand, the sulfhydryl reagent NEM did not promote mitochondrial swelling at 10^{-5}M. The NEM concentration of 10^{-4}M promoted rapid mitochondrial volume changes and NEM, 10^{-3}M, induced mitochondrial swelling which was near maximum in 4 min. In promoting mitochondrial swelling, ICN was more potent than T4 which, in turn, was more potent than NEM.

Although NEM was reported to be ineffective in blocking T4-induced swelling (Rall et al. 1963), the possibility that the concentration of NEM might be critical was investigated. Therefore, a range of NEM concentrations from 5×10^{-5} to 10^{-4}M were tested for their action on T4-induced mitochondrial swelling. The concentration of T4 selected was 5×10^{-6}M because either stimulation or inhibition of mitochondrial swelling at this T4 concentration could be measured. In Fig 2, the results of these experiments are depicted in bar graph fashion as the total optical density change after 1 hr of exposure of mitochondria to these agents. The lower concentrations of NEM examined, 5×10^{-6} and 10^{-5}M, did not induce swelling, but were inhibitory to the mitochondrial swelling induced by T4. On the other

Fig 1

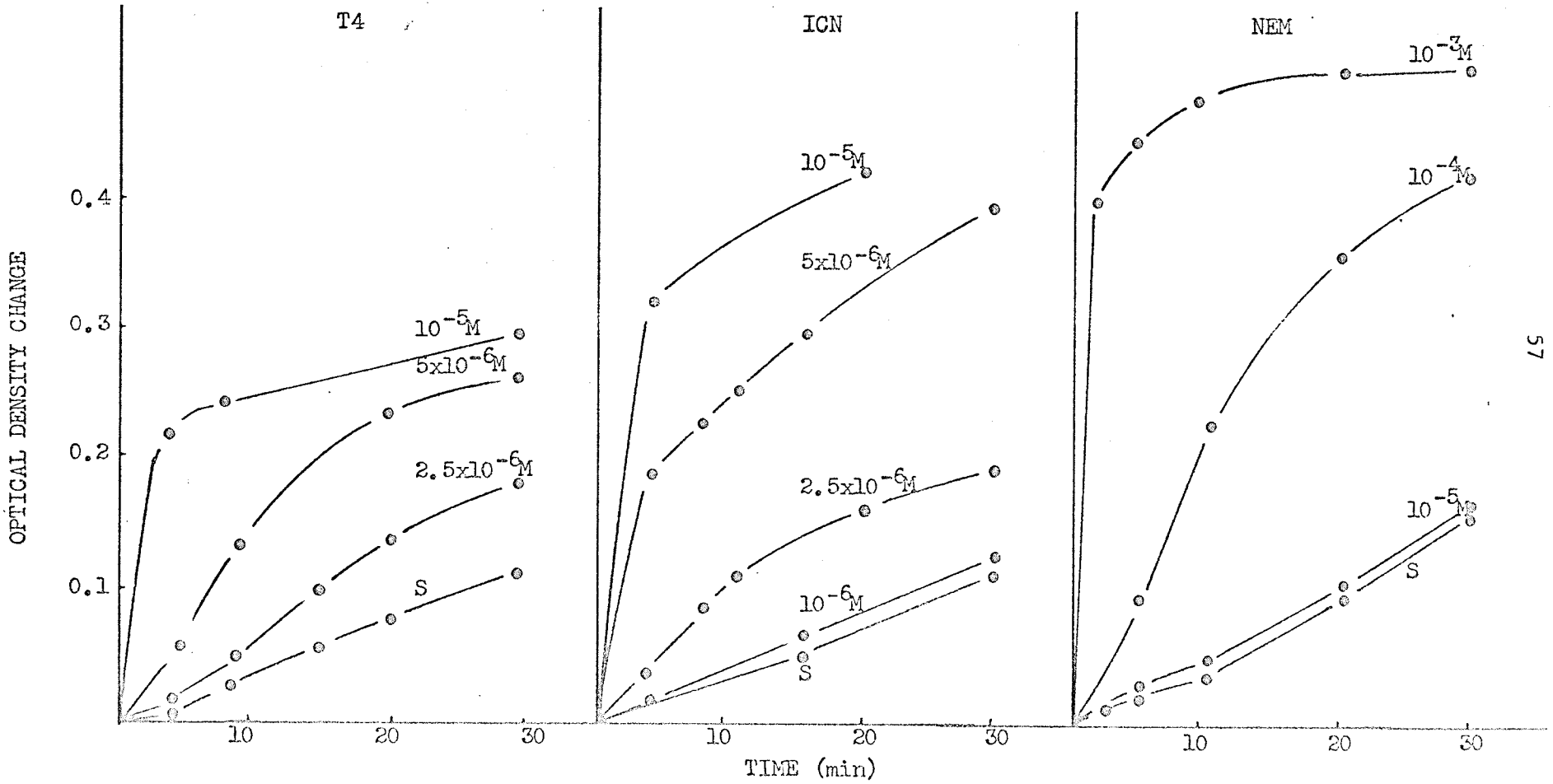

Fig 1. Effect of T4, ICN and NEM on mitochondrial volume. The suspending medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final molar concentrations. Spontaneous (S).

Fig 2

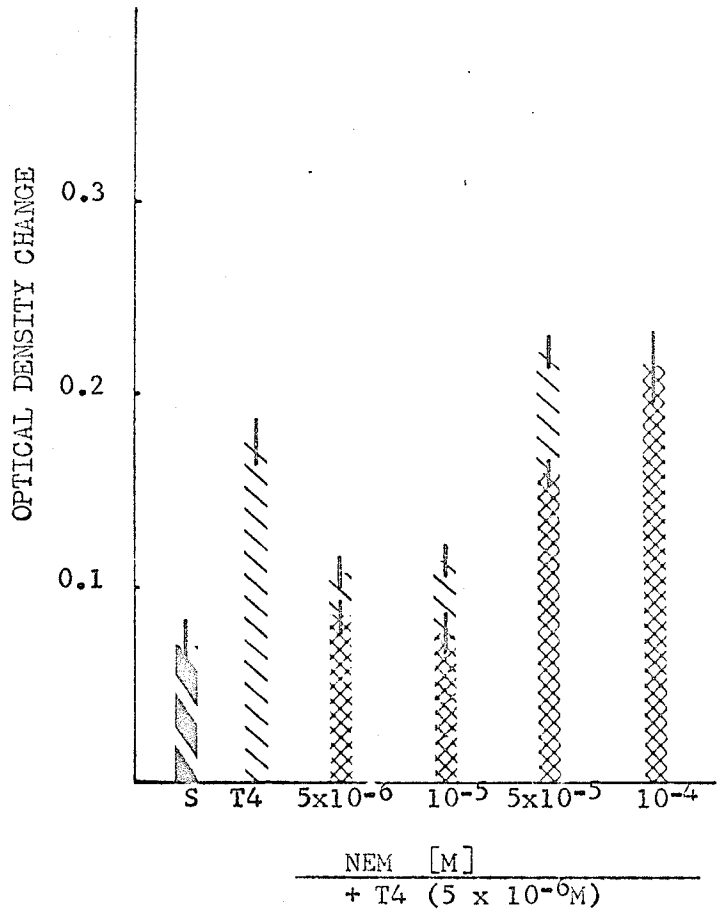

Fig 2. Effect of various concentrations of NEM on T4-induced mitochondrial swelling. Molarity in all cases is final concentration. T4 concentration, 5×10^{-6}M. The medium was 0.125 M-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Experimental time, 1 hr. Experiment is representative of 3 performed. Values are mean and SE of 3 determinations. Spontaneous (S). Crosshatched : NEM alone.

hand, mitochondrial swelling was induced by NEM, 5×10^{-5} and 10^{-4}M, but these concentrations of NEM in combination with T4 did not produce a response less than T4, by itself. Thus, NEM was able to inhibit the T4-induced mitochondrial swelling response. The NEM concentration which inhibited the T4-induced swelling was critical and approximated the swelling threshold of NEM-induced mitochondrial swelling.

The converse of the experiment depicted in Fig 2 was done. A swelling threshold concentration of NEM was examined for its influence on mitochondrial swelling induced by various concentrations of T4. These data are listed in Table 2. The concentration of NEM in all cases was 10^{-5}M. The per cent of control response increased as the concentration of T4 increased. Thus, it was demonstrated that the increase in T4 concentration can reduce the inhibition of T4-induced swelling by NEM, 10^{-5}M and at T4, 10^{-5}M, the inhibition by NEM, 10^{-5}M, was fully prevented.

It was considered that preincubation of mitochondria with NEM, 10^{-5}M, in the suspending medium would permit more NEM to react with mitochondria and result in a greater degree of inhibition of the T4-induced swelling response by NEM. The preincubation time was 15 min and the results of the experiments are illustrated in Fig 3. The NEM preincubated mitochondria displayed a spontaneous swelling similar to NEM, 10^{-5}M, and in both cases it was less than the non-NEM-preincubated controls. Both the rate and magnitude of the T4-induced mitochondrial swelling was reduced in the NEM-preincubated mitochondria. The mitochondrial swelling curve of T4 + NEM displayed a greater inhibition than when the NEM was present only during preincubation, but the per cent of inhibition of T4 + NEM was greatest in the non-preincubated control. The results of these experiments reaffirm the previous conclusion that NEM inhibits the T4-induced swelling response and demonstrate that preincubation of mitochondria with NEM, 10^{-5}M, enhances the NEM inhibition of the T4-induced swelling response.

Table 2

EFFECT OF N-ETHYLMALEIMIDE ON MITOCHONDRIAL SWELLING INDUCED BY VARIOUS CONCENTRATIONS OF THYROXINE

T4 [M]	Response to NEM + T4 as per cent of T4 control	
	10 min	30 min
$1x10^{-6}$	0.0	7.0
$2.5x10^{-6}$	20.3	16.5
$5x10^{-6}$	40.8	35.9
$1x10^{-5}$	97.3	100

The suspending medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations. NEM, 10^{-5}M.

Fig 3

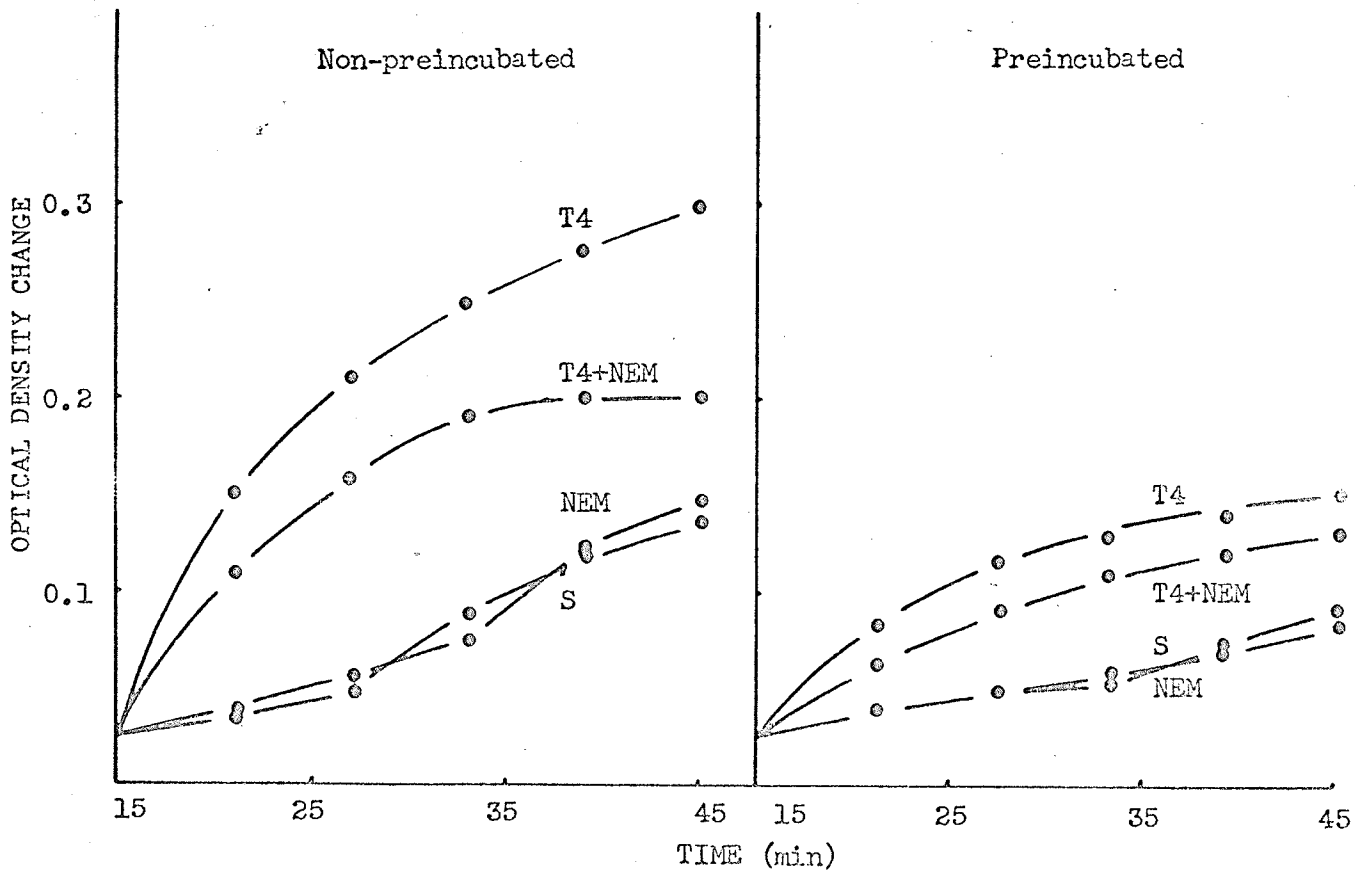

Fig 3. Effect of preincubation of mitochondria with NEM on T4-induced mitochondrial swelling. The suspending medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria were incubated 15 min with NEM, 10^{-5}M. Reagents added at 15 min. Some mitochondrial swelling occurred during the preincubation period and the curves therefore begin above the origin. Concentrations are final concentrations. NEM, 10^{-5}M; T4, 5×10^{-6}M.

In order to determine whether the inhibition of T4-induced swelling might be the result of a direct reaction between T4 and NEM, the experiment illustrated in Fig 4 was performed. NEM and T4 were incubated in the suspending medium individually and in combination prior to the addition of mitochondria. In both cases, the mitochondrial swelling curves of the preincubated and control groups were virtually identical. Therefore, no inhibition occurred as a result of a direct chemical interaction of NEM and T4.

ICN is a potent mitochondrial swelling agonist which induces swelling with characteristics similar to that with T4. NEM, 10^{-5}M, was tested to determine whether it would inhibit ICN-induced mitochondrial swelling. ICN was examined at concentrations of 2.5×10^{-6}, 5×10^{-6} and 10^{-5}M. These results appear in Fig 5. The swelling curves for each concentration of ICN and for ICN + NEM were similar. Therefore, ICN-induced mitochondrial swelling was not inhibited by NEM, 10^{-5}M.

Since NEM is a compound which exhibits a high degree of affinity for sulfhydryl groups, it was considered of interest to examine other reagents which react with sulfhydryl groups for their ability to inhibit the T4-induced mitochondrial swelling response. Fig 6 depicts the results of the experiments with $HgCl_2$, which is also a potent mitochondrial swelling-inducing agent. A threshold swelling-inducing concentration of $HgCl_2$ was determined and then tested for inhibition of T4-induced swelling. $HgCl_2$, 5×10^{-8}M, approximated the threshold swelling response and, by itself, promoted a low degree of swelling. $HgCl_2$ was able to inhibit the T4-induced mitochondrial swelling response.

Additional experiments were performed utilizing other sulfhydryl reagents and these were also tested for their ability to inhibit the T4-induced mitochondrial swelling. In each case, a swelling threshold concentration of the compound was determined and then the sulfhydryl reagent

Fig 4

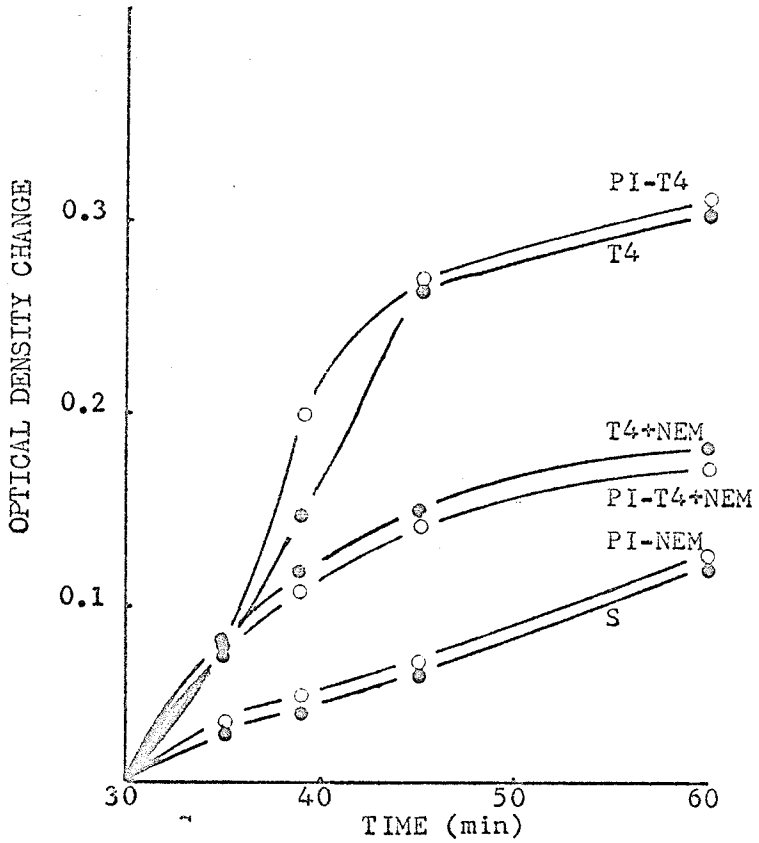

Fig 4. Effect of preincubation of NEM + T4 in the absence of mitochondria on T4-induced mitochondrial swelling. NEM, 10^{-5}M, and T4, 5 x 10^{-6}M, were preincubated for 30 min in the suspending medium. Mitochondria added at 30 min. The suspending medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Preincubated (PI). Concentrations are final concentrations.

Fig 5

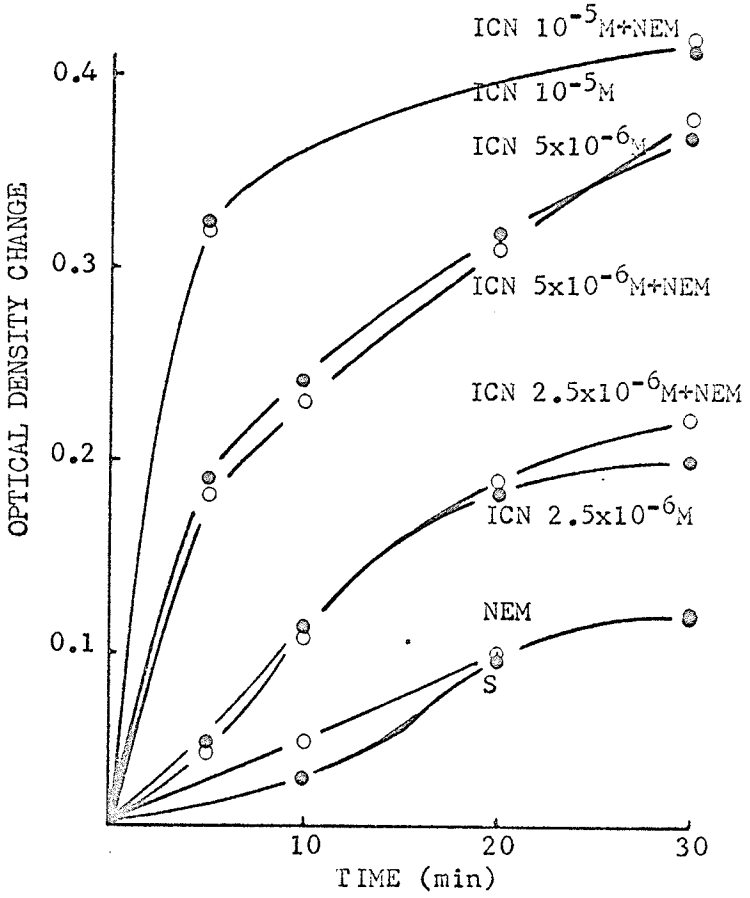

Fig 5. Effect of NEM on ICN-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, open circles, NEM, 10^{-5}M.

Fig 6

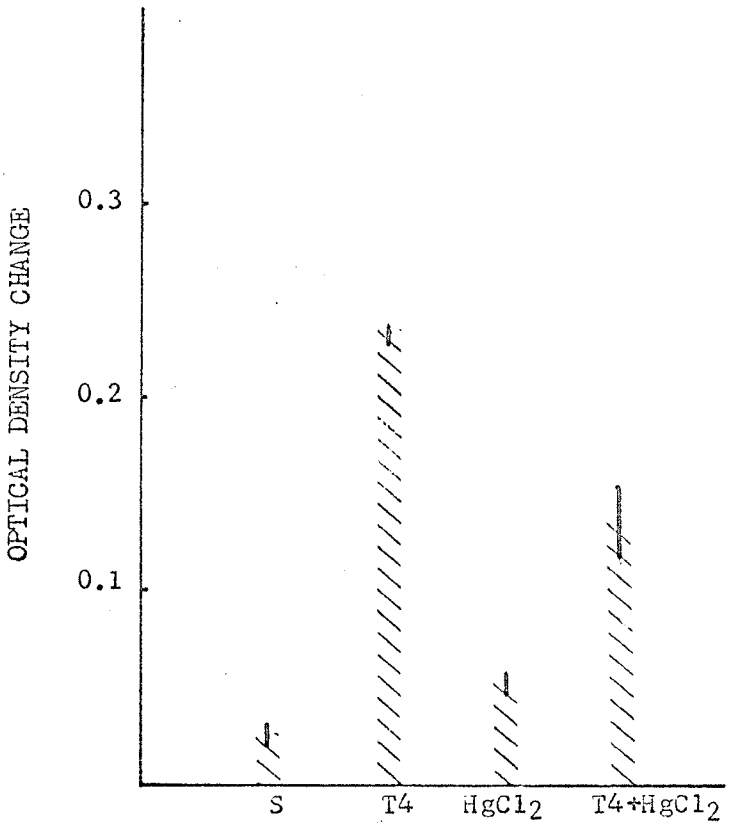

Fig 6. Effect of $HgCl_2$ on T4-induced mitochondrial swelling. The suspending medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Duration of experiment 10 min. Values are mean and S E of 3 determinations. Experiment is representative of 5. Concentrations are final concentrations, T4, 5×10^{-6}M; $HgCl_2$, 5×10^{-8}M.

was tested. Table 3 lists these compounds, which are NEM, $HgCl_2$, IA, p-HMB, and FMA. Both NEM and $HgCl_2$ inhibited the T4-induced response, whereas IA, p-HMB, and FMA did not. These experiments demonstrated that not all sulfhydryl reagents were capable of inhibiting the T4-induced mitochondrial swelling response.

Ca^{++} and PO_4 are two known inducers of mitochondrial swelling and NEM was tested for inhibition of mitochondrial swelling induced by these two compounds. NEM was utilized in a concentration of 10^{-5}M and Ca^{++} and PO_4 in concentrations of 10^{-3}M. The results of these experiments are depicted in bar graph fashion in Fig 7. NEM, 10^{-5}M, inhibited the Ca^{++}- or PO_4-induced mitochondrial swelling. The degree of inhibition was slightly greater with the Ca^{++}-induced response. These experiments demonstrate that NEM is not specifically inhibitory for T4-induced mitochondrial swelling.

SUMMARY

NEM inhibited T4-induced mitochondrial swelling response but the concentration of NEM was critical. NEM, 10^{-5}M, inhibited the T4-induced mitochondrial swelling response but, by itself, was without effect. Preincubation of NEM with mitochondria enhanced this inhibition. It appears the inhibition is at the mitochondrial level, since preincubation of T4 and NEM did not influence the T4-induced swelling response. Moreover, NEM did not inhibit the ICN-induced mitochondrial swelling response. Of several sulfhydryl reagents tested, only NEM and $HgCl_2$, in concentrations approximating their threshold swelling level, inhibited the T4-induced mitochondrial swelling response. NEM was also able to inhibit the Ca^{++} and PO_4-induced mitochondrial swelling.

Table 3

EFFECT OF SULFHYDRYL REAGENTS ON THYROXINE-INDUCED MITOCHONDRIAL SWELLING

Compound	Swelling Threshold [M]	Effect
NEM	10^{-5}	inhibition
$HgCl_2$	$5x10^{-8}$	inhibition
IA	10^{-5}	none
p-HMB	$5x10^{-6}$	none
FMA	10^{-7}	none

The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations. T4, 5 x 10^{-6}M. Sulfhydryl reagents were tested at their swelling threshold concentration. Time 8 min.

Fig 7

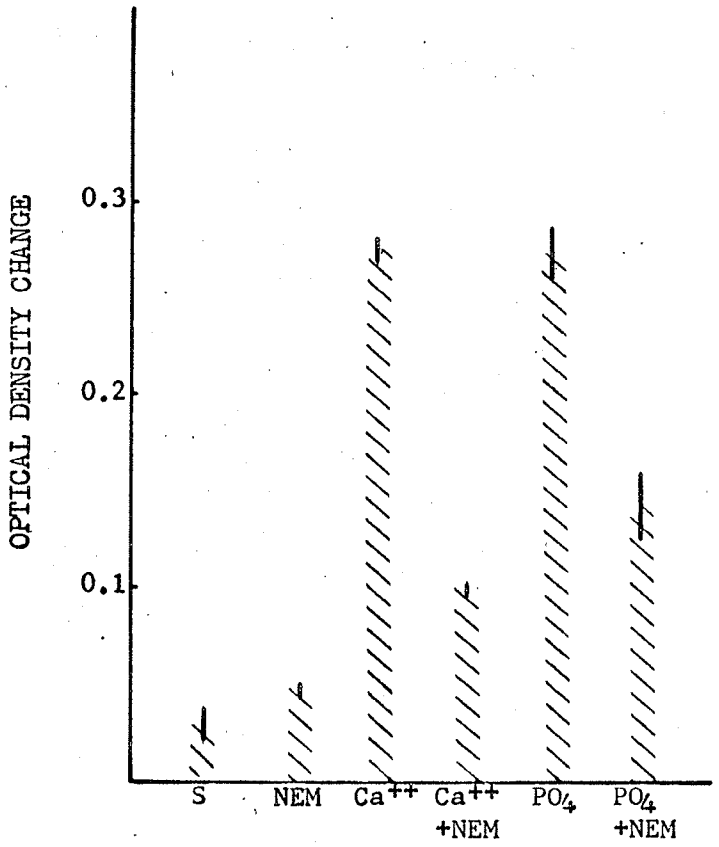

Fig 7. Effect of NEM on Ca^{++} and PO_4-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria were added at time zero. Final concentration, NEM, 10^{-5}M; Ca^{++}, 10^{-3}M; PO_4, 10^{-3}M. Values are mean and SE of 3 determinations. Experiment is representative of 5. Time, 8 min.

INTERACTION OF IODOCOMPOUNDS AND SULFHYDRYL REAGENTS IN THE INDUCTION OF MITOCHONDRIAL SWELLING

A. Effect of fluorescein mercuric acetate on mitochondrial volume and modification of its fluorescence by mitochondria.

The above experiments demonstrate that sulfhydryl reagents may modify the T4-induced swelling response, and Lehninger and Schneider (1959) suggested that sulfhydryl groups may be involved in mitochondrial swelling induced by T4. Consequently, the theory that T4 or ICN might modify the mitochondrial swelling response induced by sulfhydryl reagents by virtue of an action on sulfhydryl groups of mitochondria was formulated and investigated in this work. FMA was utilized because it is a sulfhydryl reagent and its fluorescence properties change when it reacts with sulfhydryl groups. Previously, FMA was proposed as a reagent for the assay of sulfhydryl and disulfide groups of protein (Karush et al. 1964). The basis of this assay was the quenching of fluorescence of FMA. The fluorescence changes of the FMA molecule in the presence of mitochondria might reflect reaction with mitochondrial sulfhydryl groups.

Initially, the effect of FMA on mitochondrial volume was investigated and the results of incubating various concentrations of FMA with mitochondria are illustrated in Fig 8. A FMA concentration of 10^{-7}M approximates the threshold for FMA-induced mitochondrial swelling. FMA, 5×10^{-7}M, promoted mitochondrial swelling which was immediate and rapid, and increasing concentrations produced more rapid mitochondrial swelling. At 10^{-5}M, the FMA-induced swelling was near maximal in 3 min. Hence, FMA is an extremely potent inducer of mitochondrial swelling. Fluorescein, which does not contain any Hg^{++} groups, did not promote mitochondrial swelling.

Since mitochondria contain sulfhydryl groups, the effect of mitochondria on the fluorescence of FMA was examined. Fig 9 shows the effect of changes in mitochondrial concentration on fluorescence of FMA, 10^{-5}M. The

Fig 8

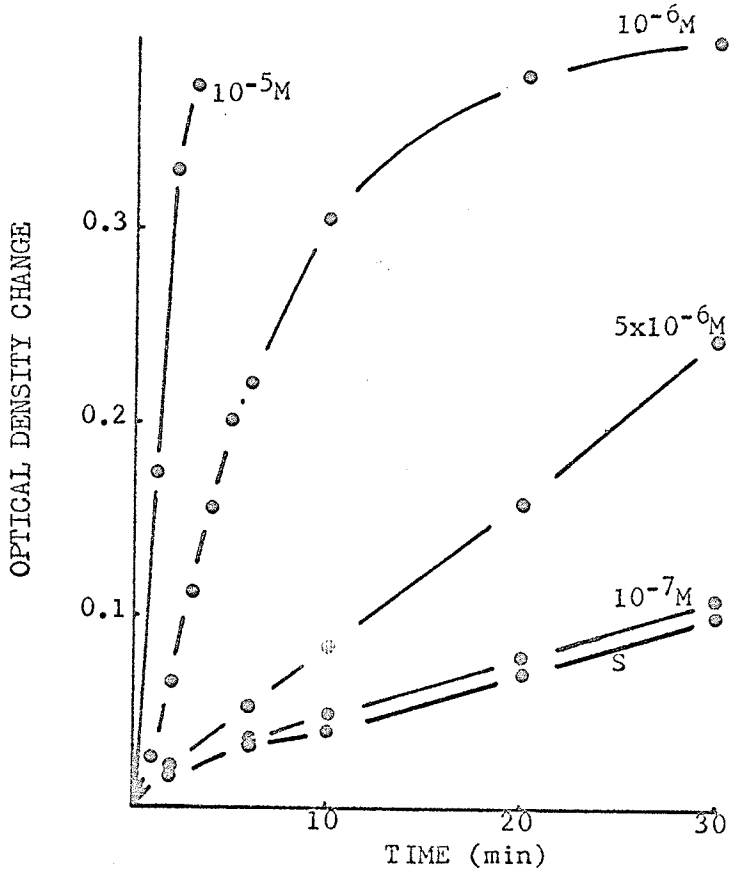

Fig 8. Effect of FMA on mitochondrial volume. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations of FMA.

Fig 9

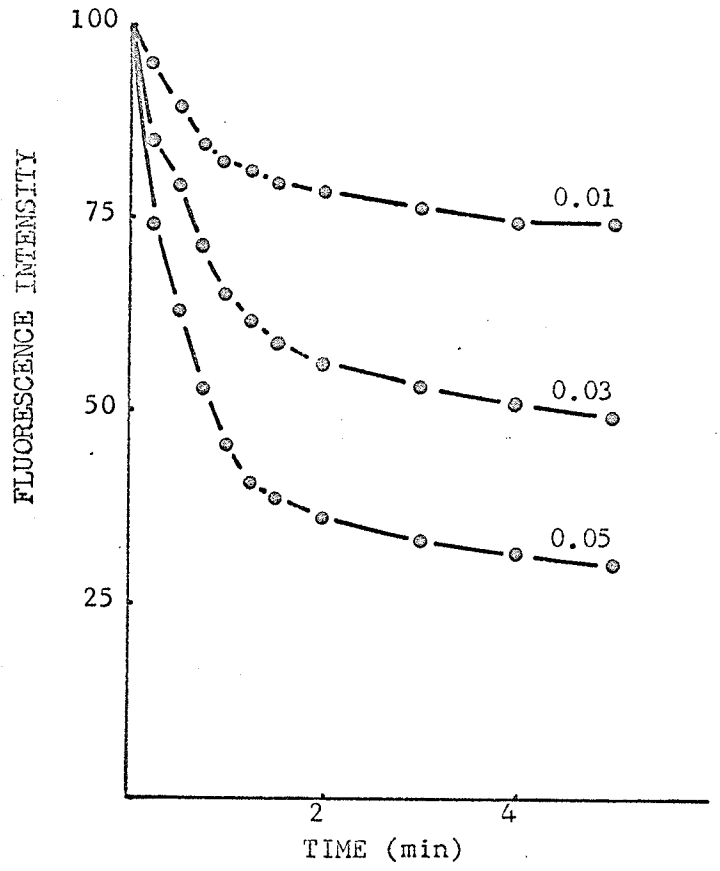

Fig 9. Effect of mitochondria on fluorescence of FMA. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 22°C. Mitochondria added at time zero. Final concentration of FMA, 10^{-5}M. Values are in ml of mitochondrial stock solution added to suspending medium. Protein 95 µg/ml in reaction mixture after addition of 0.05 ml of stock solution. FMA, absorption, 498 mµ; emission, 522 mµ.

quenching was rapid and the earliest time interval of the measurements was 15 sec at room temperature. The fluorescence intensity of FMA decreases rapidly in proportion to the concentration of mitochondria. Moreover, the fluorescence of fluorescein was not quenched by mitochondria.

In Table 4, the results of an experiment on the effect of a constant concentration of mitochondria on various concentrations of FMA are listed. The differences in the initial degree of quenching is probably due to FMA reacting with a greater portion of the added mitochondria, thus reducing the total amount of free FMA in the medium. At 5 min, the quenching is greatest with the lowest concentration of FMA, and lowest with the highest FMA concentration. This may be due to the addition of greater amounts of FMA.

The time relationships between the mitochondrial swelling curve after exposure to FMA, 5×10^{-6}M, and the quenching of its fluorescence, are depicted in Fig 10. The time course of these experiments is rapid and the changes were recorded at 15 sec intervals. In 15 sec, 40% of the total fluorescence intensity change occurred, but only 2.8% of the total volume change occurred in this same period. The changes in fluorescence intensity seem to precede the changes in mitochondrial volume.

T4, ICN, I_2 and I^- were inoubated, in the absence of mitochondria, with FMA in order to determine the effect of these compounds, individually, on the fluorescence of FMA. The results of these experiments are contained in Table 5. T4 and ICN, in the absence of mitochondria, did not cause quenching of the fluorescence of FMA, but I_2 and I^- did. The quenching of FMA's fluorescence by I_2 and I^- was immediate.

Fig 11 depicts the influence of T4 and ICN on the fluorescence intensity changes of FMA in the presence of mitochondria. T4 concentrations of 2.5×10^{-6} and 5×10^{-6}M were added to the mitochondrial suspensions with FMA, 5×10^{-6}M. Each combination of T4 with FMA produced a somewhat more

Table 4

EFFECT OF MITOCHONDRIA ON THE FLUORESCENCE OF VARIOUS CONCENTRATIONS OF FLUORESCEIN MERCURIC ACETATE

FMA [M]	Per cent quenching of fluorescence	
	15 sec	5 min
10^{-6}	70	95
2.5×10^{-6}	60	92
5×10^{-6}	36	86

The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 22°C. Mitochondria added at time zero. Concentrations are final concentrations. Protein 109 µg/ml in reaction mixture after addition of 0.06 ml of stock suspension.

Fig 10

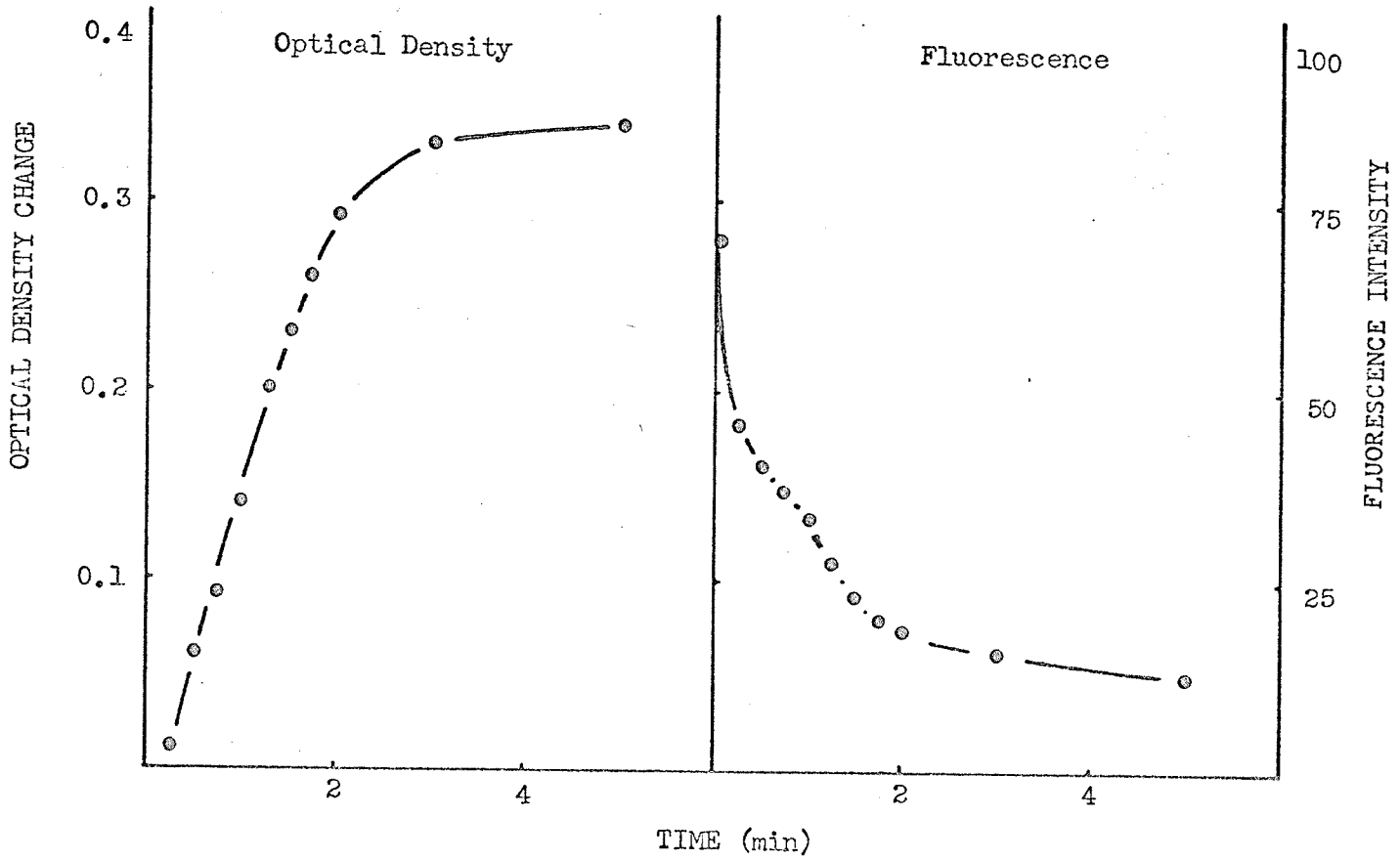

Fig 10, Comparison of FMA-induced mitochondrial swelling and the associated fluorescence intensity change. The medium was 0.125 M KCl-0.2 M Tris-HCl buffer, pH 7.4. Temperature 22°C. Mitochondria added at time zero. Final concentration of FMA, 5×10^{-6}M.

Table 5

EFFECT OF IODOCOMPOUNDS ON THE FLUORESCENCE OF FLUORESCEIN MERCURIC ACETATE

Compound [M]	Fluorometer Reading	
	15 sec	30 min
FMA + none	72	70
FMA + T4, $5x10^{-6}$	70	68
FMA + ICN, $5x10^{-6}$	70	68
FMA + I_2, $2.5x10^{-6}$	15	10
FMA + I^-, $5x10^{-4}$	17	12

The medium was 0.125 M KCl-0.02 M Tris-HCl, pH 7.4. Temperature 22°C. Compounds added at time zero. Concentrations are final concentrations. FMA, $5x10^{-6}$M. Fluorometer reading, 72 at zero time.

Fig 11

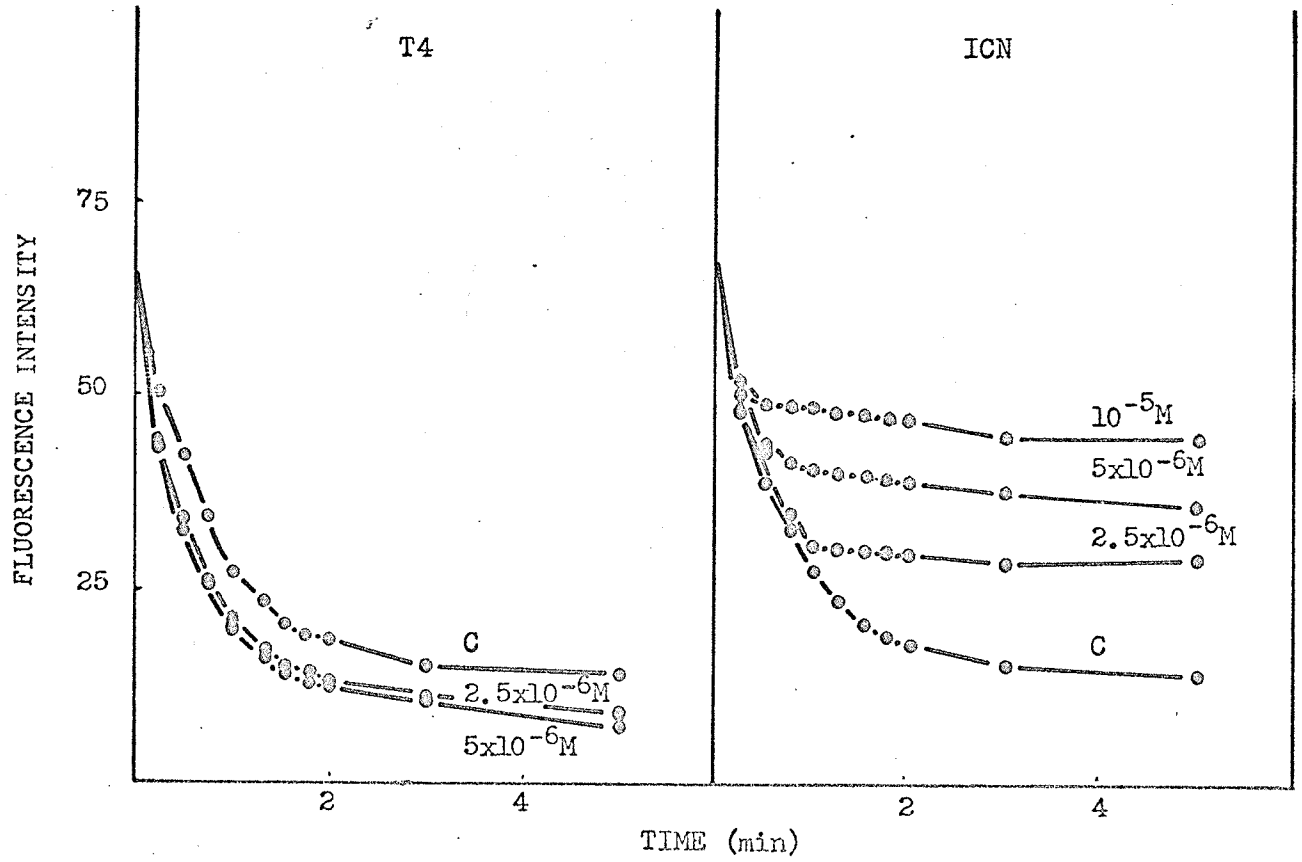

Fig 11. Effect of T4 and ICN on the fluorescence quenching of FMA by mitochondria. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 22°C. Mitochondria added at time zero. Concentrations are final concentrations. FMA, 5 x 10^{-6}M. Control (C), FMA + mitochondria.

rapid quenching of the fluorescence of FMA in the presence of mitochondria than seen with FMA alone. However, there was no apparent effect of T4 concentration on quenching of the fluorescence of FMA since the curves with all concentrations of T4 overlapped. ICN was also tested in concentrations of 2.5×10^{-6}, 5×10^{-6} and 10^{-5} for effects on the quenching of the fluorescence of FMA by mitochondria. In each of these combinations of ICN and FMA, the curves of the fluorescence intensity changes were equivalent in the initial 15 sec but then began to diverge. The magnitude of the total quenching of FMA's fluorescence was decreased with increases in ICN's concentration. From these experiments, it is apparent that T4 slightly enhanced the quenching of FMA's fluorescence by mitochondria, while ICN had inhibited it.

In the presence of mitochondria, Rall et al. (1963) demonstrated that I^- is liberated from the ICN molecule. During the course of ICN's induction of mitochondrial swelling, the compound may dissociate and give rise to CN^-, which then might interfere with the change in fluorescence intensity of FMA in the presence of mitochondria. CN^- is an inhibitor of mitochondrial swelling induced by T4, ICN and NEM. The experiments illustrated in Fig 12 indicate that CN^- can influence the mitochondrial swelling-inducing action of FMA and fluorescence intensity changes of FMA. KCN, 5×10^{-6}M, did not interfere with the swelling induced by FMA but KCN, 10^{-3}M, did. At 10^{-3}M, KCN inhibited the swelling induced by ICN + FMA to a greater extent than it inhibited the FMA-induced swelling. It was observed in these experiments that the mitochondrial swelling induced by ICN + FMA was more rapid than ICN or FMA by themselves. ICN inhibited the quenching of FMA's fluorescence by mitochondria. KCN also inhibited the quenching of the fluorescence of FMA by mitochondria with 5×10^{-6}M partially effective and 10^{-3}M KCN completely inhibitory. At equimolar concentrations, ICN was more effective at inhibiting the quenching of FMA

Fig 12

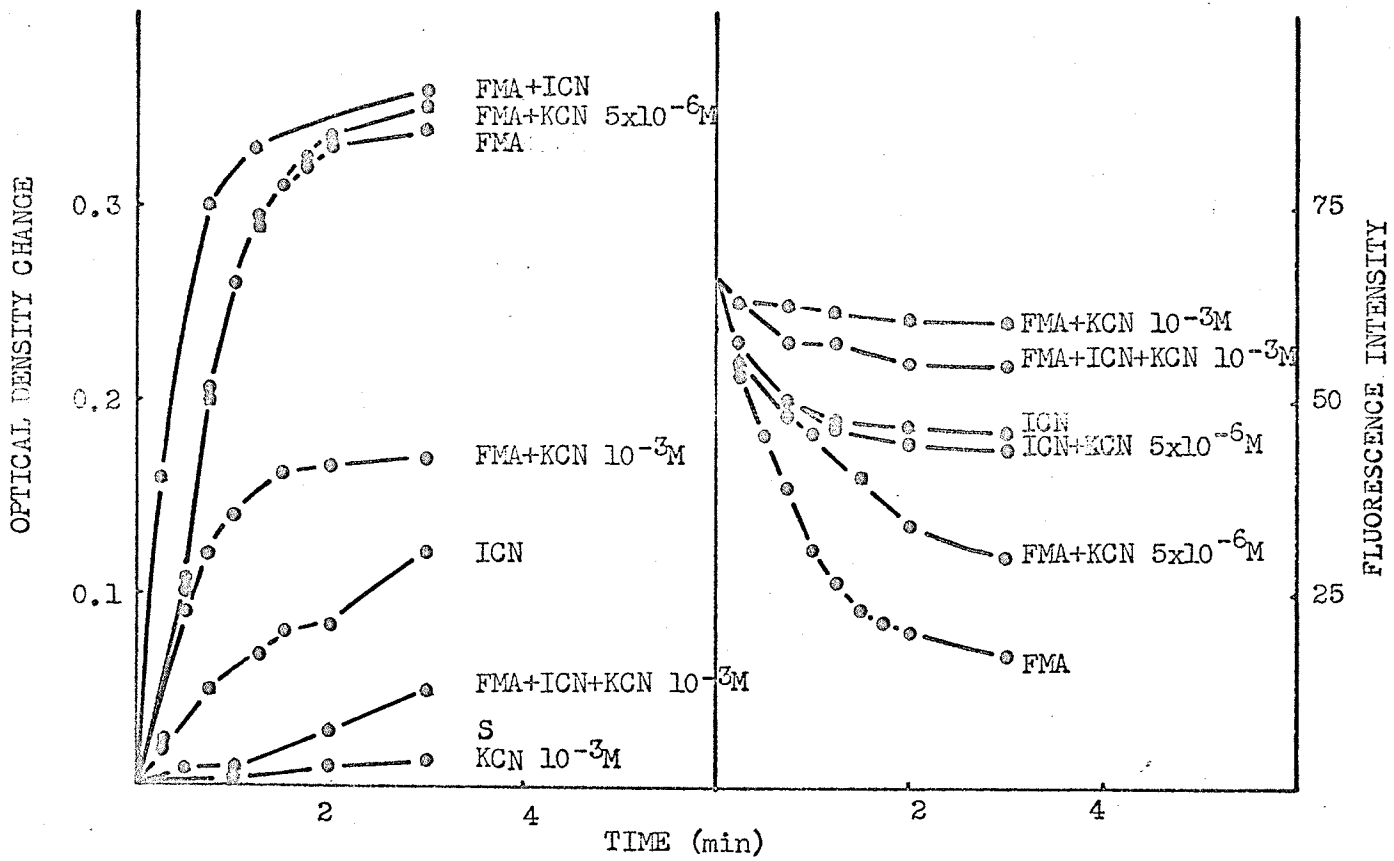

Fig 12. Comparison of the effect of KCN on ICN-and FMA-induced mitochondrial swelling with the fluorescence changes of FMA. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 22°C. Mitochondria added at time zero. Concentrations are final concentrations. FMA, 5 x 10^{-6}M; ICN, 5 x 10^{-6}M; KCN, 10^{-3} and 5 x 10^{-6}M.

than KCN. Thus it appears that ICN may block the quenching of the fluorescence of FMA by mitochondria, partly as a result of the release of CN^- from the ICN molecule.

B. Effect of iodocompounds on mitochondrial swelling induced by sulfhydryl reagents.

Since T4 and ICN influenced the changes in the fluorescence intensity of FMA by mitochondria, the following experiments with T4, ICN, I_2 and I^- were designed to determine their influence on FMA-induced mitochondrial swelling. A 5×10^{-6}M concentration of FMA was utilized, which produced maximal swelling within 5 min. The iodocompounds were utilized in concentrations which produced low degrees of swelling. Fig 13 depicts the results of these experiments. The combination of T4 and FMA produced a swelling curve which was slightly faster than that of FMA. On the other hand, the combination of ICN and FMA promoted swelling which was more rapid than that induced by FMA by itself. I_2 also potentiated the FMA-induced mitochondrial swelling and near maximal swelling occurred in 15 sec. Moreover, I^-, in a concentration which did not induce any mitochondrial swelling, also enhanced the FMA rate of swelling. Thus, the FMA-induced mitochondrial swelling was potentiated slightly by T4 and markedly by I_2, ICN and I^- in decreasing order. The magnitude of the volume change of mitochondria as judged by the optical density was not affected, but the time to reach maximum was decreased.

Since the FMA-induced mitochondrial swelling response and its augmentation by iodocompounds takes place with extreme rapidity, experiments were performed at 0°C to reduce endogenous mitochondrial metabolic effects and to slow the response in order to examine its characteristics. It was not necessary to conduct these experiments for more than 5 min, since the potentiation was evident. The results of these experiments are shown in

Fig 13

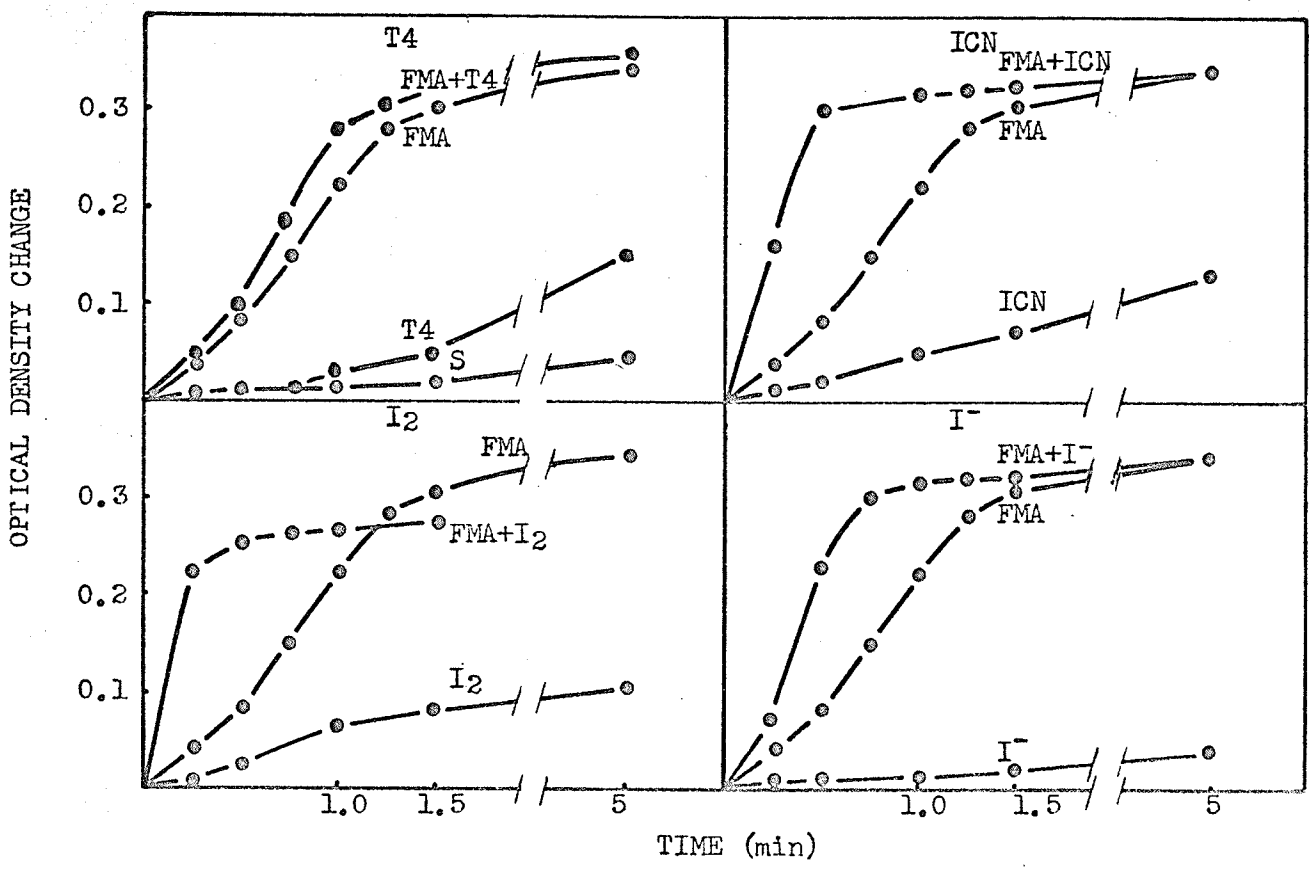

Fig 13. Effect of iodocompounds on FMA-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations. FMA, 5×10^{-6}M; T4, 5×10^{-6}M; ICN, 2.5×10^{-6}M; I_2, 2.5×10^{-6}M; I^-, 4.4×10^{-6}M.

Fig 14. The mitochondrial swelling, in response to FMA, 5×10^{-6}M, was markedly slowed at 0°C and the swelling curve had a lag period for 45 sec and then increased slowly. T4, ICN, I_2 or I^- produced little or no swelling in the concentrations utilized in these experiments. T4 + FMA induced a swelling curve similar to FMA by itself. The addition of ICN markedly potentiated the FMA-induced mitochondrial swelling, although a lag period was evident for 1 min in the ICN + FMA curve. Moreover, the combination of I_2 with FMA potentiated the FMA-induced swelling. I^-, also, potentiated the FMA-induced mitochondrial swelling and the induced swelling curve displayed a lag period for 1.5 min. Therefore, at 0°C, T4 did not influence the mitochondrial swelling induced by FMA, but I_2, ICN and I^- did potentiate the FMA-induced mitochondrial swelling with the same order of decreasing potency as at 20°C ($I_2 > ICN > I^-$).

The mitochondrial swelling curve induced by an NEM concentration of 10^{-3}M was also examined for its potentiation by T4, ICN, I_2 and I^-. These experiments were carried out at 20°C and Fig 15 shows the results. A 1 min lag period was evident in the NEM-induced mitochondrial swelling curve. T4 was without apparent effect. However, both ICN and I_2 potentiated the NEM-induced swelling. I^- was also without effect on the NEM-induced swelling curve. Thus, in these experiments, T4 and I^- were not able to potentiate the mitochondrial swelling induced by NEM, but ICN and I_2 did.

SUMMARY

FMA is a potent inducer of mitochondrial swelling. The fluorescence quenching of FMA was proportional to the mitochondrial concentrations. This quenching is rapid and precedes the changes in mitochondrial volume. I_2 and I^-, by themselves, quenched the fluorescence of FMA but T4 and ICN did not. In the presence of mitochondria T4 slightly enhanced and ICN markedly inhibited quenching. This inhibition of the quenching may, in

Fig 14

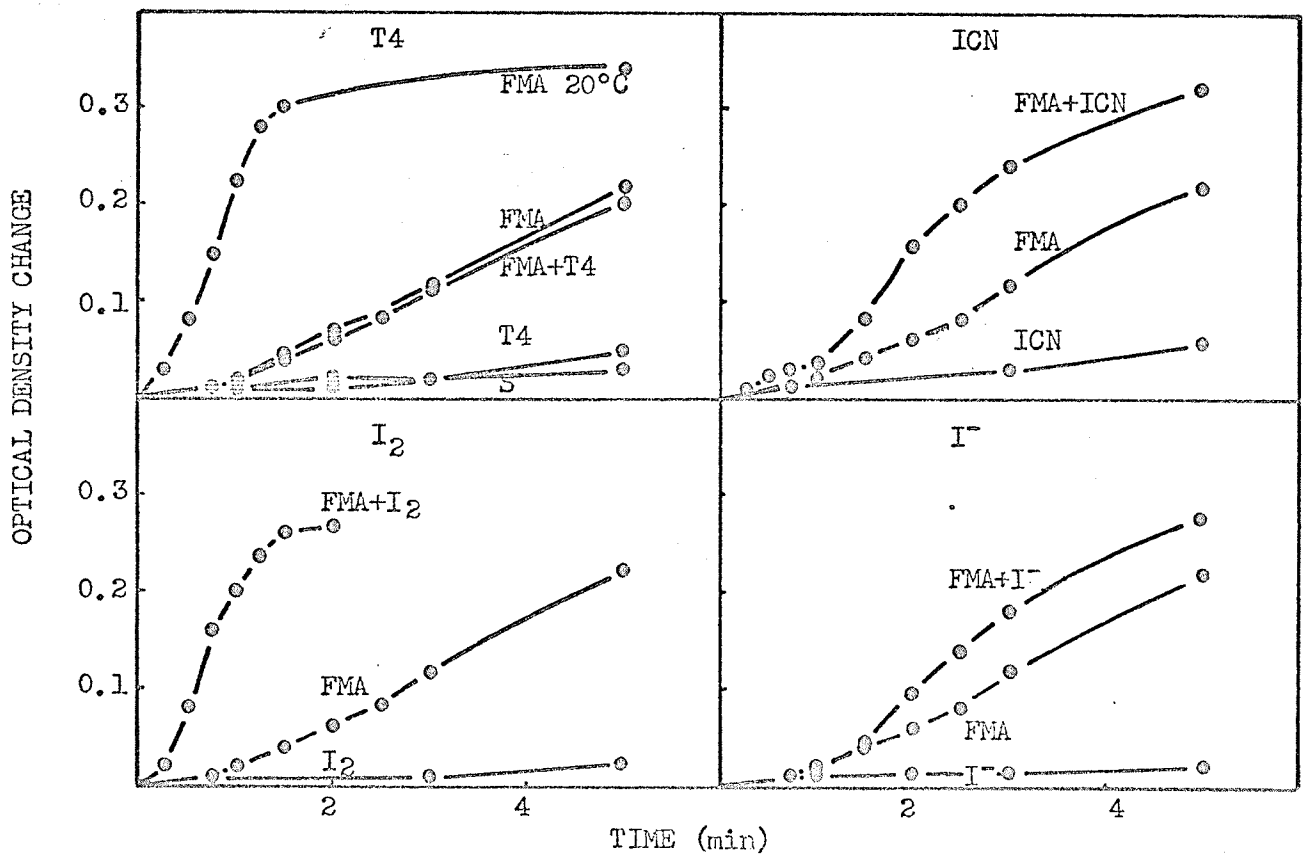

Fig 14. Effect of iodocompounds on FMA-induced mitochondrial swelling at low temperature. The medium was 0.125 M HCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 0°C. Mitochondria added at time zero. Concentrations are final concentrations, FMA, 5×10^{-6}M; T4, 10^{-5}M; ICN, 2.5×10^{-6}M; I_2, 2.5×10^{-6}M; I^-, 4.4×10^{-6}M.

Fig 15

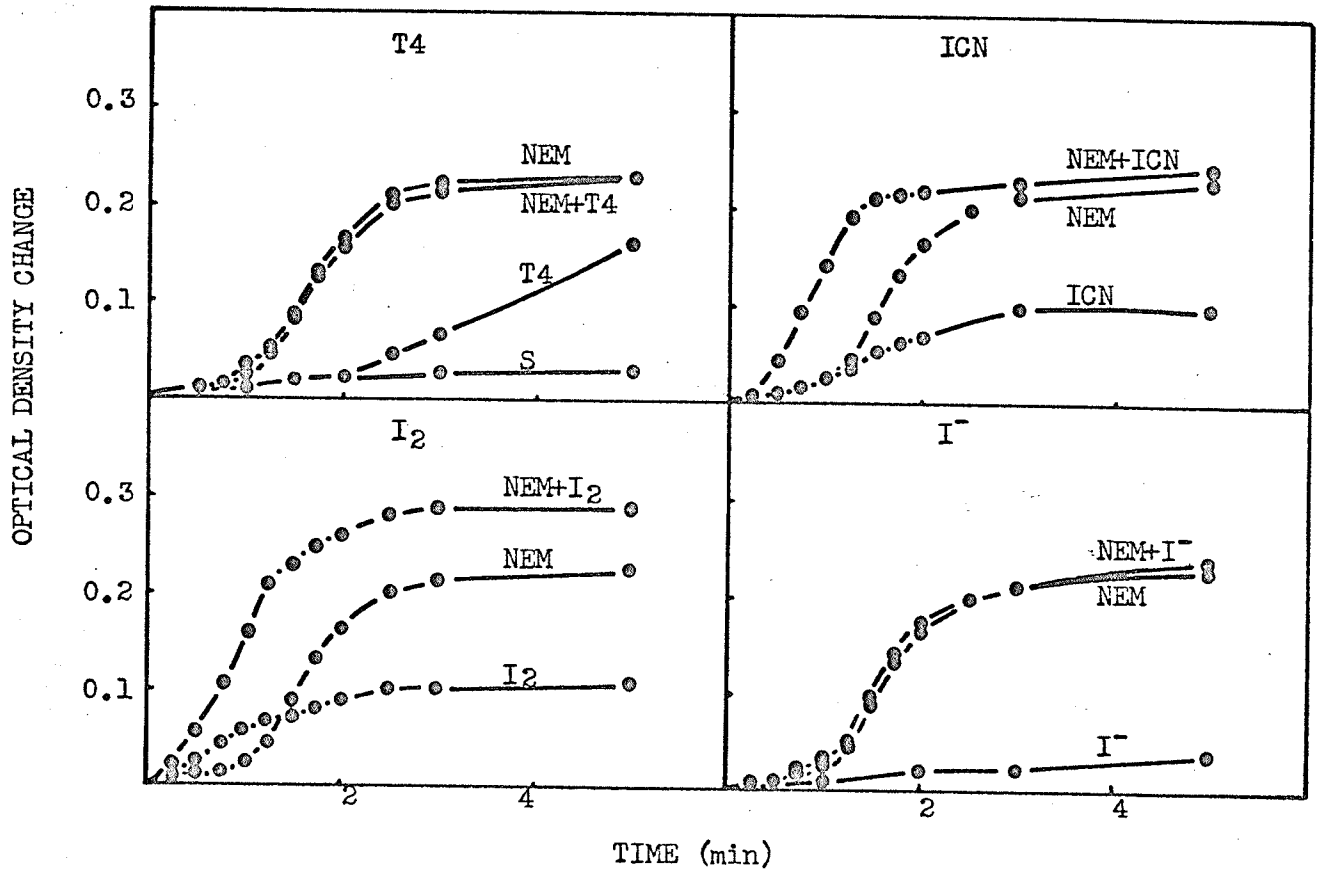

Fig 15. Effect of iodocompounds on NEM-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations. NEM, 10^{-3}M; T4, 5 x 10^{-6}M; ICN, 2.5 x 10^{-6}M; I_2, 2.5 x 10^{-6}M; I^-, 4.4 x 10^{-6}M.

part, be the result of the release of CN^- from ICN.

I_2, ICN, I^- and T4, in this order of decreasing potency, enhanced the FMA-induced mitochondrial swelling at 20°C. Mitochondrial swelling due to I_2, ICN, I^- and T4 was blocked at 0°C within the time course of the experiment. At 0°C, I_2, ICN and I^- retained their capability to enhance FMA-induced swelling, but T4 did not. Finally, at 20°C, I_2 or ICN enhanced the NEM-induced mitochondrial swelling but not T4 or I^-.

EFFECT OF PROPYLTHIOURACIL ON MITOCHONDRIAL VOLUME AND THYROXINE-INDUCED MITOCHONDRIAL SWELLING

PTU has been reported as an inhibitor of T4 deiodinase, which is of importance in the metabolism of T4, and a product of metabolism of T4 may be responsible for the observed biological effects of T4. It was considered that PTU might inhibit the T4-induced swelling response as a result of its ability to inhibit the deiodinase.

Initially, attempts were made to block the T4-induced swelling response with PTU, in vitro, but these were unsuccessful. In fact, PTU promoted swelling. The results of the mitochondrial swelling experiments with PTU are depicted in Fig 16. Little or no mitochondrial swelling was produced by a 10^{-5}M concentration of PTU, while measurable swelling occurred with PTU, 10^{-4}M. PTU, 10^{-3}M, promoted mitochondrial swelling which was rapid in onset and of large magnitude. Although PTU is able to promote rapid and large volume changes, it is of much lower potency than T4.

Both CN^- and DNP, which are known inhibitors of T4-induced swelling, were examined for their effect on PTU-induced swelling. The data of these experiments are listed in Table 6. CN^-, 10^{-3}M, inhibited the PTU-induced swelling response by 80%. DNP, 10^{-4}M, also interfered with this response, but the inhibition was only 25%. Higher DNP concentrations promoted

Fig 16

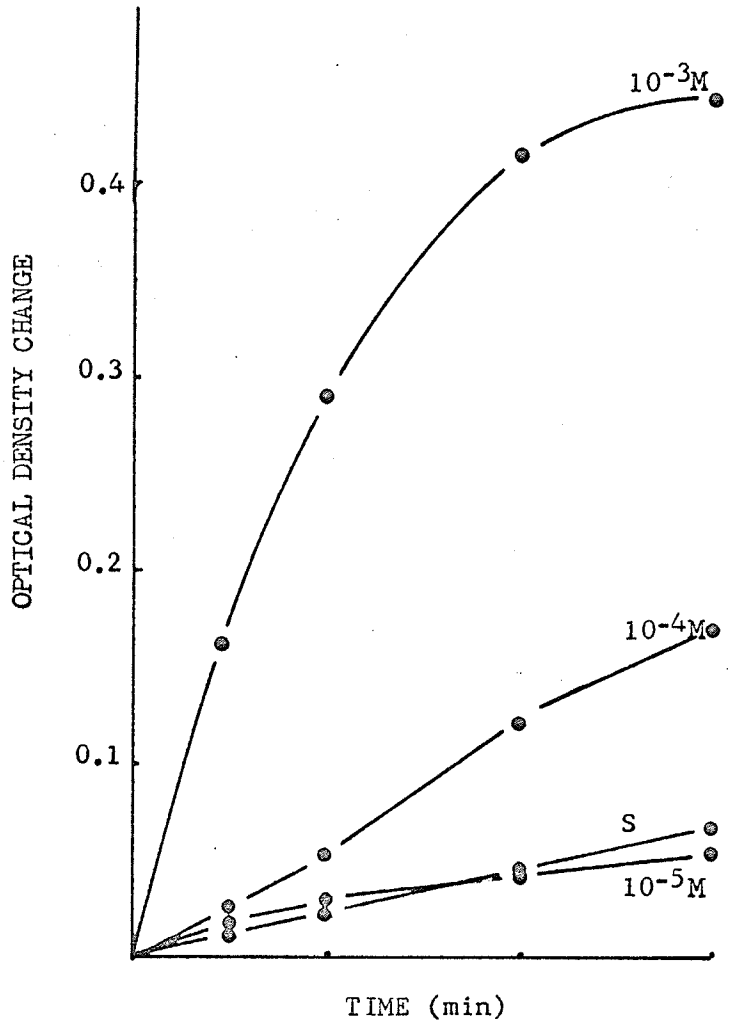

Fig 16. Effect of PTU on mitochondrial volume. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations.

Table 6

EFFECT OF CYANIDE AND DINITROPHENOL ON PROPYLTHIOURACIL-INDUCED MITOCHONDRIAL SWELLING

Compound	Per cent inhibition
CN^-, 10^{-3}M	80
DNP, 10^{-4}M	25

The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations. Experimental time, 20 min. PTU, 10^{-3}M.

mitochondrial swelling. Thus, PTU-induced mitochondrial swelling is inhibited by CN^- and DNP.

Since ATP is able to reverse the mitochondrial swelling induced by a number of agonists, its effect on swelling induced by PTU, 10^{-3}M, was determined. These results are depicted in Fig 17. The mitochondria were permitted to reach maximum volume and ATP was then added. T4, 10^{-5}M, was used as a reference. ATP, 10 mM, was added to the mitochondrial suspension at 30 min after exposure to PTU and reversal of the optical density changes was immediate and maximal changes occurred in 2 min. A similar curve was obtained with the T4 swollen suspensions but it took slightly longer to attain maximal optical density changes.

In Fig 18 are the T4-induced swelling curves of normal mitochondria and those isolated from a rat 3 hr after the administration of 15 mg of PTU. The mitochondria isolated from a PTU treated animal displayed a slower swelling response to PTU. The rate of swelling of the mitochondria of the PTU treated rat was decreased but the maximum volume change was the same as the control.

The time course of the inhibition of the T4-induced swelling response by PTU treatment was next investigated and the results appear in Fig 19. PTU, 15 mg/i.p, was administered and mitochondria were isolated 1.33, 3, and 24 hr after treatment. Since PTU administration reduced the rate of swelling, the optical density change after 9 min exposure to T4, 5×10^{-6}M, was recorded. This point is on the rising portion of the swelling curves and stimulation or inhibition could be determined. Mitochondria isolated from a rat treated with PTU for 1.33 hr displayed an inhibition of T4, 5×10^{-6}M, induced mitochondrial swelling. The inhibition was maximum 3 hr after PTU treatment, and in 24 hr, the T4-induced swelling response was approximately 70% of the control. Hence, the inhibition of the T4-induced swelling response by PTU treatment reaches a maximum inhibitory

Fig 17

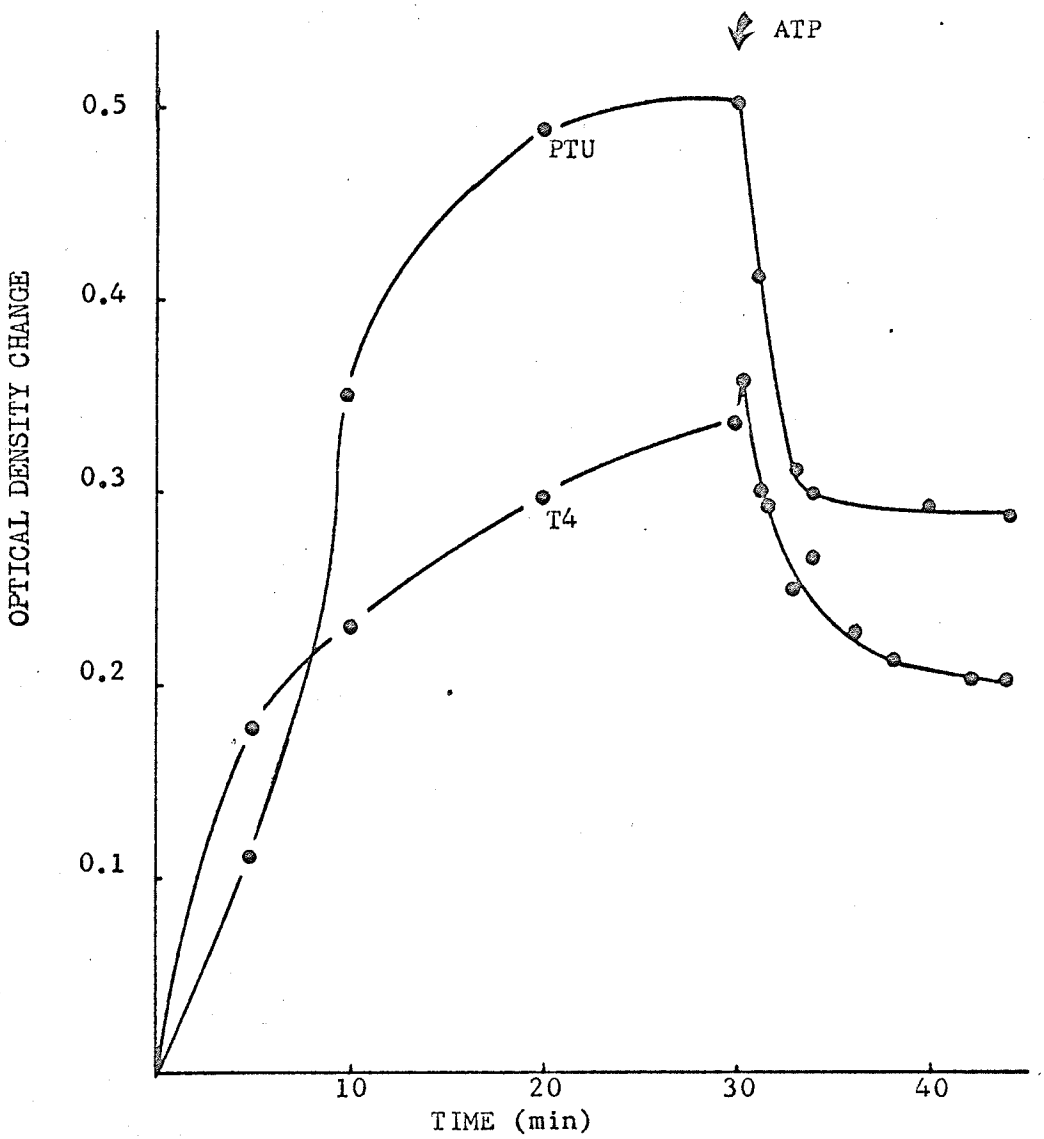

Fig 17. Effect of ATP on PTU- and T4- induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, PTU, 10^{-3}M; T4, 10^{-5}M. Arrow indicates addition 10 mM ATP.

Fig 18

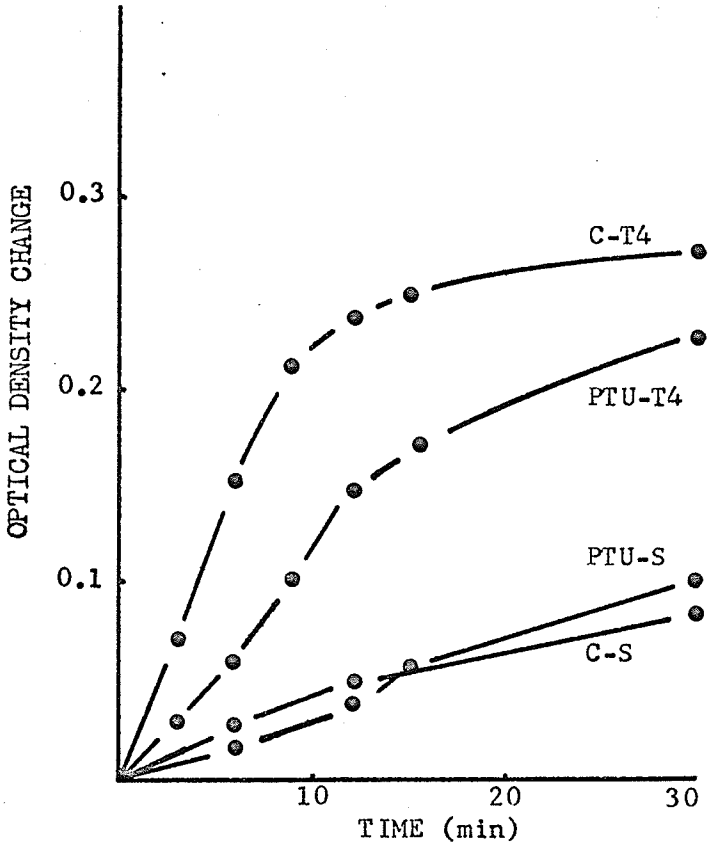

Fig 18. Effect of pretreatment of rats with PTU on T4-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, T4, 5 x 10^{-6}M. Rat treated with PTU, 15 mg, i.p. for 3 hr. Control, (C).

Fig 19

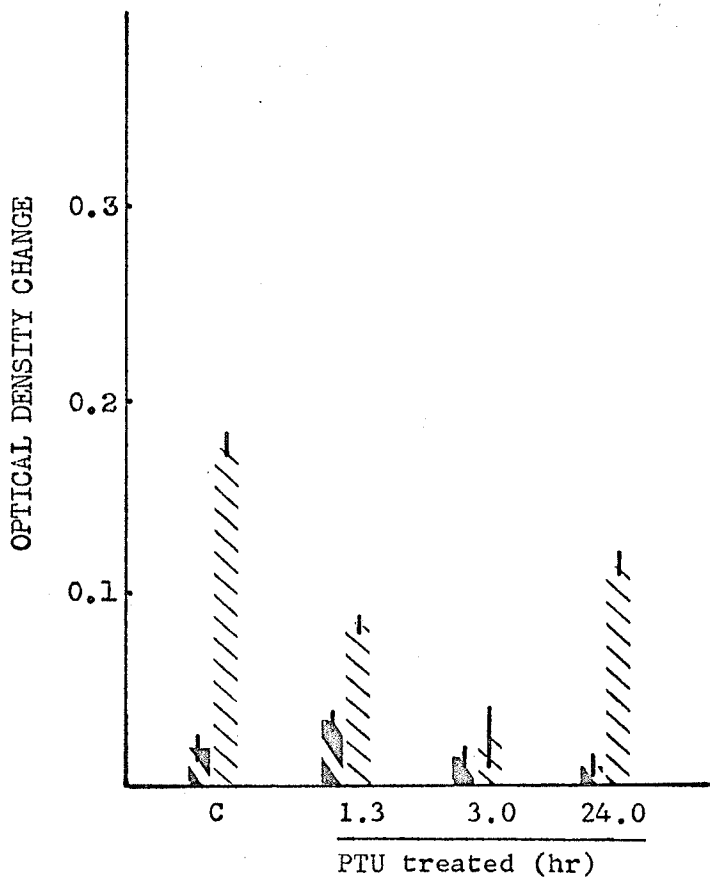

Fig 19. Effect of duration of PTU pretreatment time on T4-induced mitochondria swelling. The medium was 0.125 M KCL-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Final concentration of T4, 5 x 10^{-6}M. PTU pretreatment, 15 mg, i.p. Duration of swelling experiments 9 min. Large diagonal lines represent spontaneous swelling. Small diagonal line, with T4.

level at 3 hr and the inhibition of the T4 swelling is markedly reduced in 24 hr.

Experiments to determine the effect of chronic administration of PTU on the T4-induced mitochondrial swelling response were performed. These results are illustrated in Fig 20. Four groups of rats, consisting of saline controls, PTU, 15 mg, T4, 25 μg, as well as a group receiving both T4 and PTU. These were treated daily for 10 days and mitochondria were isolated 16 hr after the final treatment. The mitochondria were exposed to T4, 5×10^{-6}M, and the optical density changes after 6 min were recorded. The mitochondria isolated from the PTU treated rat displayed a reduced response to T4-induced swelling as did those isolated from a rat treated acutely with PTU. The mitochondria isolated from rats receiving PTU and T4, also displayed a reduced sensitivity to T4-induced swelling, while those from a rat receiving T4 were apparently normal. PTU also inhibited the T4-induced swelling response after chronic treatment of the rat. Chronic treatment did not increase the inhibition and the inhibition was of the same order of magnitude as after a single PTU treatment.

T3, ICN and I_2 were also tested to determine whether treatment of rats with PTU, 15 mg i.p 3 hr, would interfere with their induced mitochondrial swelling response. The results, reported as optical density changes in 6 min, are illustrated in Fig 21. T4- and T3-induced swelling response was reduced in the mitochondria from a PTU treated rat. On the other hand, ICN, 5×10^{-6}M, and I_2, 5×10^{-6}M, were equivalent in both the normal and PTU mitochondria.

SUMMARY

PTU promoted mitochondrial swelling. The effects of PTU could be blocked by CN^- and DNP and partially reversed by ATP. Injected PTU inhibited the swelling response to T4 by isolated liver mitochondria and

Fig 20.

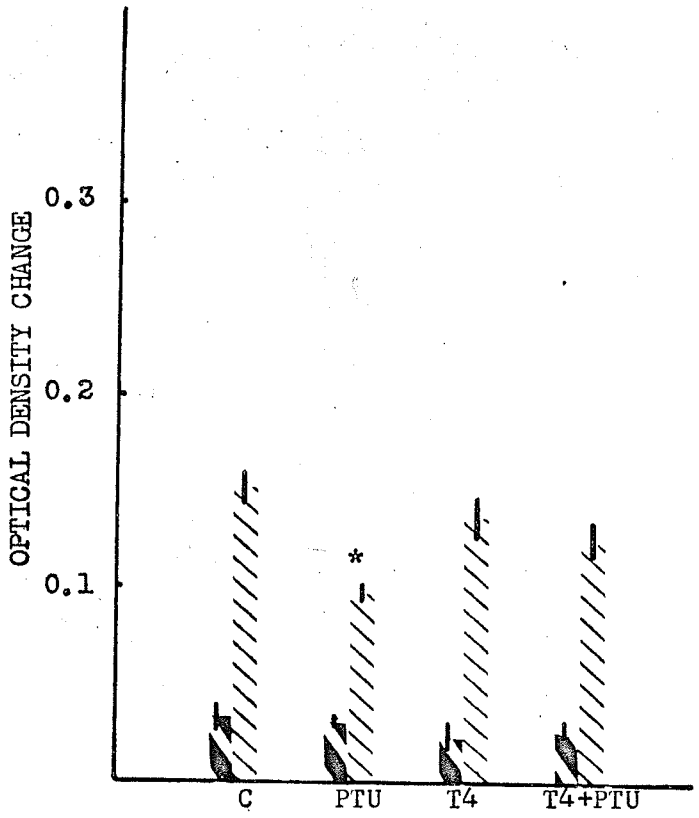

Fig 20. Effect of chronic administration of PTU on T4-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Final concentration of T4, 5×10^{-6}M. Each group of rats was injected for 10 days. Controls received saline, PTU, 15 mg, i.p./day, T4, 25 µg, i.p./day, T4+PTU, received both compounds i.p. Large diagonal lines represent spontaneous swelling. Duration of swelling experiments 6 min. Values are mean and S E of 4 experiments. *$p < 0.05$.

Fig 21

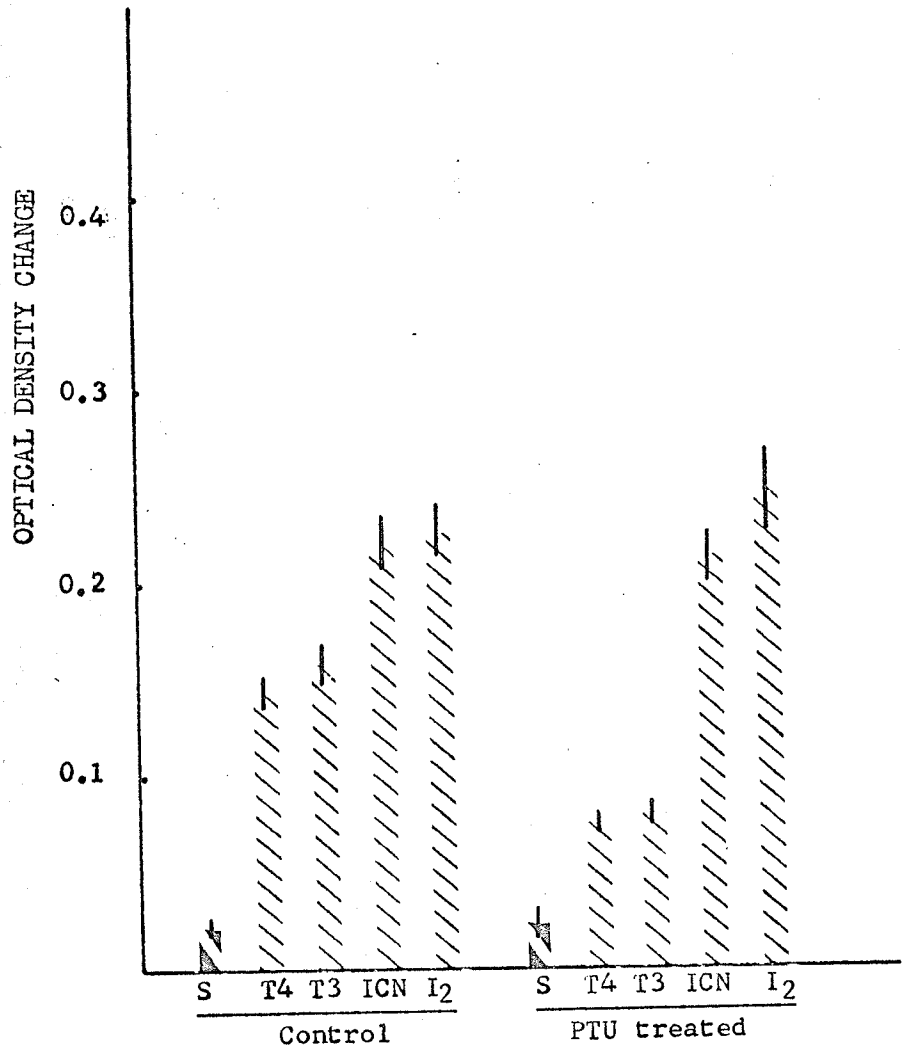

Fig 21. Effect of PTU pretreatment of rats on mitochondrial swelling induced by iodocompounds. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, T4, 5×10^{-6}M; T3, 5×10^{-6}M; ICN, 5×10^{-6}M; I_2, 5×10^{-6}M. Experimental time 6 min. PTU, 15 mg i.p., 3 hr. Values are mean and S E of 4 experiments.

this inhibition appeared to be maximum 3 hr after administration of PTU. Chronic administration of PTU also reduced the T4-induced swelling response by isolated mitochondria to the same extent as a single PTU treatment. The mitochondrial swelling response induced by T4 and T3 but not ICN or I_2, was reduced by PTU administration.

SWELLING STUDIES ON MITOCHONDRIA ISOLATED FROM THYROIDECTOMIZED RATS

It is known that mitochondria isolated from a thyroidectomized rat are less responsive to T4 than those isolated from a normal rat (Tapley, 1956; Lehninger et al. 1959; Tata, 1963). Since NEM, as well as PTU affects the T4-induced swelling response but not that of ICN, it was considered of interest to compare the swelling curves with these two agents on mitochondria isolated from normal and thyroidectomized rats. Fig 22 illustrates these effects. A 5×10^{-6}M concentration of T4 induced a swelling response by mitochondria isolated from thyroidectomized rats which at 8 min was 50% of control and at 30 min reached a level equivalent to normal. However, the swelling response induced by ICN, 5×10^{-6}M, was of greater magnitude in the mitochondria isolated from thyroidectomized rats.

Since the ICN- and T4-induced swelling displayed different characteristics in mitochondria isolated from a thyroidectomized rat, the swelling response of a number of I_2 containing compounds were examined, such as T4, T3, diiodothyronine (T2), I^-, I_2 and ICN. All substances were tested at a concentration of 5×10^{-6}M, except I^-, for which the concentration was 10^{-3}M. The results of these tests are shown in Fig 23 and the 8 min time interval was selected as the end point. Both T4 and T3 displayed a reduced activity in mitochondria isolated from a thyroidectomized rat. T2 and I^-, which are of low potency in inducing mitochondrial swelling, were equivalent to the control. ICN and I_2 were slightly more effective in inducing

Fig 22

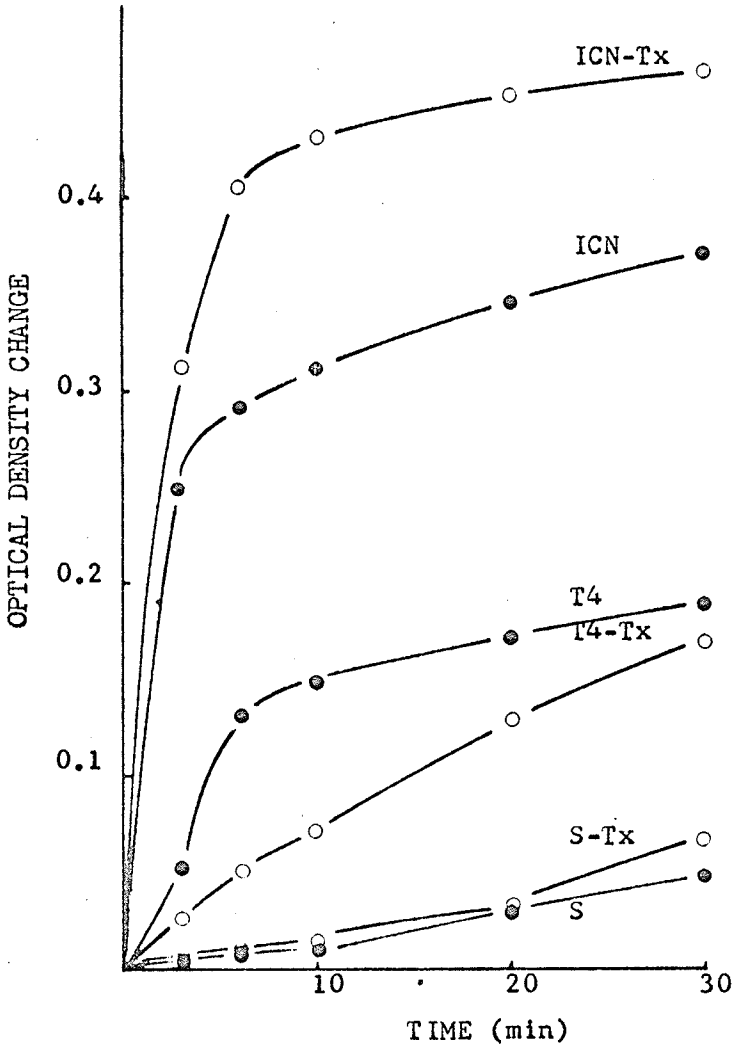

Fig 22. Effect of thyroidectomy on T4-and ICN-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, T4, 5 x 10^{-6}M; ICN, 5 x 10^{-6}M. Open circles, thyroidectomized, (Tx).

Fig 23

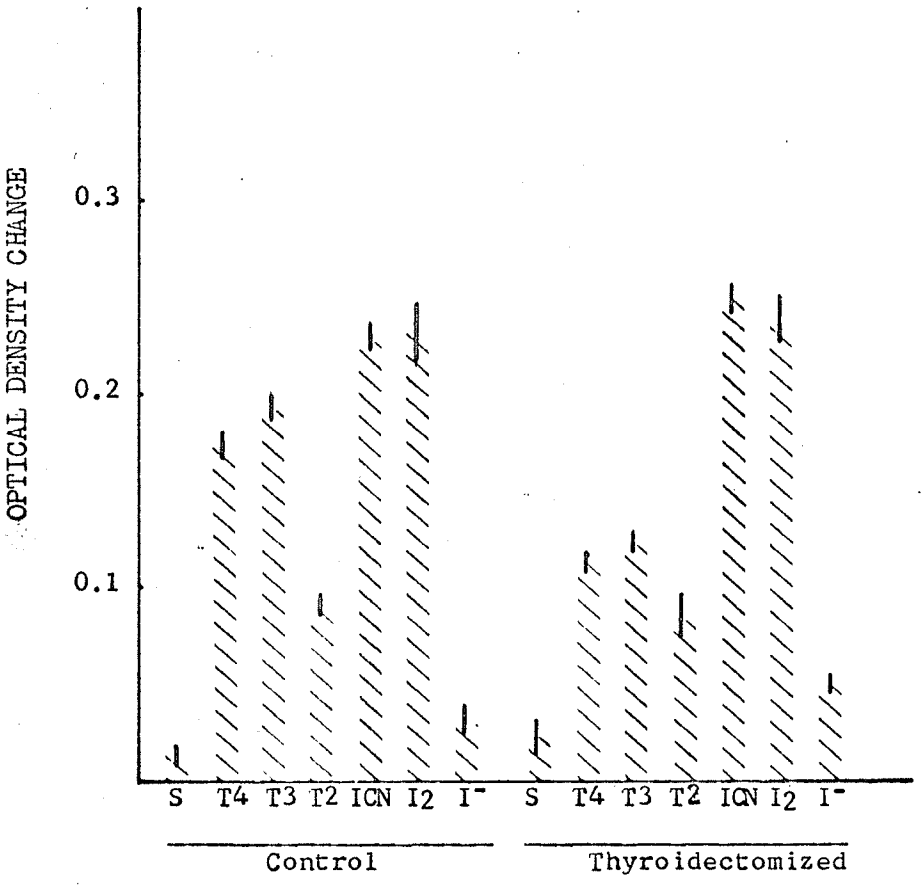

Fig 23. Effect of thyroidectomy on mitochondrial swelling induced by iodocompounds. The medium was 0.125 M KCl-0.02 M Tris-HCL buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, T4, 5×10^{-6}M; T3, 5×10^{-6}M; T2, 5×10^{-6}M; ICN, 5×10^{-6}M; I_2, 5×10^{-6}M, I^-, 10^{-3}M. Experimental time 8 min. Values are mean and S E of 5 experiments.

swelling. Consequently, thyroidectomy does not reduce the swelling response to all I_2 containing compounds but does show some specificity.

SUMMARY

Thyroidectomy reduced the mitochondrial response to T4 and T3 but not to ICN, I_2, T2 or I^-.

INTERACTION OF THYROXINE WITH VASOPRESSIN, OXYTOCIN AND INSULIN ON MITOCHONDRIAL VOLUME CHANGES

Evidence obtained in this laboratory has indicated that vasopressin, oxytocin (LaBella, 1964a) and insulin (LaBella, 1964d) can interact with T4 to modify the release of TSH. These disulfide hormones, vasopressin, oxytocin and insulin are capable of stimulating mitochondrial volume changes, and, since T4 also promotes mitochondrial volume changes, it was considered that the disulfide hormones and T4 might interact with the result being a modification of the swelling response. Fig 24 depicts the results of such an experiment. Insulin was chosen as representative of the disulfide hormones. Insulin, 10^{-6}M, induced a small degree of swelling and T4, 2.5×10^{-6} and 5×10^{-6}M promoted rapid volume changes of large magnitude. It can be seen that the combination of insulin with T4, 2.5×10^{-6}M, enhanced the rate of mitochondrial swelling. The curve plateaued and the maximum volume changes were nearly the same as those occurring in the presence of T4, 2.5×10^{-6}M. Insulin + T4, 5×10^{-6}M, also induced swelling curves showing faster rates than the corresponding curve produced by T4, 5×10^{-6}M. Insulin is capable of enhancing the T4-induced mitochondrial response.

Fig 25 displays the results of the interaction of T4, 5×10^{-6}M, with a range of concentrations of vasopressin, oxytocin and insulin. Since these compounds are capable of reducing the time for the mitochondria to reach maximum swelling, a point on the rising phase of the swelling curves

Fig 24

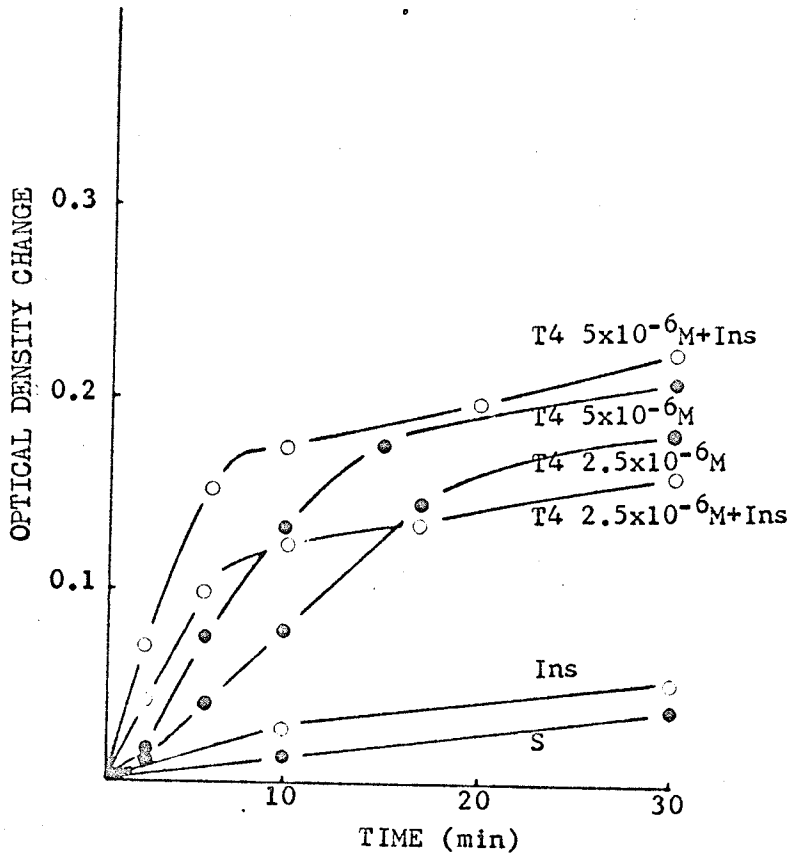

Fig 24. Effect of insulin on T4-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, open circles, Ins, 10^{-6}M.

Fig 25

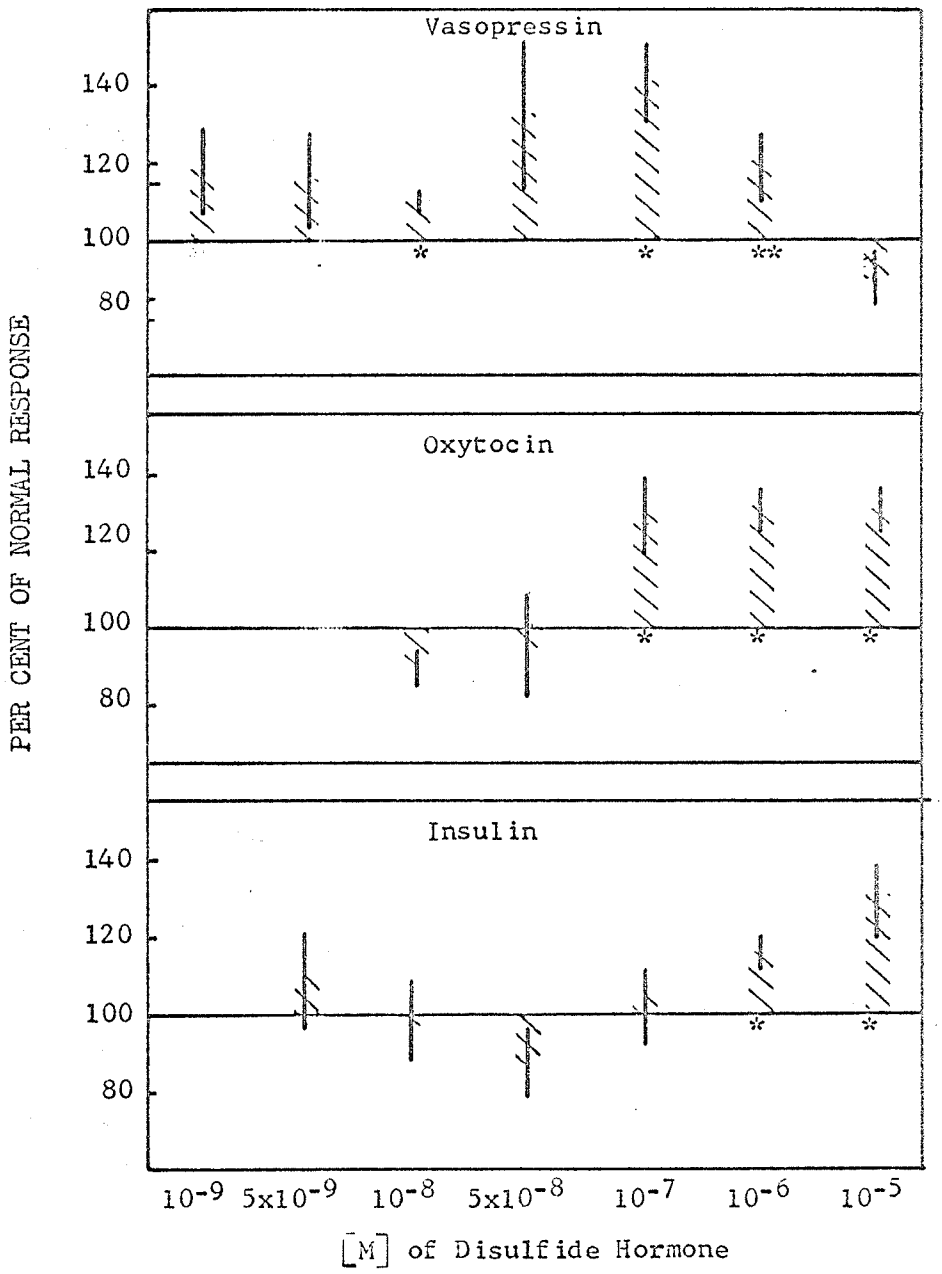

Fig 25. Effect of vasopressin, oxytocin and insulin on T4-induced mitochondrial swelling. The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, T4, 5 x 10^{-6}M. Vasopressin, mean and S E of 6 preparations, oxytocin 4 preparations and insulin 5 preparations. *p 0.05; **p<0.1. Time, 6 min.

was selected as the experimental end point. In all instances, the molar concentrations of vasopressin, oxytocin and insulin employed did not, by themselves, produce swelling over the time course of the experiments. In Fig 25, the augmentation of the T4-induced swelling is expressed as per cent of normal and the response to vasopressin is a mean of 6 mitochondrial preparations ± the standard error, to oxytocin 4 preparations, and to insulin 5 preparations. At the highest vasopressin concentration, 10^{-5}M, the T4 response was equivalent to control, while in the presence of a range of vasopressin concentrations, 10^{-6} - 10^{-9}, the T4-induced swelling response was enhanced. Oxytocin, on the other hand, increased the T4 response at concentrations of 10^{-5}, 10^{-6} and 10^{-7}M, while the response at 5×10^{-8}M was unchanged and at 10^{-8}M was slightly below the normal response. Insulin displayed a pattern somewhat similar to oxytocin. At insulin concentrations of 10^{-5} and 10^{-6}M, the response was potentiated, whereas at 10^{-7}M it was normal and at 5×10^{-8}M was slightly inhibited. Hence, the disulfide hormones show quantitative differences in the modification of the T4-induced swelling response. A potentiation effect always occurred when the appropriate concentration was selected.

The experiments listed in Table 7 show the effect of various concentrations of T4 on the augmentation of the T4-induced mitochondrial swelling response in the presence of disulfide hormones. Vasopressin and insulin were used at a constant concentration of 10^{-6}M. At a concentration of 10^{-6}M T4, no augmentation of the T4 response occurred in the presence of insulin but had occurred with vasopressin. These data indicate that the greatest enhancement of T4 action in the presence of insulin was at 2.5×10^{-6}M T4 and at T4 concentration of 5×10^{-6}M in the presence of vasopressin.

Table 7

EFFECT OF INSULIN AND VASOPRESSIN ON MITOCHONDRIAL SWELLING INDUCED BY VARIOUS CONCENTRATIONS OF THYROXINE

T4 [M]	Swelling response with disulfide hormone Per cent of T4 control	
	Insulin	Vasopressin
10^{-6}	96	132
2.5×10^{-6}	270	-
5×10^{-6}	148	180
10^{-5}	118	97

The medium was 0.125 M KCl-0.02 M Tris-HCl buffer, pH 7.4. Temperature 20°C. Mitochondria added at time zero. Concentrations are final concentrations, insulin, 10^{-6}M, vasopressin, 10^{-6}M, are constant. Time 6 min.

SUMMARY

The disulfide hormones, vasopressin, oxytocin and insulin, were able to enhance the rate of the T4-induced mitochondrial swelling response. Oxytocin and insulin exhibited similar effects on the T4-induced swelling response in their higher concentrations, 10^{-5}M, but vasopressin, 10^{-5}M, did not modify the T4 response. The concentration of T4 appeared to be important, since the concentration of 2.5 and 5 x 10^{-6}M T4 displayed the greatest augmentation by a constant disulfide hormone concentration.

DISCUSSION

ROLE OF SULFHYDRYL GROUPS IN THE SWELLING RESPONSE TO IODOCOMPOUNDS

A. Effect of NEM on T4-induced mitochondrial swelling.

The sulfhydryl content of rat liver mitochondria has been reported to be 85 to 100 mμM per mg of protein (Riley and Lehninger, 1964) and 37 ± 1 mμM of sulfhydryl groups per mg of protein (Klouwen, 1962). Different methods were used by these workers, which may account for the discrepancy between the reports. Hadler et al. (1966) studied the binding of 2,6-dichlorophenol to mitochondrial sulfhydryl groups. In their work, the reaction was inhibited by an amount of p-HMB which was in accord with the sulfhydryl content reported by Riley and Lehninger. These sulfhydryl groups are related to the normal condition of the membrane, which maintains a permeability barrier. Evidence for this are the observations that sulfhydryl reagents, such as Hg^{++}, Ag^{+}, Zn^{++}, (Dickens and Salmony, 1956; Tapley, 1956), and NEM (Neubert and Lehninger, 1962a) promote mitochondrial swelling. These compounds induce swelling, presumably as a consequence of their combination with sulfhydryl groups of the mitochondrial membrane, resulting in a decrease of the permeability barrier.

NEM is a less potent inducer of mitochondrial swelling than T4. A 10^{-5}M concentration of NEM did not produce mitochondrial swelling, but this concentration of T4 produced near maximal swelling in 5 min. Others have reported that NEM is unable to block the T4-induced mitochondrial swelling response at a concentration of 10^{-4}M of NEM (Rall et al. 1962a, 1963). However, NEM, in this work, inhibited the T4-induced swelling response at a critical concentration , 10^{-5}M, i.e. a concentration approximating the threshold level of NEM-induced swelling. In accord with Rall et al. (1962a, 1963), an NEM concentration of 10^{-4}M did not inhibit T4-induced mitochondrial swelling response in this experiment, but, by itself, promoted swelling as seen in Fig 1. It appears that some of the sulfhydryl groups of mitochondria, at least, are essential for the mitochondrial

swelling induced by T4.

The NEM inhibition of the T4-induced swelling response could be enhanced or overcome. Preincubation of NEM with the mitochondria enhanced this inhibition. It is likely that a greater amount of NEM combines with the mitochondrial sulfhydryl groups during the time of preincubation. Conversely, increasing the T4 concentration overcame the inhibition of T4-induced swelling by NEM, which may be the result of T4 competing and preventing the NEM conjugation with certain sulfhydryl groups.

It was considered possible that a direct chemical antagonism might occur between T4 and NEM, since NEM has been reported to react with the amino group of amino acids and T4 is an amino acid. However, high concentrations of the reactants are required for this reaction (Benesch and Benesch, 1962). Solutions of a mixture of T4 and NEM were monitored spectrophotometrically at the absorption maximum of NEM in 0.125 M KCl - 0.02 M Tris-HCl buffer. No observable change in absorption occurred over a 30 min period. In another experiment, which is illustrated in Fig 4, T4 and NEM were preincubated in the suspending media prior to the addition of mitochondria. No inhibition of the T4-induced swelling response was noted under these conditions and, also, no precipitate in the suspending media was observed. Thus, it appears that no direct reaction of NEM and T4 occurs in the suspending medium, and antagonism of T4 by NEM occurs at the level of the mitochondrion.

The addition of ATP to swollen mitochondria causes their contraction or, if added prior to the event of volume change, will inhibit swelling. NEM, when added to swollen mitochondria, did not promote mitochondrial contraction, which indicates that the NEM inhibition of the T4 response is not due to contraction of mitochondria by NEM.

The mitochondrial swelling response induced by ICN was uninfluenced by NEM. Therefore, some property is not shared by T4 and ICN in the

induction of mitochondrial swelling. Roche et al. (1962) had suggested that iodocompounds promote swelling as a consequence of the formation of I^+, which is transient and reversible. According to this theory, for T4 to exert its swelling-inducing effects deiodination must occur with the formation of I^+. Evidence indicates that T4 deiodinase can apparently remove I_2 from T4 and produces I^+ in the process (Galton and Ingbar, 1961). ICN would not require the mediation of an enzyme to produce I^+. Hence, the property not shared between ICN and T4 may be the requirement of an enzyme by T4 in order to form I^+, the latter species then exerting the mitochondrial swelling-inducing action.

NEM, 10^{-5}M, did not display any inhibitory influence toward the spontaneous mitochondrial swelling. This is interesting because Lehninger et al. (1959) noted a number of similarities between the spontaneous and T4-induced swelling and suggested that T4 speeds up the intrinsic mitochondrial processes, resulting in a more rapid swelling. If spontaneous and T4-induced mitochondrial swelling are basically similar, then NEM must be acting at some point in the T4-induced swelling response prior to any change in volume. Perhaps this point of activity may be at the level of T4 deiodinase, prior to the production of I^+, which would hasten the oxidation of sulfhydryl groups.

In the examination of other sulfhydryl reagents such as $HgCl_2$, p-HMB, IA and FMA for their ability to block the T4-induced swelling, only $HgCl_2$ was also able to block the response. Since all of the examined sulfhydryl reagents are capable of inducing swelling and since only selected concentrations of NEM and $HgCl_2$ were able to block the T4 response, it appears that only a portion of the total mitochondrial sulfhydryl groups may be involved in T4-induced swelling and blocked by selective concentrations of NEM or $HgCl_2$. This could be possible since more than one kind of sulfhydryl group are present in mitochondria. Riley and Lehninger (1964)

differentiated two classes of sulfhydryl groups in mitochondria while measuring sulfhydryl changes during mitochondrial swelling, (1) rapidly reacting groups and (2) slowly reacting groups. The rapidly reacting sulfhydryl groups represented 12% of the total mitochondrial sulfhydryl groups and reacted within 40 sec, whereas the slowly reacting groups represented 88% of the total and required 60 min for full reaction. Moreover, Strittmatter (1959) studied the reaction of sulfhydryl groups of proteins with sulfhydryl reagents and differentiated four classes of sulfhydryl groups based on their reactivities with p-HMB and NEM.

NEM and $HgCl_2$ interfered with the T4-induced mitochondrial swelling response and the interference may be at the level of T4 deiodinase. In conjunction with this idea, it is worthwhile to note that Stanbury (1960) and Tata (1960) reported that preparations of T4 deiodinase were inhibited by $HgCl_2$ *in vitro*. Larsen *et al.* (1955) observed that $HgCl_2$ inhibited the deiodinase of rat kidney slices.

One hypothesis which attempts to explain the manner in which T4 promotes mitochondrial swelling proposes that U-factor is the causal agent. The release of U-factor, a mixture of long chain fatty acids, from mitochondria accompanies both spontaneous and T4-induced mitochondrial swelling. In order to test whether U-factor is involved with the inhibitory effects of NEM, two mitochondrial swelling agonists were selected, Ca^{++}, the effects of which are accompanied by a release of U-factor, and PO_4, the effects of which are not. However, NEM was inhibitory to both Ca^{++}- and PO_4-induced swelling. It seems that NEM is not acting as a result of an interference with U-factor's release or effects. The observation that PO_4-induced swelling was interfered with is evidence against this. Perhaps these agonists all have an involvement of sulfhydryl groups in the swelling mechanism, i.e. sulfhydryl groups other than those in deiodinase. It is not unlikely that NEM would have several sites of action at which

it might influence mitochondrial volume changes. Furthermore, the addition of NEM to swollen mitochondria will block the ATP-induced mitochondrial contraction (Rall et al. 1962a, 1963). Consequently, NEM is somewhat unique, since only a few substances, such as oligmycin A, azide and atractylate, inhibit both swelling and contraction of mitochondria. These latter compounds are inhibitors of oxidative phosphorylation and, as a result of the overt differences in their inhibition of swelling and contraction, Lehninger (1962a) suggested that at least a portion of the oxidative phosphorylation mechanism is necessary for contraction, but perhaps not the same portion involved in swelling. Thus, the possibility that a compound may act at more than one site in the mitochondrial swelling-contraction mechanism exists. Finally, Lehninger and Schneider (1959) had reported that a combination of GSH and T4 produced a smaller response than either compound by itself. They suggested that these agonists may share common sulfhydryl groups in their respective pathways for exerting their swelling-inducing effects.

B. Interaction of iodocompounds and sulfhydryl reagents.

FMA is an agonist of mitochondrial swelling, with a potency approximating that of $HgCl_2$. Its ability to promote swelling is apparently due to the presence of Hg^{++} groups in the molecule, since fluorescein, itself, does not cause mitochondrial swelling. CN^- is able to inhibit the FMA-induced swelling. This probably reflects a requirement for some respiratory chain activity. Thus, the effects of FMA on mitochondrial volume appear to be basically similar to those of certain other sulfhydryl reagents.

The FMA molecule is highly fluorescent and when it reacts with sulfhydryl groups, its fluorescence is quenched (Karush et al. 1964). Its fluorescence was quenched proportionally to the concentration of

mitochondria in these experiments. This quenching effect is probably due to its combination with sulfhydryl groups of mitochondria via the Hg^{++}. The fluorescence of fluorescein, which does not have the Hg^{++} groups, is not quenched by mitochondria. However, a comparison of the time course of the curve of FMA-induced mitochondrial swelling and its quenching of its fluorescence revealed that the changes in fluorescence precede the changes in volume. FMA is apparently reacting with the sulfhydryl groups before the mitochondrial volume changes are initiated.

It was observed that T4 enhanced both the mitochondrial swelling induced by FMA, as well as the quenching of fluorescence of FMA by mitochondria. The increase in mitochondrial swelling may be due to two agonists acting in the same direction, with the result that swelling is enhanced. The enhancement of the quenching of the fluorescence of FMA may be the result of (a) increased reactivity of sulfhydryl groups in the presence of T4, and (b) a greater exposure of sulfhydryl groups in the course of swelling. In this work, a more rapid rate of quenching of the fluorescence of FMA was noted with swollen mitochondria than with unswollen, which may be the result of exposure of an increased number of sulfhydryl groups at the surface. On the other hand, ICN was extremely potent in enhancing the FMA-induced mitochondrial swelling but, at the same time, ICN inhibited the quenching of the fluorescence of FMA by mitochondria. The blocking of quenching in the presence of ICN may be the result of the formation of CN^- from ICN, since the addition of CN^-, itself, was able to block the quenching. ICN is probably split into I^+ and CN^- in the presence of mitochondria. ICN, when incubated by itself with FMA, did not quench its fluorescence.

In the experiments comparing the effect of CN^-, 5×10^{-6}M, and ICN, 5×10^{-6}M, on the mitochondrial swelling induced by FMA and the fluorescence of FMA, CN^- was not as effective as ICN in preventing the quenching

of FMA's fluorescence by mitochondria. Perhaps this may be due to some CN^- associating with Hg^{++} prior to the addition of mitochondria, thus reducing the amount of Hg^{++} available for reacting with mitochondrial sulfhydryl groups. It was reported by Rall et al. (1963) that ICN remains nearly undissociated in aqueous media for hours but the addition of mitochondria to the suspension caused a rapid breakdown of the ICN molecule. Rall et al. (1963), reported that $I^{131}CN$ and $I_2{}^{131}$ in the presence of mitochondria released I^{-131} into the suspending medium and that 1 to 3% of the I^{-131} released from the parent molecule was associated with the mitochondria centrifuged from suspension.

The influence of T4, ICN, I_2 and I^- on FMA-induced mitochondrial swelling was qualitatively similar. Each of these compounds potentiated the effects of FMA, 5×10^{-6}M, at 20°C. Their capacity to enhance the FMA-induced swelling did not parallel their own capacity to induce swelling. The order of potency for inducing mitochondrial swelling is I_2, ICN, T4 and I^-, while the order for enhancing FMA-induced swelling is I_2, ICN, I^- and T4. It may be that the presence of a form of I_2 might be responsible for this enhancement of FMA-induced swelling, possibly the formation of I^+. I_2 can oxidize sulfhydryl groups and this may result in an increase in the reactivity of mitochondrial sulfhydryl groups, thus producing a more rapid rate of FMA reaction. Thus, the evidence indicates that sulfhydryl groups of mitochondria may have a role in the swelling induced by iodocompounds, in addition to their previously suggested role in maintaining the integrity of the mitochondrial membrane.

At 0°C, the swelling response to FMA, 5×10^{-6}M, was slowed. This reduction in rate could be expected since decreases in temperature are known to reduce the mitochondrial swelling responses (Lehninger, 1959a). I_2, ICN, and I^-, in that order, potentiated the FMA response at 0°C, but T4 was ineffective.

It may be that at 0°C, T4 deiodinase is not functioning or is functioning at a low order of activity. Under conditions which T4 deiodinase may be inactive, i.e. 0°C, T4 is not able to potentiate, whereas I_2 and ICN can potentiate FMA-induced mitochondrial swelling since they would not require the enzymatic formation of an active species.

I_2 and ICN, but not T4 and I^-, enhanced the mitochondrial swelling by NEM. The potentiation of FMA-induced swelling by I_2 and ICN was greater than their potentiation of NEM-induced mitochondrial swelling. This could be a reflection of an order of sensitivity of the sulfhydryl groups associated with the swelling induced by reagents which react with sulfhydryl groups. FMA and $HgCl_2$ are reagents, whose potency at inducing mitochondrial swelling is considerably greater than NEM. FMA and $HgCl_2$ had a mitochondrial swelling threshold level of 10^{-7} and 5×10^{-8}M, respectively, and for NEM the level was 10^{-5}M.

EFFECT OF PROPYLTHIOURACIL ON MITOCHONDRIAL VOLUME AND THYROXINE-INDUCED MITOCHONDRIAL SWELLING

The present work indicates that PTU induces rapid and extensive, but not irreversible, mitochondrial swelling. Rapid swelling was promoted by a PTU concentration of 10^{-4}. The fact that CN^- inhibited PTU-induced swelling suggests that the effect of the former may be due to the inhibition of the respiratory chain. Furthermore, DNP was able to partially block the effect of PTU on mitochondrial swelling, which indicates that PTU possesses different characteristics in its induction of swelling than GSH. DNP was not able to inhibit the GSH-induced mitochondrial swelling (Hunter et al. 1959), but does block swelling by T4 (Tapley, 1956).

Mitochondrial swelling is induced by a number of specific agents, of which the thiols and disulfides form distinct groups (Neubert and Lehninger, 1962b). PTU is a sulfur containing compound but apparently

does not exhibit all of the characteristics of thiols. The thiol and disulfide effects on mitochondria depend on their concentration and structure. Neubert and Lehninger (1962b) suggests that thiols, as well as disulfides react with "specific, structure-controlling sulfhydryl or disulfide groups, or both, in critical portions of the mitochondrial membrane". They further suggested that mitochondrial swelling takes place when free sulfhydryl groups are oxidized, substituted, or when certain disulfide groups are reduced. Low concentrations of GSH and GSSG, which do not cause swelling by themselves, are potent mitochondrial swelling-inducing agents when combined, presumably by interacting with mitochondrial sulfhydryl and disulfide groups.

The PTU-induced mitochondrial swelling was reversed by a combination of ATP + Mg^{++} + serum albumin. This fact points out another difference between PTU- and GSH-induced swelling. The GSH-induced swelling requires ATP + Mg^{++} + serum albumin + C-factor. Therefore, PTU does not apparently cause a loss of C-factor from mitochondria or, if such a loss occurs, it is probably not extensive.

The inhibition of the T4-induced mitochondrial swelling response of mitochondria isolated from rats which received PTU may be due to an inhibition of T4 deiodinase. The deiodination of thyroid hormones, as determined by the amount of inorganic I^- liberated via a T4 deiodinase, has been demonstrated to be a major pathway in the metabolism of the thyroid hormones (Pitt-Rivers and Tata, 1959). This enzyme had wide distribution in the body (Stanbury, 1960) and intracellular studies on the localization of deiodinase have led to reports which localize activity in mitochondria (Yamazaki and Slingerland, 1959; Yamamoto *et al.* 1960; Wolff, 1960; Numez *et al.* 1964). Furthermore, the thyroid state influences deiodinase activity. Larson *et al.* (1955) demonstrated that tissue deiodinating activity was sensitive to thyroid function and had reported that kidney

slices from rats made hyperthyroid by cold exposure or the feeding of desiccated thyroid had an elevated deiodinase activity, and those from hypothyroid rats had a lower activity than normal. Ingbar and Freinkel (1955) reported similar effects in man. Tata (1960) observed that T4 deiodinase activity was less than normal in the hypothyroid state.

Peripheral deiodination of thyroid hormones has been reported to be inhibited by thiouracil derivatives including PTU (Hogness et al. 1954; Van Arsdel and Williams, 1956; Jagiello and McKenzie, 1960; Jones and Van Middlesworth, 1960; Escobar del Rey and Morreale de Escobar, 1961; Van Middlesworth and Jones, 1961; Braverman and Ingbar, 1962). In these studies the experimental animals were treated with perchlorate to prevent the cycling of I^- or were thyroidectomized, and a decrease in urinary I^- excretion was taken to be a reflection of deiodinase inhibition. Inhibition, in vitro, of deiodinase by PTU has also been reported (Braverman and Ingbar, 1961). Moreover, the reduced mitochondrial swelling with T4 observed in mitochondria isolated from PTU treated rats is not due to hypothyroidism since, in the acute situation, sufficient time has not elapsed for a hypothyroid state to develop. After administering a single dose of PTU, the inhibition was maximal in 3 hr and returned to near normal levels in 24 hr. This is in accord with the metabolism of PTU. It is rapidly metabolized and in 24 hr the amount of drug remaining in the rat is only 25% of the administered dose (Williams and Kay, 1947).

The administration of PTU to rats chronically also produced an inhibition of the T4-induced mitochondrial swelling response. However, the inhibition of the T4-induced mitochondrial swelling response was of the same order of magnitude as after a single PTU dose. These results are somewhat analogous to those of Larson et al. (1955). These authors had reported that thiouracil administered to rats for four weeks inhibited T4 deiodinase activity to the same extent as thyroidectomy. They also

observed that T4 deiodinase activity was inhibited after four days of thiouracil treatment, thus suggesting a direct effect on the enzyme and *in vitro* addition of thiouracil inhibited the enzyme. These results are akin to the observations of Stasilli *et al.* (1960) that thiouracil is capable of interfering with the calorigenic action of T4 as determined by the rate of oxygen consumption of the whole animal. The rats receiving thiouracil required ten times the dose of T4 to give equivalent calorigenesis.

Experiments were performed to determine whether PTU would block the T4-induced swelling response when both agents were added to isolated mitochondria. No blocking effect could be detected with a wide range of PTU concentrations. However, PTU, itself, at 10^{-4}M or higher, promoted mitochondrial swelling.

Presumably, PTU is acting on an enzyme in or on the mitochondrial membrane to modify the T4 effects, as well as on the membrane itself to modify the permeability barrier of the mitochondria. Other evidence obtained in this laboratory (LaBella, 1964c) indicates that PTU may have a direct effect on anterior pituitary tissue to release the thyroid stimulating hormone. This indicates that PTU possesses a stimulatory effect on pituitary, in addition to its inhibitory effect on deiodinase. This is not meant to imply that in both instances the mechanism of action is the same but the similarity of effects indicates the usefulness of the mitochondrion as an experimental membrane model.

After administration to the rats, PTU was not able to inhibit the mitochondrial swelling induced by ICN and I_2 but it did inhibit the response to T4 and T3. This can be construed as evidence supporting the concept that T4 deiodinase can play a part in T4-induced mitochondrial swelling since ICN and I_2 would not require deiodinase activity to give rise to I^+ which could be responsible for the mitochondrial swelling action of the iodocompounds.

SWELLING STUDIES ON MITOCHONDRIA ISOLATED FROM THYROIDECTOMIZED RATS

Hypothyroidism, in animals, is associated with a marked reduction in the basal metabolism rate and in young animals with a reduction in both growth rate and maximum. There is also a decreased function of organ systems, as well as mental and physical sluggishness. This decreased activity is also evident at the subcellular level. Mitochondria isolated from a hypothyroid rat are more resistant to swelling than those isolated from a normal rat, while those isolated from a hyperthyroid rat swell more readily than normal (Aebi and Abelin, 1953; Tapley, 1956). Furthermore, Tapley (1956) and Tapley and Cooper (1956) demonstrated that mitochondria isolated from a hypothyroid rat were less sensitive to swelling-inducing agents such as T4, Ag^{+}, Ca^{++} and succinate than normal. Tata et al. (1963) also found this reduced sensitivity to swelling-inducing agents in isolated mitochondria of hypothyroid rats, as well as to hypo-osmolarity.

Thyroidectomy leads to a reduction in sensitivity to some agonists but not all. T4 and T3 exhibited a reduced effect on the swelling of mitochondria isolated from a thyroidectomized rat, whereas T2, I^{-}, I_2 and ICN did not. Thus, it seems that the capability of the swelling mechanism(s), per se, is not markedly influenced; however, some step may be involved prior to the action of T4 and T3 on the swelling mechanism, which is inhibited by thyroidectomy. This may reflect the necessity that T4 and T3 undergo a molecular transformation before they are able to exert their full activity, and the site of this effect may be T4 deiodinase. Thyroidectomy did not reduce the T2-induced swelling. Since the I_2 on the iodothyronine inner ring is less labile than on the phenolic ring (Roche et al. 1952) and since a low degree of swelling was observed, this may be due to a direct effect of T2 rather than deiodination. In this work, the response to Ca^{++} and PO_4 but not to FMA and n-butyl 3,5-diiodo-4-hydroxybenzoate was reduced by thyroidectomy. Thus, it appears that

thyroidectomy does not reduce the swelling response to all agonists.

I_2 and ICN did not display a reduction in potency in promoting swelling of mitochondria isolated from a hypothyroid rat, whereas T4 did. Consequently, these results are in accord with the concept that the I^+ may be responsible for the hormonal effects and that deiodinase activity is associated with the effects of T4.

On the other hand, although a deiodinase may be necessary for T4 to exert its effects, there is the possibility that it may be bypassed in the presence of high concentrations of T4. Tata (1964) considers that T4 can act on different receptor sites. At low or physiological concentrations, T4 acts at physiological receptor sites, but in high concentrations at additional extraphysiological receptor sites. He suggests that this type of response could result from saturation of serum binding sites, thus permitting the more rapid entry of the thyroid hormone to the extraphysiological sites. Furthermore, the sites reached in the presence of high concentrations of hormones may be different from the sites normally reached. An example is that the administration of low doses of T4 produces a hyperthyroid-like state after a lag period of 12 to 18 days, but high doses of T4 produces the hyperthyroid-like state without such a lag phase. T4 may have an indirect and a direct action.

INTERACTION OF THYROXINE WITH VASOPRESSIN, OXYTOCIN AND INSULIN ON MITOCHONDRIAL VOLUME CHANGES.

Experimental evidence obtained in these studies demonstrated that T4 and the disulfide hormones, vasopressin, oxytocin and insulin, are capable of interacting to modify the T4-induced swelling response. In attempting to establish an experimental basis for this interaction, several possibilities were examined. T4 avidly binds to mitochondria (Klemperer, 1955; Tapley and Basso, 1959) and it has been suggested that binding of T4 to

mitochondria may be necessary before swelling is induced (Tapley and Basso, 1959), but Tata (1964) suggests that the strong binding sites may not be concerned in swelling and their saturation is responsible for the high T4 concentrations necessary to induce swelling. The possibility that mitochondrial binding of T4 might be altered was investigated in this work. However, no observable differences from controls were noted. If specific sites on the mitochondrial membrane are associated with T4 activity, any local alteration might be small and masked by the large amount of T4 bound to mitochondria. In addition, the time course of these experiments was short, 6-8 min, and no deiodination could be detected in the incubation mixture.

While the basis of the enhancement of T4-induced swelling by disulfide hormones has not been established, several possibilities exist. Both T4 (Klemperer, 1955; Tapley, 1956; Lehninger et al. 1959; Mattey and Green, 1963) and the disulfide hormones (Leaf, 1952; Lehninger and Neubert, 1961) have been shown to act on membrane systems to increase the transference of water. Since T4 and the disulfide hormones independently promote mitochondrial swelling and both appear to be capable of influencing sulfhydryl groups, this may be the level of their interaction. Mitochondrial sulfhydryl groups have been subdivided into two classes based on their reactivity in the course of swelling (Riley and Lehninger, 1964). Under the influence of vasopressin, the number of mitochondrial sulfhydryl groups were reduced (Riley and Lehninger, 1964) but T4 had no effect. Moreover, the interaction of disulfide compounds with sulfhydryl groups is well known (Boyer, 1959; Jensen, 1959) and a sulfhydryl-disulfide interchange has been invoked to explain the swelling produced by the combination of subthreshold concentrations of GSH and GSSG (Neubert and Lehninger, 1962b) and GSH and the disulfide hormones, vasopressin, oxytocin and insulin (Lehninger and Neubert, 1961). The literature

relating to the influence of T4 on sulfhydryl groups is sparse. Lehninger and Schneider (1959) reported that a combination of T4 and GSH produced a smaller response than each compound by itself. It has been reported that the sulfhydryl groups of liver proteins are reduced in number (Goldshtein and Gotovtzeva, 1958) in T4 treated rats and rabbits, and a possible T4 protection of sulfhydryl groups of succinic dehydrogenase in vitro has been proposed (Kripke and Bever, 1956). In the present work, NEM and $HgCl_2$ decreased T4-induced mitochondrial swelling, and the mitochondrial swelling induced by FMA and NEM was potentiated by iodocompounds. Thus it is possible that these agonists, the disulfide hormones and T4, might be interacting via the mitochondrial sulfhydryl groups.

In studying this interaction, T4 was utilized in a constant concentration and concentrations of the disulfide hormones were varied from 10^{-9} to 10^{-5}M. These disulfide concentrations are high relative to the effective concentrations employed in other systems, e.g. 10^{-11}M vasopressin will release TSH, in vitro (LaBella, 1964a), and toad bladder is also sensitive to low vasopressin concentrations (Leaf, 1962). Although the concentrations are high, this fact does not necessarily detract from the use of the mitochondrion as a model membrane system.

The interaction between T4 and disulfide hormones appears to be biphasic. The highest concentration of vasopressin utilized was 10^{-5}M and it did not enhance the T4-induced swelling, whereas lower concentrations were stimulatory. On the other hand, insulin and oxytocin augmented the T4 effect at the highest concentration but were not so effective as vasopressin in the lower concentrations. It may be that this is the result of changes in T4 deiodinase activity, since it is known that the T4 deiodinase activity can be influenced by substances capable of reacting with sulfhydryl groups. A similar biphasic effect was obtained in another experimental situation. LaBella (1964b) reported that the highest

vasopressin concentrations utilized were inhibitory to the release of TSH and the lower ones stimulatory. On the other hand, oxytocin and insulin were stimulatory to TSH release in high concentrations and not as effective in lower. Such striking similarity between different systems does not necessarily mean they can be interpreted in a like manner, but it does reinforce the concept that the mitochondria are a good model membrane system. T4 and vasopressin have also been reported to interact to increase the transfer of water across the toad skin (Marusic and Torretti, 1964). Cash et al. (1964, 1966) reported that small amounts of metal ions, such as Ca^{++} and Zn^{++}, were able to potentiate the T4-induced swelling, but this potentiation was not evident until 30 min. In the present experiments, the experimental time interval is less than 10 min. Furthermore, the modification of the T4 response by the disulfide hormones appeared to be biphasic. Cash et al. also reported that ashed preparations of vasopressin retain the ability to promote mitochondrial swelling. This is probably not a problem in these experiments since swelling due to disulfide hormones alone did not occur at the concentration employed.

SUMMARY AND CONCLUSIONS

A. SUMMARY OF EXPERIMENTS WITH N-ETHYLMALEIMIDE

1. NEM, 10^{-5}M, which approximated the threshold level for NEM-induced swelling, inhibited the T4-induced swelling.

2. NEM, 10^{-5}M, by itself, was without influence on the spontaneous mitochondrial volume changes.

3. Preincubation of mitochondria with NEM, 10^{-5}M, enhanced the inhibition of T4-induced swelling.

4. Increases in the concentration of T4 progressively reduced the inhibition by NEM.

5. NEM was without influence on the ICN-induced mitochondrial swelling response.

6. NEM and $HgCl_2$ in their threshold swelling concentration had inhibited the T4-induced swelling response.

7. Ca^{++}- and PO_4-induced swelling was also inhibited by NEM, 10^{-5}M.

B. CONCLUSION FROM EXPERIMENTS WITH N-ETHYLMALEIMIDE

NEM is probably acting at the level of sulfhydryl groups of mitochondria. The effects of NEM on T4-induced swelling may be the result of an interference with T4 deiodinase and I^+ may be responsible for the swelling effect of T4 and ICN.

C. SUMMARY OF EXPERIMENTS WITH FLUORESCEIN MERCURIC ACETATE

8. FMA promoted mitochondrial swelling and its fluorescence was quenched by mitochondria.

9. The quenching was rapid and preceded the mitochondrial volume changes.

10. In the absence of mitochondria, T4 and ICN did not quench the fluorescence of FMA, but I_2 and I^- did.

11. In the presence of mitochondria, T4 enhanced and ICN inhibited the quenching of FMA's fluorescence by mitochondria.

12. FMA-induced mitochondrial swelling, at 20°C, was potentiated by I_2, ICN, I^- and T4, in that decreasing order of potency.

13. At 0°C, I_2, ICN and I^- potentiated FMA-induced mitochondrial swelling, but T4 did not.

14. NEM-induced mitochondrial swelling was also potentiated by ICN and I_2, but not by T4 or I^-.

D. CONCLUSIONS FROM EXPERIMENTS WITH FLUORESCEIN MERCURIC ACETATE

FMA probably promotes mitochondrial swelling as a consequence of its reaction with sulfhydryl groups. The inhibition by ICN of the quenching of FMA's fluorescence may in part be due to the release of CN^- from the ICN molecule. It was also concluded that the FMA-induced mitochondrial swelling potentiated by T4, ICN, I_2 and I^- and NEM-induced swelling potentiated by ICN and I_2, may be the result of an increase in the reactivity of the sulfhydryl groups of the mitochondrial membrane by the iodocompounds.

E. SUMMARY OF EXPERIMENTS WITH PROPYLTHIOURACIL

15. PTU promoted mitochondrial swelling, and it was inhibited by CN^- and DNP.

16. The addition of ATP reversed the PTU-induced swelling.

17. Mitochondria isolated from a PTU treated rat displayed a reduced swelling response to T4, which was maximum in 3 hr.

18. Chronic administration of PTU also reduced the response to T4 but this was similar to that response obtained after a single PTU administration.

19. The mitochondrial swelling response to T4 and T3 was reduced in mitochondria isolated from a PTU treated rat, but not to ICN or I_2.

F. CONCLUSIONS FROM EXPERIMENTS WITH PROPYLTHIOURACIL

PTU may be interfering with the T4-induced swelling by inhibiting T4 deiodinase but similar *in vitro* effects were not demonstrated.

G. SUMMARY OF EXPERIMENTS WITH THYROIDECTOMY

20. Thyroidectomy reduced the response to T4 and T3, but not to T2, ICN, I_2 and I^-.

H. CONCLUSIONS FROM EXPERIMENTS WITH THYROIDECTOMY

These effects may be the result of a reduced T4 deiodinase activity in thyroidectomized rats.

I. SUMMARY OF EXPERIMENTS WITH DISULFIDE HORMONES

21. The disulfide hormones, vasopressin, oxytocin and insulin, in concentrations which did not promote mitochondrial volume changes over the experimental time course, modified the T4-induced mitochondrial swelling response.

22. Oxytocin and insulin exhibited a similar pattern of effects but vasopressin was active at lower concentrations.

23. The concentration of T4 appeared to be of importance, since the augmentation of the response in the presence of T4 was optimal at 2.5×10^{-6}M.

J. CONCLUSIONS FROM EXPERIMENTS WITH DISULFIDE HORMONES

This interaction between T4 and the disulfide hormones is probably occurring at the level of the mitochondrial sulfhydryl groups.

K. OVERALL CONCLUSIONS

The results of this thesis indicate that the mitochondrial sulfhydryl groups are critical in the swelling mechanism of T4-induced swelling. Since T4, but not ICN-induced swelling, is inhibited by NEM,

PTU administration and thyroidectomy, it is concluded that T4 deiodinase activity is involved in the course of T4-induced swelling and that the transiently formed I^+ might be responsible for exerting the swelling-producing effects.

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