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ROLE OF SODIUM ION IN IODIDE TRANSPORT
BY CULTURED THYROID CELLS

by

NANDALAL BAGCHI

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "ROLE OF SODIUM ION IN IODIDE TRANSPORT BY CULTURED THYROID CELLS", submitted by Nandalal Bagchi in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Investigations have been performed to study the influence of Na^+ on thyroidal iodide transport.

A system consisting of bovine thyroid cells cultured as monolayers was employed for the studies. The cells were found to actively accumulate iodide but did not bind the transported ion into organic compounds. Iodide transport was augmented when the cells were grown in the presence of TSH.

The process of iodide influx into thyroid-cells was found to have a specific and absolute requirement for Na^+. The rate of iodide influx was proportional to the Na^+-concentration of the medium from 0 - 150 mM. The rate of iodide influx followed Michaelis-Menten kinetics and was characterized by an increase in K_m without a significant change in V_m when the Na^+-concentration of the external medium was decreased.

Studies on the iodide efflux process revealed that it obeyed first order kinetics. The presence of Na^+ in the external medium had an inhibitory effect on the rate of iodide efflux. Addition of iodide, or anions which are competitive inhibitors of iodide transport, to the external medium abolished such inhibitory effects of Na^+. These anions did not modify the rate of iodide efflux when added to Na^+-free media.

Metabolic inhibitors and cardiac glycosides abolished net iodide transport into thyroid cells, suggesting an important role for Na^+-K^+-dependent, ouabain-sensitive ATPase in iodide transport. The inhibitors had no effect on the rate of iodide influx into thyroid cells. However, the rate of iodide efflux was augmented by these

agents provided that Na^+ was present in the medium. This increase in rate of efflux was observed after a lag period of 20-25 minutes.

A kinetic model for iodide transport has been formulated in an attempt to explain the experimental observations. The model postulates that during transport iodide ion is bound with Na^+ to a mobile carrier molecule contained in the thyroid cell membrane.

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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
BSS	Balanced salt solution
Control Thyroid Cells	Thyroid cells cultured without the addition of TSH to the growth medium
cpm	Counts per minute
DIT	Diiodotyrosine
EDTA	Ethylenediaminetetraacetic acid
g	Centifugal force relative to gravity
K_m	Apparent Michaelis constant
KRP	Krebs Ringer phosphate
MIT	Monoiodotyrosine
SEM	Standard error of mean
Tris	Tris (hydroxymethyl) aminomethane
TSH	Thyroid stimulating hormone
TSH Grown Cells	Thyroid cells cultured with the addition of TSH to the growth medium
T_3	Triiodothyronine
T_4	Thyroxine
V_m	Initial velocity at infinite substrate concentration

CHAPTER I

INTRODUCTION

Transport phenomena involve crossing by the metabolite of a very specialized part of the cell, the cell or plasma membrane. The definition of a cell membrane varies according to the manner of investigation, physiologic, anatomic (including electron microscopic), or biochemical. However, for the purpose of the present discussion the cell membrane may be defined as the external limiting region of the cell which functions as a permeability barrier. Its chemical composition varies from cell to cell but consists primarily of lipids (cholesterol and phospholipids) and proteins. It is about 75-100 A° thick (Robertson, 1964). Various techniques of investigation (e.g., electron microscopy, X-ray crystallography, etc.) have suggested a definite structural arrangement of its components. As an approximation it can be described as a lipid bilayer (Davson and Danielli, 1952; Robertson, 1959) with hydrophobic chains located at the centre and polar groups directed outwards. The protein component coats each surface of the lipid bilayer. Various specialized areas of membrane may well exist. For example, in some regions protein pores and plugs may traverse the membrane forming a continuous non-lipid region from one face of the membrane to the other. Simplified models such as these, however, ignore the dynamic structural arrangement of the membrane constituents which may undergo rapid turnover and reversible changes, possibly corresponding to phase transitions.

Since the cell membrane is lipoprotein in nature with a hydrophobic interior, it presents a major barrier towards the movements of most metabolites (e.g., ions, sugars, amino-acids, etc.) which

exist in aqueous solution on either side of the membrane. Permeation of such a structure by water-soluble substances would involve breaking of hydrogen bonds, which had formed between these substances and water molecules in solution, and then diffusion through the lattice formed by the hydrocarbon chains in the membrane. Transport would thus be affected by such factors as molecular size (or weight) of the permeants, and their ability to form hydrogen and hydrophobic bonds. The experimental diffusion rates found for many compounds have been consistent with such factors (Stein, 1967, pp. 74-77). It is also possible that penetration of the membrane by water and certain ions may occur in part through water-filled pores extending through the lipid bilayer.

A. Mechanisms of Specific Transport

Living membranes exhibit a much higher degree of specificity in response to various permeants than they would if they consisted solely of a lipid bilayer. Thus membrane permeation rates for a wide range of substances have been found to be appreciably higher and have shown a higher degree of specificity towards the particular substrate than would be expected on the basis of the properties discussed above, e.g., molecular weight, bonding abilities, etc. The types of transport that have been described for such systems include such diverse processes as facilitated diffusion, which involves movement of substrate along its electrochemical gradient without expenditure of cellular energy; "downhill active transport," which is similar to facilitated diffusion except for a requirement for cellular energy; and finally, "active transport," which involves movement of a substrate against its electrochemical gradient at the expense of cellular energy. For each

of these processes various specific transport mechanisms involving the cell membrane have been postulated.

1. Pore Models

The various pore models advanced to explain specific transport processes assume that small hydrophilic regions exist in the membrane extending from interface to interface. Substrates would diffuse through these regions rather than between the hydrophobic side chains of the lipid molecules. Specificity of transport in such a system would depend on the specific binding properties of enzyme-like proteins which line and form the pore. In addition, some models postulate substrate-induced variations in pore diameter to explain the different rates of transport discussed for various substances. Because simple pore models cannot account for such phenomena as exchange diffusion and counterflow (Stein, 1967, pp. 143-146), unidirectional pores have been postulated. However, even unidirectional pores do not account for the action of non-penetrating inhibitors (which block both influx and efflux) or the phenomenon of accelerative exchange diffusion (Stein, 1967, p. 150). On the whole, pore models have not gained wide acceptance.

2. Mobile Carrier Models

The mobile carrier models, proposed to explain specific transport processes, imply existence of a hypothetical carrier molecule in the cell membrane. The carrier forms a complex with the substrate, the whole complex then diffusing across the membrane (Willbrandt and Rosenberg, 1961). According to this hypothesis, the final carrier-substrate complex must translocate. The intermediate forms, and the free carrier itself, however, may not be able to translocate, or may do so at different rates. The substrate alone would permeate at a negligible rate when not combined with the carrier. The carrier-

substrate interaction may be catalyzed by a specific enzyme, present at one or both surfaces of the membrane. Alternatively, the carrier molecule might itself possess such catalytic properties. The latter possibility is doubtful in view of the small size which the carrier presumably would have in order to diffuse through the membrane at an appreciable rate. In spite of some deficiencies, the carrier hypothesis is probably the most acceptable one available at present.

3. Specialized Transport Systems

Existence of specialized transport systems has been postulated (Kleinzeller and Kotyk, 1961) and transport models based on these have been advanced. In such systems lipoproteins are postulated to be firmly anchored to the cell membrane. These proteins possess a hydrophilic interior and an outer shell of hydrophobic groups, a direct contrast to the usual structure of globular proteins. The substrate moves by simple thermal diffusion through the protein plug to its hydrophilic interior. It is postulated that the specific substrate then combines with an active centre in the protein which undergoes a conformational change and initiates the movement of the substrate-active centre complex towards the opposite face of the membrane. When chemical equilibrium is again established with the medium on the opposite face, the complex dissociates and the substrate diffuses through the protein plug to the aqueous phase. The active centre, freed of the substrate, returns to its previous conformation. Such models utilize the concept of substrate-induced conformational changes in an enzyme (Koshland, 1960). They have provided a reasonable explanation for the mechanism of action of both facilitated diffusion and cotransport (discussed below) processes.

B. Role of Na^+ Ion in Transport Processes

While there is a great deal of uncertainty concerning the mechanism of specific transport processes, an important advance in this area of knowledge has occurred in recent years with the recognition of the important role played by sodium ion in the cellular transport of many substrates. A number of recent studies clearly demonstrated that the presence of Na^+ in the external medium stimulated the active transport of many substrates, often to a marked degree. Such effects of Na^+ were noted as early as 1902 when Reid suggested that Na^+ was a stimulant of glucose absorption in the small intestine. The more recent studies began in 1958 when Riklis and Quastel demonstrated that the absorption of glucose by guinea pig small intestine was markedly dependent on the presence of Na^+ in the solution bathing the mucosal surface. In the years following, Na^+-dependence has been reported for the transport of a number of metabolites in a wide variety of tissues. Though to date most emphasis has been on the role of Na^+, other cations, notably potassium, also have been found to influence the transport of various metabolites. However, the effect of such ions is not yet as well defined as that of Na^+; indeed in some cases it appears to be minimal.

Na^+-dependent transport processes are of widespread occurrence in nature, not only in vertebrates, e.g., mammals, birds, fish, amphibians, etc., but also in many lower forms. Sugar absorption by the small intestine of sea cucumber (Holothurian) Thyone depends on Na^+ (Farmanfarmaian, 1968), and similar effects of Na^+ have been reported for the uptake of glycine across the body wall of aquatic annelids (Stephens, 1964). A marine bacterium, Pseudomonad B-16, has

been shown to exhibit Na^+-dependent glucuronate uptake (Payne, 1960). Na^+-dependent metabolite uptake has been reported also for marine fungi (Siegenthaler *et al*, 1967) and algae (Sistrom, 1960). On the other hand, with a few exceptions, most terrestrial bacteria do not show such Na^+-dependent transport processes.

The influence of Na^+ on the transport of various monosaccharides in mammalian tissue has been the subject of extensive studies. Na^+-dependence has been observed in epithelial tissues, e.g., small intestine and kidney, and in the choroid plexus of dogs. In all cases these tissues were capable of concentrating sugars against an electrochemical gradient, i.e., they exhibited active transport. In other systems where sugars entered cells down an electrochemical gradient by the process of facilitated diffusion, as in striated muscle, no effect of external Na^+ was observed (Parrish *et al*, 1964). So far Na^+ has been implicated in all processes capable of bringing about transport of sugars across animal cell membrane against concentration differences. The only exception to this generalization has been the uptake of 2-deoxy-D-glucose and 2-deoxy-D-galactose against concentration differences by rabbit kidney cortex slices; these have been found to be independent of the concentration of Na^+ in the external medium (Kleinzeller *et al*, 1967a and 1967b).

The transport of amino acids by various mammalian tissues is another system that has been extensively investigated with respect to Na^+-dependence. In contrast to the limited number of tissues which exhibit Na^+-dependent sugar transport, amino acid transport has been observed to be Na^+-dependent in a wide variety of tissues, both epithelial and non-epithelial including small intestine, kidney, striated

muscle, brain, Ehrlich ascites cells, and red blood cells. In some cells the Na^+-dependent transport of amino acids has been found even in isolated organelles such as nuclei and mitochondria. In each case, the Na^+-dependent process was capable of bringing about amino acid transport against a concentration difference which suggested that the important factor in determining Na^+-dependence was the nature of the transport process rather than the nature of the tissue or the cell. Such dependence was not observed if amino acid entry took place by a process of facilitated diffusion, as in the case of glycine and alanine uptake by mature rabbit erythrocytes (Wheeler and Christensen, 1967b). However, all "uphill" amino acid transport may not be Na^+-dependent. Thus lysine accumulation by ascites cells (Christensen and Liang, 1966) and by epithelial cells of the toad bladder (Thier, 1968) were reported to be independent of extracellular Na^+. Another factor which may determine whether the transport of an amino acid is Na^+-dependent seems to be the charge on the amino acid. The anionic amino acids have the greatest Na^+-dependence, the cationic ones the least, while neutral amino acids exhibit intermediate dependence (Schultz, 1969).

Na^+-dependence has been detected in a few other transport systems in mammalian tissues. Transport of ascorbic acid, creatinine, thiamine and acetate into brain cells has been found to be Na^+-dependent (Quastel, 1965). Furthermore, the transport of uric acid, myoinositol, p-amino hippurate and Ca^{++} into kidney cells has been reported to be Na^+-dependent (Berndt and Beechwood, 1965; Hauser, 1969, Vogel *et al*, 1965; Vogel and Stoeckert, 1967). Observations suggesting similar dependence have also been made for the transport of sulphate, uracil, phosphate, Ca^{++} and bile salts in the small intestine (Anast

et al, 1965; Csaky, 1961; Harrison and Harrison, 1963; Holt, 1964; Martin and DeLuca, 1968).

All recognized Na^+-dependent transport processes have several common features. A decrease in the Na^+-concentration of the extracellular medium is associated with a reduction in both net transport and influx of the metabolite concerned. Na^+-dependence may be absolute in some cases with no active transport occurring in the absence of Na^+. In any case it is generally specific in nature, and in most situations other monovalent ions such as K^+, Li^+, Rb^+, $Tris^+$, NH_4^+, $choline^+$, or neutral molecules such as mannitol and sucrose exhibit little or no activity when substituted for Na^+. Also, wherever it has been examined, entry of the metabolite into the cell has been found to be accompanied by an additional entry of Na^+ into the cell in a definite stoichiometric ratio. Examples of this include alanine entry into rabbit small intestine (Curran *et al*, 1967) and glycine entry into pigeon red blood cells (Wheeler and Christensen, 1967a).

Such observations have given rise to the concept of co-transport. According to this theory, metabolites become associated with Na^+ in some step or steps of their transport, with the result that both species are transported together into the cell. The phenomenon of cotransport may be well explained by certain mobile carrier models if the assumption is made that the carrier molecule combines with both Na^+ and the metabolite, and that the resulting ternary complex crosses the membrane.

C. The Na^+ Gradient Hypothesis

The finding that many active cellular transport processes are Na^+-dependent, when considered in the light of the well-known

asymmetric distribution of sodium across animal cell membranes, led to the suggestion that Na^+ asymmetry itself might provide the energy required for the observed active transport of other solutes. Riggs, Walker and Christensen (1958) first suggested such a role for Na^+ asymmetry. In addition, they regarded K^+ asymmetry across the cell membrane as another possible energy source. Crane (1962) proposed the "Na^+-gradient hypothesis" as a mechanism for active sugar transport in the small intestine, and this concept was later extended to other Na^+-dependent transport systems. This hypothesis is based on the assumption that movement of solute from one side of the membrane is influenced by the Na^+ concentration on the same side. Thus influx of the solute into the cell will be dependent on the extracellular Na^+-concentration, and similarly efflux of the solute will be dependent on the local Na^+ concentration on the inner side of the membrane. Since the intracellular Na^+ concentration is normally considerably lower than the extracellular concentration, influx will exceed efflux, resulting in the accumulation of the solute against a concentration difference. Thus, as long as asymmetry of Na^+ distribution is maintained, this potential energy source would be available for the active transport of any solute whose bidirectional movements are influenced by the local Na^+ concentration.

It must be emphasized that the contribution of coupled transport to the overall movement of Na^+ across the cell membrane is only a small fraction of the total. Most of the Na^+ movements across the cell membrane occur independently, perhaps by a combination of leak into the cell and active transport (the so-called Na^+-pump) out of the cell (Tosteson and Hoffman, 1960). The former follows the concentration gradient of Na^+ and is perhaps "passive". The latter involves the

Na^+-K^+-dependent, ouabain-sensitive adenosine triphosphatase (ATPase), a membrane-bound enzyme, which exchanges extracellular K^+ for intracellular Na^+. Because operation of the Na^+-pump requires ATP, the maintenance of the Na^+-gradient across the cell membrane is energy dependent. However, according to the sodium gradient hypothesis, no direct link between metabolic energy and solute transport would be necessary. Instead, the metabolic energy would be invested in the active Na^+-extrusion mechanism (i.e., in the operation of the "sodium pump"), and the resulting low intracellular Na^+ concentration would serve as a transducer of this energy input for other transport purposes.

Such an arrangement would be of obvious advantage to the energy economy of the cell. Without such coupling, only a fraction of the energy of the exergonic chemical reaction (i.e., ATP hydrolysis) would be converted to useful osmotic work (i.e., transport of Na^+ against electrochemical gradient), and the rest would be dissipated in the form of increased internal entropy. The coupling mechanism would permit the downhill flow of Na^+ into the cell to provide the driving force for the transport of other solutes against their respective electrochemical gradients. Thus the amount of useful work obtained per mole of Na^+ transported would be significantly increased. Without such a coupling mechanism, each individual active transport process would have to be linked to exergonic chemical reactions which would decrease the overall efficiency with which chemical energy was converted to osmotic work (i.e., the work of transport).

There has been abundant experimental evidence in support of the sodium gradient hypothesis. Thus the diminution of net transport and of influx of various solutes caused by a reduction of extracellular

Na^+ concentration, examples of which have been given in the previous sections, confirms the predictions of the hypothesis. However, the most striking verification of the hypothesis came from the work of Vidaver (1964) who studied a system consisting of lysed, and subsequently restored, pigeon red blood cells, the latter having been prepared with solutions of varying Na^+ and glycine concentrations. In this system it was possible to adjust the intracellular concentrations of Na^+ and glycine as required and to avoid problems arising out of intracellular compartmentation. After suspension of these cells in various media, Vidaver clearly demonstrated that the direction of net transport of glycine was determined, not by intracellular or extracellular Na^+ concentration alone, but by the difference between the two. A study of the effect of various metabolic inhibitors (e.g., NaCN, 2,4-dinitrophenol) and cardiac glycosides (e.g., ouabain) on the Na-dependent transport processes has led to further support of the sodium-gradient hypothesis. The metabolic inhibitors are known to abolish ATP production and thus render the "sodium pump" ineffective. Ouabain achieves the same result by its direct inhibition of active Na^+-extrusion. The result, in both cases, is a failure of the active Na^+ extrusion mechanism which secondarily produces a gradual rise in the intracellular concentration of Na^+ due to the continuing passive "leak" of Na^+ into the cell from the external medium. This, according to the hypothesis, would increase the rate of efflux of any solute which shows Na^+-dependent transport and would lead, as a result, to a decrease in net transport of the solute. The predictions have been amply verified experimentally. Thus alanine efflux across the brush border of ileum has been shown to be stimulated by ouabain on direct measurement (Hajjar *et al*, 1970). In

another system glycine efflux from ascites cells also was shown to be accelerated by ouabain (Eddy, 1967). The effect of ouabain was generally found to occur only following a lag period, which would be expected because the rise in the intracellular concentration of Na^+ due to the "leak" would be quite gradual.

D. Role of Na^+ in Iodide Transport

The role of Na^+ in the active transport of iodide by the thyroid gland has been the subject of a number of studies. These have suggested a significant involvement of Na^+ though the precise mechanism remains unclear. Alexander and Wolff (1964) found that the equilibrium ratio of iodide (tissue/medium) in sheep thyroid slices increased linearly when the Na^+ concentration of the medium was raised from 0 to 170 mM. Shishiba and Solomon (1967) showed that amphotericin B, a membrane active agent, increased the concentration of Na^+ and concomitantly reduced the concentration of K^+ by bovine thyroid slices, and that this was accompanied by a decrease in the tissue/medium ratio for iodide. Wolff and Maurey (1958) demonstrated virtually complete inhibition of thyroidal iodide concentration with various cardiac glycosides including ouabain; this finding was confirmed by other investigators (Lee and Ingbar, 1965). Wolff and Halmi (1963) showed that there was a correspondence between the iodide transport activity and the Na^+-K^+-dependent, ouabain-sensitive ATPase activity of whole homogenates or subcellular fractions prepared from thyroids of different species. They concluded that enzyme activity was connected indirectly with transport activity. Turkington (1962) reported the stimulation of such enzyme activity in an isolated thyroid cell membrane preparation by thyroid-stimulating hormone (TSH) an

agent that also stimulates iodide transport activity. Iff and Willbrandt (1963) showed that depletion of Na^+ from the external medium increased iodide efflux from beef thyroid slices. Addition of ouabain to the medium had a similar effect except that the increased efflux occurred after a lag period. Scranton and Halmi (1965) also observed an increased iodide efflux from rat thyroid lobes on addition of ouabain to the medium. From such observations it has become apparent that Na^+ plays an important role in thyroidal active iodide transport, and that the process involves in some way the Na^+-K^+-dependent, ouabain-sensitive ATPase activity of the tissue. However, it has not been established whether Na^+ influences net iodide transport by its effect on influx or efflux or both.

A major difficulty in using thyroid tissue for investigation of iodide transport, by methods suitable in other systems, has been the structural complexity of the thyroid gland. The gland is composed of a number of follicles, each of which consists of a central space occupied by a colloid-like material surrounded by a single layer of thyroid epithelial cells. Since the cells separate the colloid space from the extracellular space, they possess a polarity, one cell membrane (the basal) facing the extracellular space and the other (the apical) facing the colloid space. Influx of iodide into the colloid spaces of the gland thus involves crossing of both these membranes in series by the extracellular iodide which is derived from the circulating iodide pool. Efflux of iodide from the gland involves a retracing of this pathway in the reverse direction. Thyroidal iodide transport in the intact gland is thus transcellular. This complexity makes experimental data, especially those derived from kinetic studies, difficult to analyze

because the respective contributions of the two membranes are not known although the basal membrane has generally been considered as the one primarily involved in active transport. In addition, technical difficulties prevent a separate determination of the iodide concentrations of the cells and the colloid space.

The foregoing difficulties associated with the use of the intact thyroid gland as an experimental system were circumvented in the present investigation by the utilization of cultured thyroid cells. Such cells retain their biological functions during growth and have been used for transport studies in several recent investigations (Chapter III). Cultured cells are non-polar and are arranged in a two-dimensional array. Iodide movements occur across a single membrane in these cells and are thus transmembrane rather than transcellular. Measurements of iodide influx and efflux in such systems are comparatively simple, and the interpretation of the resultant data is relatively straightforward. The initial objective of the present study was to develop a system of thyroid cells in tissue culture, which would be functional at least with respect to active iodide transport properties. Furthermore, it was necessary to establish that the properties of the transport system exhibited by the cells should be basically equivalent to those seen in the gland *in vivo*.

The present thesis describes experiments which were performed to establish a suitable thyroid cell culture system possessing the characteristics mentioned above. Studies were undertaken using the thyroid cell cultures to elucidate the effect of extracellular Na^+ on the rate of influx of iodide into thyroid cells. The effect of extracellular Na^+ on the rate of efflux of iodide from such cells was

then investigated. Further studies were undertaken to determine how the effect of Na^+ on iodide influx and efflux was modified by competitive inhibitors of iodide transport, metabolic inhibitors and cardiac glycosides. Finally, a model of iodide transport has been postulated in an attempt to explain on a molecular basis the data obtained from the various studies.

CHAPTER II

THE PREPARATION OF THYROID CELLS IN MONOLAYER CULTURE

A. Introduction

Establishment of a thyroid cell culture system in which the cells have lost their follicular arrangement but are still functional, at least with respect to active iodide transport, was the initial object of the present investigation. The frequent use of monolayer cultures of thyroid cells has provided considerable information on the properties of such cultures (the literature pertaining to this subject is reviewed in the next chapter). On the other hand, reports which describe retention of functional characteristics by other forms of culture have been infrequent. These initial considerations led to the choice of a monolayer culture system for the present studies. Bovine thyroid tissue was selected in view of its availability, even though the literature contained no reports at that time about the functional properties of a bovine thyroid monolayer system.

The culture procedure involved two main steps:

(i) Disaggregation of thyroid tissue to obtain a cell suspension.

(ii) Culture of the isolated cells in a suitable medium on a glass or plastic surface.

Disaggregation was achieved by physical disruption of the tissue by chopping to a fine size (2 mm x 2 mm) after which trypsin was used to digest the intercellular material. Such enzymatic disaggregation has been achieved by others using trypsin, pancreatin, collagenase and papain (Rinaldini, 1958; Siegel, 1971), either alone or in combination. An alternative, non-enzymatic mode of tissue disaggregation in common use involves chelating agents, such as EDTA, which remove divalent

cations which seem to be essential for maintaining the integrity of the tissue. However, as is generally true of tissues which have a low-fibrous content, use of trypsin alone proved to be adequate for the disaggregation of thyroid tissue.

The trypsinization of thyroid tissue was carried out by a continuous technique, first described for kidney tissue (Rappaport, 1956) and later adapted for thyroid tissue (Tong *et al*, 1962). This involved exposing the tissue to a continuous flow of fresh trypsin solution while at the same time an equal volume of cell suspension was being withdrawn from the reaction vessel to a reservoir where trypsin action was terminated. This ensured that newly isolated cells did not remain in the reaction vessel for long, and thereby minimized cell damage due to prolonged exposure to trypsin. The continuous technique, besides ensuring minimal cell damage, possessed the further advantages of being relatively simple from a technical standpoint and also capable of handling large amounts of tissue.

B. Materials

1. Earle's Balanced Salt Solution (BSS)

This solution had the following composition (in grams per liter):

NaCl	6.80
KCl	0.40
$CaCl_2$	0.20
$MgSO_4.7H_2O$	0.10
$NaH_2PO_4.H_2O$	0.125
Glucose	1.00
$NaHCO_3$	2.20

Distilled water to 1000 c.c.

The final pH of the solution was 7.4

2. Trypsinizing Solution

This was a 0.25% solution of 1:250 trypsin (Difco Laboratories) in Earle's BSS, adjusted to pH 7.8 by the addition of a few drops of 7 N NaOH. The solution also contained 100 units/ml of penicillin G and 100 μg/ml of Streptomycin sulphate, both purchased from Glaxo-Allenburys.

3. Culture Medium

This was prepared as follows:

Eagle's Minimum Essential Medium (Eagle, 1959)	100 ml
Glutamine (200 mM)	1 ml
Fetal Calf Serum	20 ml
Penicillin G	100 units/ml
Streptomycin Sulphate	100 μg/ml
Mycostatin (Squibb)	20 units/ml

With the exception of mycostatin, all other reagents were purchased from Baltimore Biological Laboratories. Penicillin and streptomycin were supplied together as a lyophilized mixture. The final pH of the medium was 7.4. When indicated, 70 milliunits of TSH (Nordic Biochemicals) were added to the medium.

4. Bromsulphthalein Reagent

The composition was:

Bromsulphthalein (5%) (BDH Pharmaceuticals)	1 ml
1 N HCl	100 ml
1 M citric acid	50 ml
Distilled water to	250 ml

5. Trypsinizing Flask (Tong *et al*, 1962)

This was a modified Erlenmeyer flask (1 liter capacity) with three longitudinal indentations at the sides designed to promote turbulence when the contents were stirred by a magnetic stirrer. The outlet was at the side of the flask. A perforated disc at this outlet prevented large particles from passing on to the tubes (Fig. 2.1).

6. Tubing

Tubing for connections was made of pure gum rubber.

7. Culture Vessels

These were either 250 ml capacity plastic culture bottles (Falcon Plastics) or short Leighton tubes (Bellco Glass, Inc.) accommodating 9 mm x 35 mm glass coverslips (Bellco Glass, Inc.).

8. Sterilization

The balanced salt solution and all apparatus were sterilized by autoclaving at 275°F for 40 minutes under a steam pressure of 20 lbs. per square inch.

C. Methods

1. Disaggregation of Thyroid Tissue into Isolated Cells

(i) Fresh bovine thyroid glands, obtained from local abbatoirs, were collected in Earle's BSS in polyethylene bags cooled in crushed ice.

(ii) At the laboratory, the glands were trimmed of extraneous tissue, cut with a pair of scissors into small pieces (1 cm x 1 cm), rinsed several times in Earle's BSS and finally minced to 2 mm x 2 mm (approximately) pieces using a mechanical tissue chopper of the McIlwain-Buddle type (McIlwain and Buddle, 1953). Chopping was done in a cold

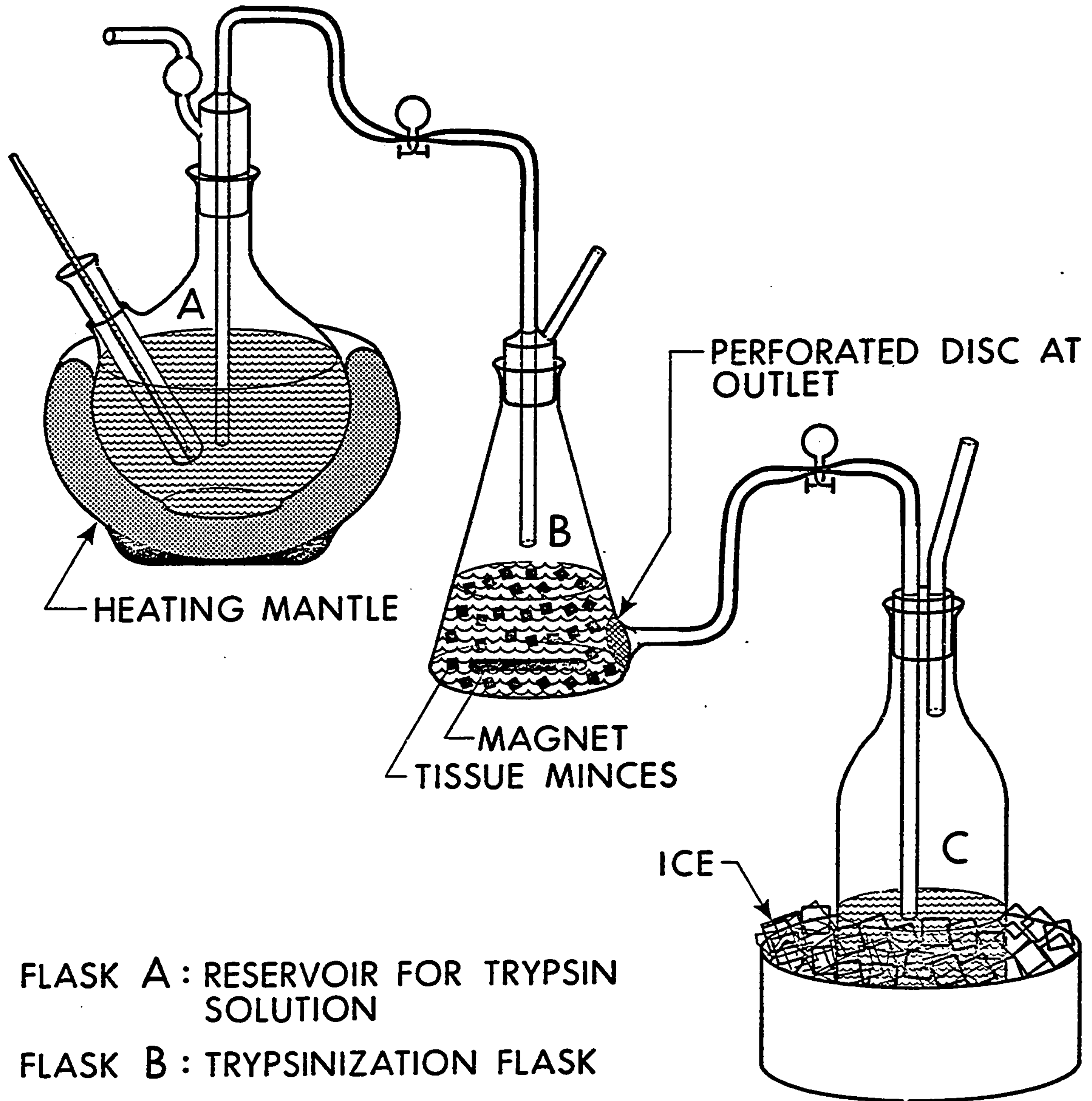
room at 4°C. Sterile technique was introduced at this step and maintained during the succeeding steps. The process required approximately two hours from the time of collection of tissue to the time the mincing was completed.

(iii) Trypsinization was carried out in the apparatus shown in Fig. 2.1. Approximately 50 grams of minced thyroid tissue were suspended in 150-200 ml of trypsin solution in the trypsinizing flask B. The suspension was stirred continuously by a silicone-covered magnet. Fresh trypsin solution from flask A (2000 ml capacity), warmed to 37°C by a heating mantle, was introduced continuously and dropwise into the trypsinizing flask by siphon action, the rate being controlled by a pinch-cock on the rubber tubing connecting flasks A and B. The magnet speed was adjusted to cause slight cavitation but no foaming at the surface of the fluid.

(iv) The effluent from vessel B, which contained the isolated cells in suspension, was collected in a reservoir consisting of a 4-liter bottle C. The reservoir, which initially contained 200 ml of calf serum, was cooled in crushed ice throughout the procedure. The serum, which has considerable antitryptic activity, as well as the cooling, served to arrest any continuing trypsin action in the fluid collected in the reservoir. The drainage from flask B to reservoir C was also continuous, and its rate was controlled by regulating the rate of inflow of trypsin solution into the flask B.

(v) Continuous trypsinization of the minced thyroid tissue was carried on for approximately five hours during which time the

Figure 2.1. Apparatus for trypsinization of thyroid tissue.



FLASK C : RESERVOIR FOR CELL SUSPENSION

tissue was exposed to 3-3.5 liters of trypsin solution. During the first two hours the cell content of the effluent from flask B was low, but thereafter it increased appreciably. By the end of five hours the reaction flask contained mainly masses of disorganized, fibrous tissue.

(vi) The fluid from reservoir C was filtered successively through 28-, 60- and 150-mesh stainless steel sieves and was then centrifuged at 4°C at 150 x *g* for 10 minutes. This yielded approximately 1 ml of cells as a pellet. Such low yields were not unexpected in view of the follicular architecture of thyroid tissue, in which cellular elements constitute only a small proportion of the total tissue mass. The cell pellet was resuspended in Earle's BSS and recentrifuged as before. This washing step served to eliminate most of the adherent trypsin solution.

2. Culture of Cells

Cells obtained as described above were suspended in 60-80 ml culture medium to yield approximately 10^6 cells/ml. Microscopic examination of the suspension showed that most of the cells were present as single cells or as small aggregates of 2-6 cells. The cell suspension was then transferred to culture flasks (15 ml per flask) or Leighton tubes bearing coverslips (1.5 ml per tube) which were then incubated at 36°C after the vessels has been tightly capped. After 12-16 hours most of the cells had attached to the surface. At this time the supernatant medium containing dead or damaged cells, red blood cells and cellular debris, all of which may be toxic to the surviving cells, was discarded and fresh medium was introduced. Thereafter the medium was changed

every 3-4 days.

It was found that if the culture flasks were tightly capped to retain the carbon dioxide liberated by cellular metabolism, gassing of the culture system by 95% oxygen - 5% carbon dioxide mixture was not necessary (Paul, 1970, p. 57).

3. Suspension of Cells Grown in Monolayer Culture

The cells which had been grown as monolayer cultures were suspended by one of two methods:

(i) Gentle scraping with a rubber "policeman". The resulting flakes were then gently sucked up and down a pipette a few times in order to break them up.

(ii) Incubation with 0.1% trypsin in Earle's BSS for 5 minutes, followed by incubation in Ca^{++}-Mg^{++}-free BSS for approximately 30-45 minutes at 37°C.

4. Cell Counting

The suspended cells were counted in a hemocytometer (Neubauer type) without any further dilution and without staining.

5. Staining

For morphological studies the monolayers or coverslips were stained with a standard hematoxylin-eosin stain. In such preparations the nuclei were stained blue while the cytoplasm appeared red (plates 2.1 and 2.2).

6. Estimation of Cell Proteins (Paul, 1970, p. 356)

Coverslips with adherent cells were incubated at 37°C for one hour with 1 ml of 1 N NaOH to digest the cells (2 ml of NaOH were used when the cells had been grown in the presence of TSH and so contained larger amounts of protein). 2.5 ml of bromsulphthalein reagent was

added to 1 ml of the digest and the mixture, after thorough shaking, was centrifuged for 10 minutes at room temperature at top speed in a clinical centrifuge. Seven ml of 0.1 N NaOH was then added to 0.5 ml of the supernatant in a separate test tube; after mixing the optical density was read at 580 mμ in a Beckman (Kintrac VII) spectrophotometer. The decrease in optical density from that obtained for a protein-free solution was proportional to the amount of protein in the sample. A standard curve was prepared using known amounts of protein standard solution (Armour Pharmaceutical Co.), which was a solution of crystalline bovine albumin containing 10 mg protein nitrogen per ml. The curve was linear over the range of 10-100 μg protein. When required, the same method was used for the determination of protein in specimens other than cells grown on coverslips.

D. Results and Discussion

1. Formation of a Cellular Monolayer

Usually the thyroid cells, which had been obtained by the continuous trypsinization of bovine tissue, were found to have attached to the surface in about 12 hours. By the second day of incubation the attached cells had assumed an elongated shape which marked the beginning of the stage of proliferative growth. As growth continued the cells formed islands which ultimately became confluent forming one continuous sheet of cells. The time required to form a complete monolayer depended on various factors, the most important of which appeared to be the density of the cells in the inoculum and the percentage of cells which were viable initially. The latter was estimated to be 50-60% as determined by measuring the proportion of cells of the inoculum that excluded trypan blue stain (Paul, 1970, p. 356). In most cases a complete monolayer

formed in 4-5 days. The addition of TSH demonstrably hastened the process of monolayer formation.

2. Appearance of the Monolayers

The monolayers appeared to be composed exclusively of epithelial-type cells of uniform appearance (plates 2.1 and 2.2). Each cell had abundant cytoplasm with a centrally placed round or oval nucleus with 2-4 nucleoli. No evidence of follicle formation or of any other type of special grouping was seen. The appearance of the monolayer was the same in both TSH-treated and untreated cultures. The cells were maintained as primary cultures for as long as four weeks during which time the medium was changed twice a week. Throughout the four-week period the morphology of the cells did not appear to change significantly, and there was only minimal desquamation of cells from the surface.

Follicle formation in thyroid monolayer cultures had been reported frequently. Some investigators (Siegel, 1971; Malette and Anthony, 1966) have reported it in cultures grown both with and without TSH, while others (Kerkof *et al*, 1964a; Lissitzky *et al*, 1971) have reported it only if TSH, dibutyryl cyclic 3',5'-adenosine monophosphate or theophylline was added to the culture medium. Lack of follicle formation in the present culture system may be related to the partial loss of differentiated function of the cultured cells which will be described in the following chapter.

3. Effect of TSH on Cellular Multiplication

The results which are summarized in Table I were obtained by using a single population of trypsinized thyroid cells for the inoculation of two groups of culture tubes. In one group 70 mU/ml of TSH was added to the culture medium, while the second group without such additions

TABLE 2.1

Effect of TSH on Number and Protein Content of Thyroid Cells in Culture

Type of Cells	A. Amount of Protein Per Coverslip (μg)	B. Protein Content Per Cell (ng)	C. Number of Cells Per Coverslip ($x\ 10^{-4}$)*
1. Control thyroid cells	121 ± 16	0.88 ± 0.02**	13.7
2. TSH-grown cells	263 ± 23	0.94 ± 0.02	27.9

Thyroid cells were grown for 5 days as monolayer cultures with or without the addition of TSH to the medium (see text). One-half of each group of cells was used for the determination of the average protein content per cell. For this analysis the thyroid cells were suspended by the use of 0.1% trypsin, followed by Ca^{++}-Mg^{++}-free BSS as described in the text. After the cells were counted, an aliquot of the suspension was centrifuged and its protein content was determined in the usual manner. These data enabled the calculation of the results presented in Column B. The remaining one-half of each group of cells was used for the determination of the total protein per coverslip (Column A).

*Obtained by dividing the value in Column A by that in Column B.

**Mean of 4 determinations ± SEM.

served as control. After 5 days of growth, the average amount of protein recovered per coverslip was more than doubled in the TSH-treated group. However, the amount of protein per cell did not differ markedly between the two groups. The results suggested that the presence of TSH in the culture medium stimulated multiplication of thyroid cells without greatly altering the size of the daughter cells. The stimulatory action of TSH on thyroid cell multiplication is quite similar to the thyroid cell hyperplasia seen following the administration of antithyroid drugs to iodide-deficient animals (Wollman and Breitman, 1970) in which endogenous levels of TSH are believed to be elevated.

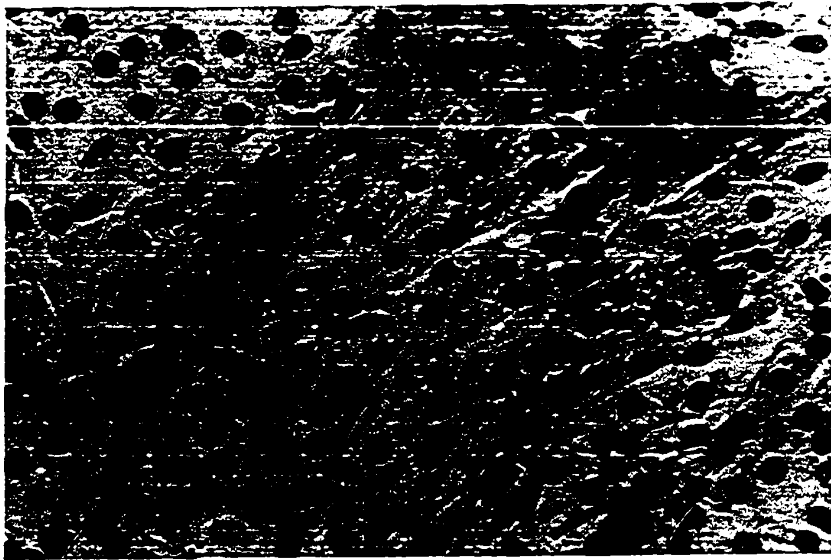


Plate I. Photomicrograph of 5-day old monolayer culture of control thyroid cells.

Stained with hematoxylin and eosin x 460.

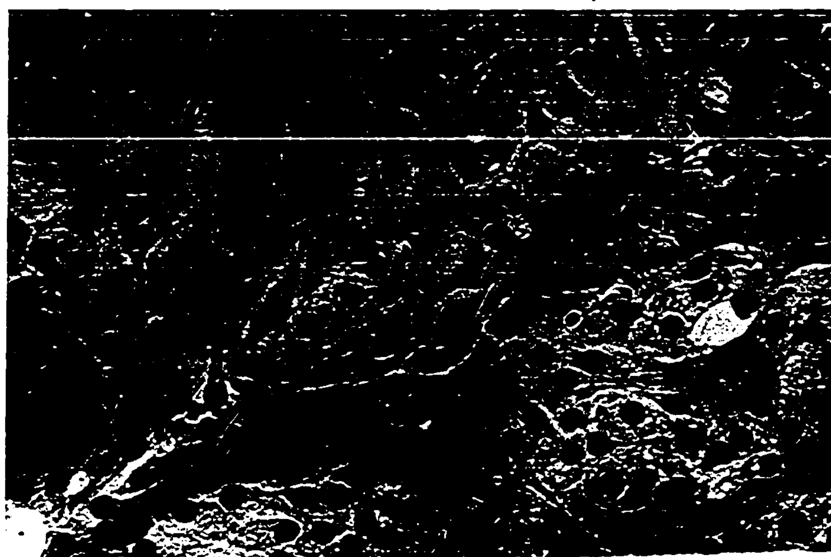


Plate II. Photomicrograph of 5-day old monolayer culture of TSH-grown thyroid cells.

Stained with hematoxylin and eosin x 460

CHAPTER III

IODINE METABOLISM IN THYROID CELLS IN MONOLAYER CULTURE

A. Introduction

The metabolism of iodine by the normal thyroid gland occurs in several distinct steps. Iodide ion is first actively transported into the gland where it then becomes covalently bound to a specific thyroidal protein (thyroglobulin). The iodoprotein so formed is ultimately hydrolyzed yielding the active hormones, triiodothyronine (T_3) and thyroxine (T_4) as well as the intermediates, monoiodotyrosine (MIT) and diiodotyrosine (DIT). These steps are shown in a schematic form in Fig. 3.1. All these functions are stimulated by thyroid-stimulating hormone (TSH), a glycoprotein secreted by the anterior pituitary gland. A number of investigators have reported that thyroid cells grown in monolayer culture either retain both active transport and binding functions, or lose them both completely.

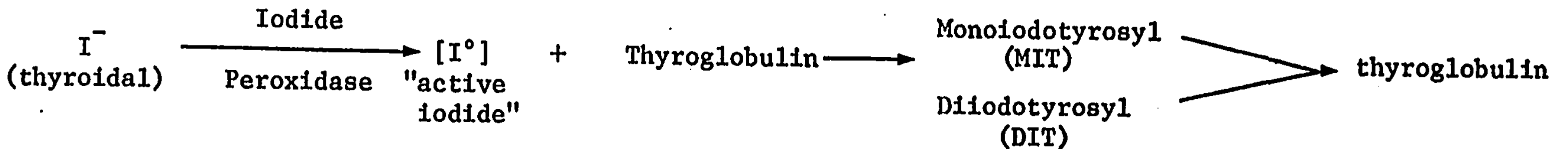
Pulvertaft *et al* (1959) studied human thyroid cells in monolayer culture and reported that the cells concentrated iodide to a small extent; MIT was the predominant, or sometimes exclusive, product of organification.

Hilfer (1962) cultured 16-day old chick embryo thyroids in monolayers and found that these cells could form MIT and DIT but not T_3 or T_4. In contrast, Spooner (1970) described the formation of both T_3 and T_4 by the same type of cells grown in monolayers using culture times of up to 20 days, with a medium different from that utilized by Hilfer.

Tong *et al* (1962) and Kerkof *et al* (1964b) investigated iodide metabolism in sheep thyroid monolayer cultures and demonstrated

I. Transport of Iodide

II. Organic Binding of Iodide



III. Release of Hormones

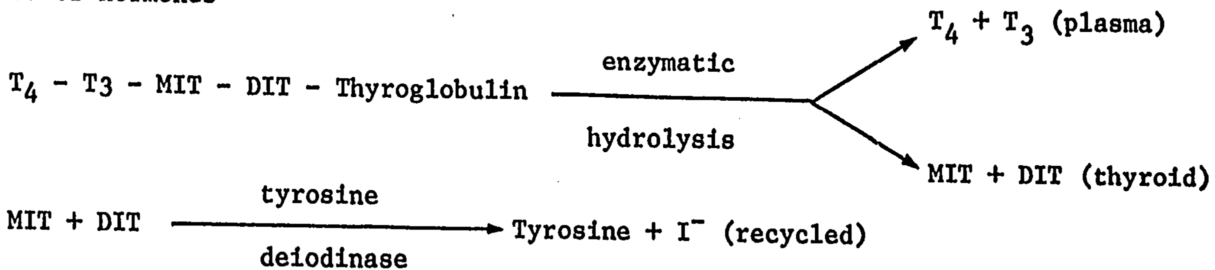


Figure 3.1. Sequence of iodine metabolism in the thyroid gland.

complete retention of both active transport and organification functions. Furthermore, they showed that addition of TSH to the culture medium led to a marked enhancement of both these functions.

Rabin *et al* (1966) studied hog thyroid cells in monolayer culture and detected the formation of iodotyrosines during the first 5 days, and iodothyronines during the first 3 days of culture. Iodide transport function was lost sometime between the first and the fifth day of culture.

Siegel (1971) studied steer thyroid cells in monolayer culture and concluded that these cells retained the ability to iodinate proteins, although the exact nature of the iodinated compounds was not determined.

Lissitzky *et al* (1971), using pig thyroid cells in monolayer culture, showed that iodide ion was concentrated and metabolized to organic compounds only if TSH was present in the culture medium. In the absence of TSH, such functions were lost between the first and second day of culture.

With few exceptions the retention of active iodide transport function by cultured thyroid cells has not been investigated in detail. Thus it is possible that some culture systems, in which hormone formation was not detected, might have still retained the ability to actively transport iodide ion.

On the basis of the data from the above studies, it was clear that there was considerable variation in the function of different thyroid cell culture systems. This probably reflects the different species of animal chosen, the culture conditions employed and other factors. The present investigation was thus directed towards defining more precisely the extent of iodine metabolism occurring in the cell

culture system. One series of experiments was designed to ascertain whether the cells retain the function of active iodide transport, while a second set of experiments was performed to detect the formation, if any, of iodoproteins or their derivatives in these cells. Finally, experiments were designed to define the optimal conditions (e.g., age of culture) under which cultured cells should be grown for iodide transport studies.

B. Materials and Methods

1. Cells

(i) Thyroid Cells

In general, 5- to 6-day old monolayer cultures of bovine thyroid cells were used for the experiments unless otherwise specified.

(ii) HeLa Cells

Stock HeLa cell cultures, kindly supplied as a suspension by Dr. P. McConnachie, were diluted with the culture medium to a concentration of 100,000 cells/ml and cultured in Leighton tubes or plastic flasks under conditions similar to those employed for culturing thyroid cells. The HeLa cells formed extensive monolayers by 5-6 days of culture and were used at this stage for the experiments.

2. Measurement of ^{131}I Metabolism of Cultured Cells by Incubation with ^{131}I

All incubations were carried out in Krebs-Ringer phosphate buffer (pH 7.4) of composition:

NaCl (154 mM)	100 parts
KCl (154 mM)	4 parts

$CaCl_2$ (110 mM)	3 parts
$MgSO_4$ (154 mM)	1 part
0.1 M Phosphate Buffer	20 parts

(The 0.1 M phosphate buffer was prepared by adding 20 ml of 1N HCl to 26.81 *g* of Na_2HPO_4, $7H_2O$ and then diluting the solution to a final volume of one liter.)

Appropriate concentrations of sodium iodide (NaI) and ^{131}I (obtained as carrier-free $Na^{131}I$, Charles Frosst and Co.) were added to this medium. When iodide transport function alone was to be measured, 1-methyl-2-mercapto-imidazole (methimazole, Eli Lilly and Co.) was added to the medium to a final concentration of 2 mM. This agent is known to effectively block further thyroidal metabolism of transported iodide (Werner and Ingbar, 1971). When iodoprotein formation was to be measured, this agent was omitted from the medium. Incubation of cells on converslips was carried out in 50 ml capacity beakers using 5 ml of medium. In the case of cultures grown in flasks, the growth medium was discarded and the cells were washed with three consecutive changes of KRP medium at 37°C. Following this, the incubation was started by adding 10 ml of the appropriate medium. In each case, incubation was carried out for one hour at 37°C with gentle shaking in a Dubnoff metabolic shaking incubator. After incubation, the adherent medium was removed from the cells by washing them with 0.9% NaCl solution. For cells grown on coverslips, this was achieved by removing each coverslip from its incubation medium with fine-toothed forceps and then dipping it twice in each of four consecutive beakers containing approximately 70 ml of the washing solution at room temperature (Tsan

and Berlin, 1971). The entire rinsing was completed in about 10 seconds and appeared to remove adherent extracellular medium adequately, as determined by measuring the radioactivity of the fourth wash fluid. Washing of cells grown in plastic flasks was performed by draining the incubation medium from the flask and then washing the cells with four quick, consecutive changes (15 ml each) of 0.9% NaCl solution. The adequacy of the wash was again checked by determining the radioactivity in the fourth wash fluid.

3. Determination of Protein-Bound Iodine Formation by Cultured Thyroid Cells

Cells grown in flasks were incubated for one hour at 37°C in 10 ml KRP buffer containing 1.0×10^{-5} M sodium iodide and 0.1 µCi/ml of ^{131}I (carrier-free), after which they were washed with 0.9% saline, collected by scraping with a rubber "policeman" and then homogenized in 1 ml of 0.9% saline in a Potter-Elvehjem homogenizer. The protein fraction of the homogenate was isolated by the following procedure (Greenberg, 1970). Each homogenate was treated with 4 volumes of 10% trichloracetic acid (TCA) at room temperature. Following centrifugation, the precipitate was washed by resuspension in a series of solvents: twice in 10 ml of 5% TCA, once in 8 ml ethanol, once in 8 ml 3:1 ethanol:ether at 60°C for 3 minutes, and finally in 10 ml ether. During the first resuspension with 5% TCA, the mixture was heated at 90°C for 15 minutes to eliminate the nucleic acids from the precipitate. The treatments with ethanol and ether were designed to extract the lipid fraction of the precipitate. After the washed precipitate had been dried in air at room temperature its ^{131}I content was determined by counting in a Nuclear-Chicago well-type scintillation counter. The

iodide concentration of the precipitate was calculated from the known specific activity of ^{131}I in the medium. Finally, the precipitate was dissolved in 2.0 ml 1 N NaOH in preparation for the estimation of its protein content (Chapter II). The results were expressed as moles of iodide bound per mg of protein.

4. Gel Filtration Chromatography

A 100 cm x 1 cm column of coarse G-25 Sephadex (100-300 μ particle size) was employed, with 0.1 N NaCl as the solution used for packing and washing of the column. Filtration was carried out at 4°C. One ml of cell homogenate, prepared after incubation of thyroid cells with sodium iodide and ^{131}I as described above, was layered on the top of the column. In addition, a 0.5 ml aliquot of the culture medium taken at the end of the incubation period was similarly filtered through another Sephadex column. Fractions of 3 ml were collected, examined for absorbancy at 280 mμ in a Spectrophotometer (Beckman Kintrac VII) and assayed for ^{131}I radioactivity in a well type scintillation counter.

5. Ascending Paper Chromatography

Homogenates from both control thyroid cells and TSH-grown cells were obtained after incubation of the cells with NaI and tracer as described above, except that Tris buffer, pH 8.5, (Tris-0.04 M, NaCl-0.15 M, $CaCl_2$-0.014 M, Methimazole-0.002 M) was used instead of 0.9% NaCl solution during homogenization. After the addition of 5 mg of Pronase (Calbiochem grade B, 45,000 P.U.K./gm) per ml and one drop of toluene the homogenate was incubated for 8 hours at 37°C. Aliquots (200 μl) of each hydrolyzate, each containing at least 1 μg of ^{127}I, were then chromatographed on 3 cm-wide strips of Whatman No. 3 MM paper using two different solvent systems:

(i) n-butanol-2N acetic acid (1:1) and

(ii) n-butanol-ethanol-2N ammonia (5:1:2)

Aliquots (100 μl) of the culture medium taken at the end of the incubation period were similarly chromatographed. Following chromatography for 16-18 hours at room temperature the strips were air-dried and scanned for radioactivity in a strip scanner (Actigraph III, Nuclear Chicago). Various standards (^{131}I-iodide, ^{131}I-labelled T_3 and T_4, non-labelled MIT and DIT) were chromatographed similarly in both solvents for determination of their R_f values. The positions of the radioactive bands were identified by scanning as above. The bands corresponding to MIT and DIT were made visible by spraying the paper strips with 0.2% ninhydrin solution in n-butanol (Mann Spraytec Reagent, Mann Research Laboratories).

6. Measurement of Cell/Medium Concentration Ratios for Iodide

Cells were harvested from 250 ml culture flasks by scraping with a rubber "policeman", suspended in 5 ml KRP medium containing 0.002 M methimazole, 1.0×10^{-5} M sodium iodide and 0.01 μCi/ml of ^{131}I (carrier-free), and incubated at 37°C for one hour with gentle shaking in a Dubnoff metabolic shaking incubator. At the end of the incubation period the suspension was transferred to 6.5 ml graduated, tapered centrifuge tubes (Shevky-Stafford type) and centrifuged at room temperature for 5 minutes at 50 x *g*. The volume of packed cells was read to the nearest 0.01 ml. After the supernatant had been drawn off, the radioactivity of both the cell pellet and an aliquot of the supernatant was measured. The results were expressed as cell/medium (C/M) ratios where:

$$C/M = \frac{^{131}I \text{ cpm per ml of packed cells}}{^{131}I \text{ cpm per ml of medium}}$$

7. Measurement of Iodide Accumulation by Thyroid Cells

Coverslips bearing monolayer cultures of thyroid cells were incubated under the same conditions described above for the C/M measurements except that a higher concentration of ^{131}I was used (1 μCi/ml). The specific activity of ^{131}I in the medium was calculated from the known iodide concentration and radioactivity of the medium. At the end of incubation, each coverslip was washed as described earlier and placed in a counting tube for the measurement of its radioactivity. Subsequently the amount of protein on each coverslip was determined. The results were expressed as moles of iodide accumulated per mg of cell protein.

C. Results

1. The Formation of Protein-Bound Iodine by Cultured Thyroid Cells

Table 3.1 summarizes the results of experiments performed to determine the formation of protein-bound iodine by thyroid cells grown as monolayers either in the presence or in the absence of thyrotropin in the medium. The amount of protein-bound iodine formed under similar experimental conditions by freshly isolated bovine thyroid cells and by HeLa cells was also determined. The latter types of cells were chosen for the control experiments because the first was known to be capable of forming iodoprotein (Tong *et al*, 1962), while the second was incapable of doing so. The results indicated that protein-bound iodine formation was negligible for cultures of thyroid cells grown under the conditions of this study, regardless of whether TSH was present or absent in the growth medium. The same negative results were obtained with the HeLa cell cultures. On the other hand, freshly isolated thyroid cells formed significant amounts of protein-bound iodine under the same experimental conditions indicating that

TABLE 3:1

Formation of Protein Bound Iodine by Various Cells Incubated with ^{131}I-labelled NaI

	Cell Type	Protein Bound Iodine (moles per mg of cell protein x 10^{11})*
1.	Control thyroid cells**	0.12 ± 0.02
2.	TSH-grown thyroid cells**	0.14 ± 0.02
3.	HeLa cells**	0.14 ± 0.02
4.	Freshly isolated thyroid cells	5.83 ± 0.52

Five-day-old cells cultures (**) or freshly isolated cells were incubated with ^{131}I-iodide at 37°C for one hour. Following incubation, the cells were homogenized. The protein content of the homogenate was estimated by the methods described in the text. The iodide content of the proteins was calculated from the ^{131}I count of the protein sample and the known specific activity of ^{131}I in the incubation medium.

*Mean of four determinations ± SEM.

(i) the composition of the incubation medium was suitable, and

(ii) the thyroid cells, at the time of inoculation, were functionally capable of forming iodoproteins.

Since the suspension of freshly isolated thyroid epithelial cells was contaminated with other cells such as red blood cells, and with cellular debris, the ability of the epithelial cells alone to form iodoproteins was probably much higher than that indicated by the data shown.

2. Gel Filtration Chromatography

The results, which are summarized in Fig. 3.2, show the profiles of radioactivity and absorbance at 280 mμ of eluates obtained by the filtration of cellular homogenates through Sephadex G-25. Proteins were recovered in eluates collected between 15 ml and 21 ml, as shown by measuring the absorbance of the fractions at 280 mμ. The ^{131}I activity was recovered in the fractions collected between 36 ml and 60 ml. The complete absence of radioactivity in all fractions corresponding to the protein peak marked by the absorbancy peak at 280 mμ indicated the absence of iodinated cellular proteins in the homogenate. Furthermore, the observed peak of radioactivity coincided with the position of iodide, as determined by filtering a sample of ^{131}I-labelled sodium iodide through the column under the same conditions. About 99% of the total radioactivity of the hydrolyzate was recovered in this iodide peak suggesting that at least 99% of the iodide taken up by the cells during incubation was present as inorganic iodide.

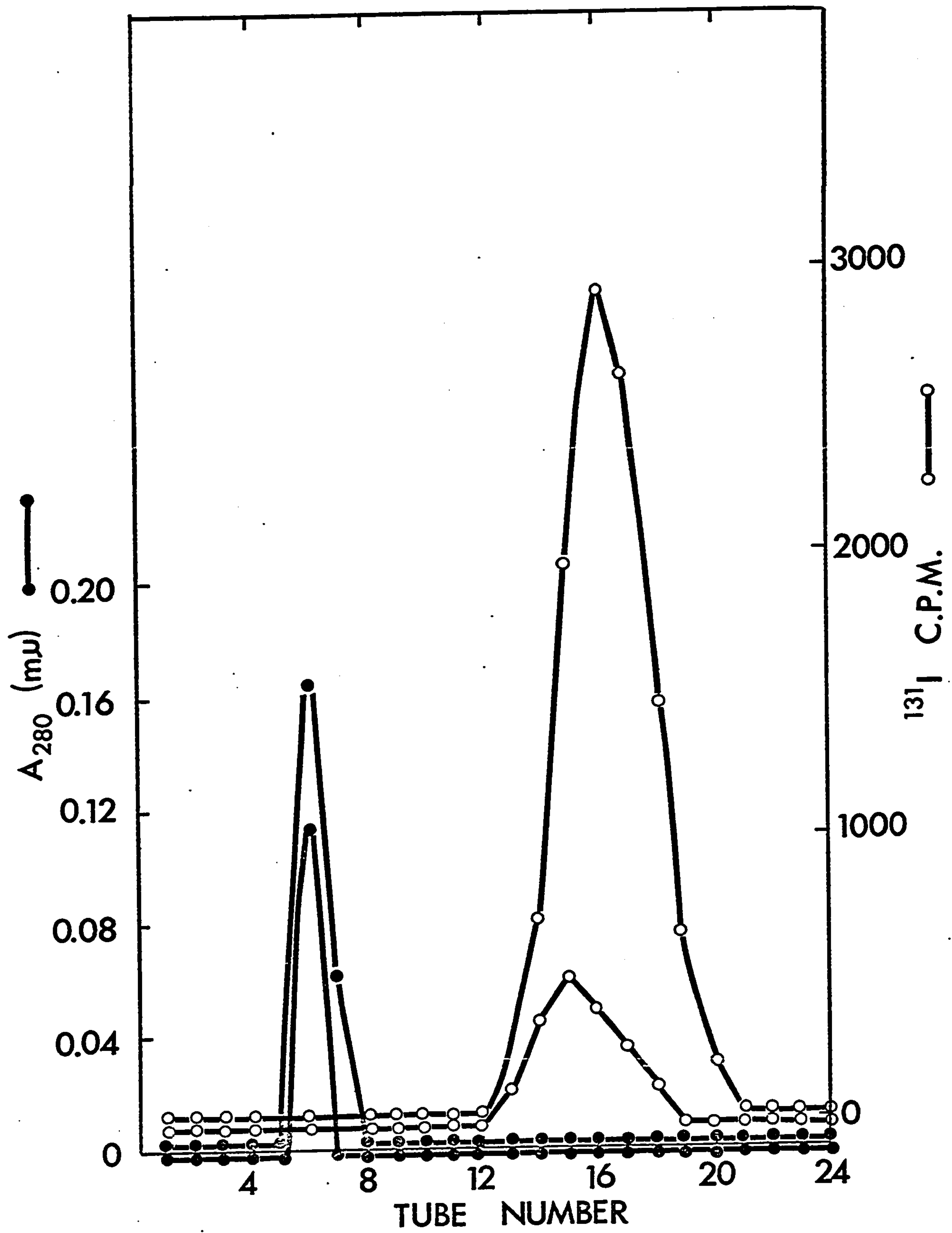
When 0.5 ml of the medium removed at the end of the incubation period was layered on the column, the eluates once more showed a single radioactivity peak corresponding to iodide. Recovery was again 99%.

Figure 3.2. Filtration through Sephadex G-25 of homogenates prepared from cultured thyroid cells which had been incubated with ^{131}I.

Five-day-old monolayer cultures of thyroid cells grown with or without the addition of TSH to the growth medium, were incubated with ^{131}I-iodide as described in the text. Following the incubation, the cells were homogenized and the homogenate was analyzed by filtration through a column packed with G-25 Sephadex. 3 ml fractions of eluate were collected in each tube.

Upper Curves: TSH grown cells

Lower Curves: Control thyroid cells



0.20
0.16
0.12
0.08
0.04
0
A_{280} (mμ)
3000
2000
1000
0
^{131}I C.P.M.
4
8
12
16
20
24
TUBE NUMBER

These results suggest that the thyroid cells did not release any iodinated proteins into the medium during the incubation.

3. Ascending Paper Chromatography

Paper chromatograms, prepared from the hydrolyzed thyroid homogenates and culture media at the end of incubation, showed a single peak of radioactivity with an R_f value identical with that of iodide ion (0.31 in the butanol-acetic acid system, 0.25 in the butanol-ethanol-ammonia system). An occasional minor peak representing 1% or less of the total radioactivity was seen at the origin. However, no peaks corresponding to the iodotyrosines or iodothyronines were observed on any of the strips.

4. C/M Ratios (Table 3.2)

The observed cell/medium ratio of radioactivity for HeLa cells was close to unity confirming that these cells were incapable of concentrating iodide from the medium. Under the same experimental conditions the system of control thyroid cells had a C/M ratio of approximately 3.2 showing that the cells possessed the ability to establish a concentration gradient for iodide between themselves and the medium. This ability was still higher for thyroid cells grown in presence of TSH for which the C/M ratio had a mean value of 11.0. These C/M values for thyroid cells are underestimated to some extent since a proportion of the cell volume as measured corresponded to extracellular space. Also, the presence of any non-viable cells (which could result from the trauma sustained during scraping of the monolayers with a "policeman") would tend to lower the actual C/M ratio.

5. Iodide Accumulation by Thyroid Cells

The amount of iodide accumulated per mg of cell protein in cultured cells under different conditions is summarized in Table 3.3.

TABLE 3.2

Cell/Medium (C/M) Iodide Concentration Ratios for Various Cultured Cells Incubated with ^{131}I-iodide

	Type of Cell	C/M Ratio*
1.	Control thyroid cells	3.15 ± 0.19
2.	TSH-grown thyroid cells	11.0 ± 1.1
3.	HeLa cells	0.90 ± 0.04

Five- or six-day-old cells cultured in flasks were incubated in KRP media containing ^{131}I-iodide for 1 hour at 37°C. Following this, the cells were collected from the flasks by gentle scraping with a policeman. The volume of the cells and the radioactivity of the cells and a 1 ml aliquot of the medium were determined by methods described in the text. From these data C/M ratios were calculated.

*Mean of 4 determinations ± SEM.

TABLE 3.3

The Accumulation of Iodide by Various Cultured Cells Incubated with ^{131}I-iodide

	Type of Cell	Iodide Accumulation (moles/mg of cell protein x 10^{10})*
1.	Control thyroid cells	2.22 ± 0.09
2.	TSH grown thyroid cells	23.8 ± 2.2
3.	HeLa cells	0.27 ± 0.01

Five- or six-day-old cell cultures were incubated in KRP media containing ^{131}I-iodide of known specific activity for 1 hour at 37°C. Iodide accumulation was measured by methods described in the text. The control and TSH-grown cells were derived from the same population of freshly isolated cells.

*Mean of 5-6 determinations ± SEM.

The iodide accumulated by thyroid cells was several times higher than that found in HeLa cells under similar conditions. Further, iodide accumulation was several-fold greater in thyroid cells grown in the presence of TSH compared to the control thyroid cells. These results are consistent with the results described above for C/M measurements on similar cells.

Virtual abolition of iodide accumulation by the thyroid cells resulted from the addition of ClO_4^- or CNS^- to the incubation medium (Table 3.4). This finding was in complete agreement with the known action of these agents as competitive inhibitors of iodide transport (Wyngaarden *et al*, 1953). Marked depression of iodide accumulation was seen also when the cells were incubated with inhibitors of cellular energy metabolism, such as 2,4-dinitrophenol or sodium cyanide. This suggested that the iodide accumulation process was dependent upon cellular energy production. The marked inhibition of iodide accumulation occurring in the presence of ouabain and the reversal of this inhibitory effect by 20 mM KCl was of considerable interest, because it suggested a possible link between the function of the "sodium pump", (the Na^+-K^+-dependent membrane ATPase) and the iodide accumulation process.

6. Variation in Iodide Accumulation Activity with Age of Cell Culture

The amount of iodide accumulated by control thyroid cells grown for 4,7,10,14,21 and 28 days, all cells originating from the same common inoculum, is shown in Table 3.5. Data are given for cells incubated both in the presence and in the absence of ClO_4^- (1.0×10^{-4} M). The purpose of the experiment was to establish whether there was any change in iodide accumulation activity with age of culture, and also

TABLE 3.4

Effect of Various Inhibitors on Iodide Accumulation by Thyroid Cells

Condition†	Iodide Accumulation per mg of Cell Protein (moles x 10^{10})**	
	A. Control Thyroid Cells	B. TSH Grown Thyroid Cells
1. Control	2.05 ± 0.24	31.3 ± 3.0
2. 4°C	0.31 ± 0.04	0.33 ± 0.03
3. Addition of 0.1 mM ClO_4^-	0.28 ± 0.02	0.34 ± 0.02
4. Addition of 0.1 mM CNS^-	0.30 ± 0.01	0.33 ± 0.02
5. Addition of 10 mM NaCN*	0.42 ± 0.01	1.57 ± 0.17
6. Addition of 0.1 mM 2,4-dinitrophenol	0.43 ± 0.02	2.25 ± 0.29
7. Addition of 5 x 10^{-5} M ouabain	0.40 ± 0.02	1.88 ± 0.20
8. Addition of 5 x 10^{-5} M ouabain in presence of 20 mM KCl*	1.96 ± 0.20	28.6 ± 3.1

Iodide accumulation was measured as described in the legend to Table 3.3 under the conditions given above. The data in columns A and B were obtained with two different cell populations.

*Isoosmolar amounts of NaCl were withdrawn from the medium where additions of NaCN or KCl were made.

**Mean of 5-6 determinations ± SEM.

†All concentrations denote the final concentration of the reagent in the incubation medium.

TABLE 3.5

The Variation with Age of Culture of the Ability of Cultured Thyroid Cells to Accumulate Iodide

Age of Culture	Iodide Accumulation (moles/mg cell protein x 10^{10})		Ratio of A/B
	A. In Absence of ClO_4^{-}*	B. In presence of ClO_4^{-}**	
1. 4 days	2.97 ± 0.10	0.34 ± 0.04	8.7
2. 7 days	3.10 ± 0.17	0.31 ± 0.03	9.9
3. 10 days	2.42 ± 0.21	0.41 ± 0.03	5.9
4. 14 days	0.97 ± 0.03	0.37 ± 0.03	2.6
5. 21 days	0.85 ± 0.11	0.31 ± 0.02	3.2
6. 28 days	0.63 ± 0.08	0.40 ± 0.04	1.6

The cell cultures were incubated with ^{131}I-iodide under conditions described in the legend to Table 3.3 with or without the addition of 0.1 mM $KClO_4$ to the incubation medium. The cells were obtained by culturing the same population of freshly isolated cells for different periods of time, without the addition of $KClO_4$ to the growth medium.

*Mean of 5 determinations ± SEM.

**Mean of 3 determinations ± SEM.

to establish optimal conditions for future experiments. Because the level of iodide accumulation that occurred with thyroid cells incubated in the presence of ClO_4^- was found to be similar in magnitude to that seen for non-functional tissues (e.g., HeLa cells), the ClO_4^- studies served as controls during the evaluation of iodide accumulation activity of thyroid cells of different ages. The results show that the iodide accumulation activity of uninhibited thyroid cells remained high and essentially constant for the first 10 days or so of culture after which the activity declined and settled to a lower level. Even at 28 days, however, the cultures accumulated significantly more iodide than the corresponding perchlorate controls.

D. Discussion

The experiments described above have shown that bovine thyroid cells grown in monolayer culture retain the ability to establish a concentration gradient for iodide between cells and incubation medium. This ability was markedly enhanced in cells grown in the presence of TSH. Because there was some variation (usually within 2- to 3-fold) in transport activity between different batches of cultured cells, it was necessary for most experiments to use cells grown from the same common inoculum in order to make comparative studies.

It was of interest that the amount of iodide accumulated by thyroid cells incubated in the presence of the ions ClO_4^- or CNS^-, or at 4°C, or in the presence of ouabain or inhibitors of energy metabolism (e.g., NaCN, 2,4-dinitrophenol), was always of the same magnitude and comparable to that seen in HeLa cells. This constant base-line level probably represents iodide accumulated by "passive diffusion", or other non-energy dependent entrance of iodide into the cell. Under the

same experimental conditions, the cells were found to be capable of establishing ratios for iodide of 1:1 across the cell membrane confirming that even when active iodide transport was inhibited, the cells accumulated some iodide equivalent to the level of iodide in the medium.

Since the cell interior is electrically negative with respect to the extracellular medium, the establishment of a concentration gradient for the negatively charged iodide ion between the cell and the medium must involve movement of iodide ion against its electrochemical gradient. Such a process would involve the expenditure of cellular energy and would be expected to be markedly inhibited, as was observed experimentally, by NaCN, 2,4-dinitrophenol or cold (4°C). Thus the experimental conditions under which iodide was accumulated by the cultured cells satisfied the usual criteria for a process of active transport (Davis, 1969). The term active transport, as used above, is not a strict thermodynamic definition which would require the additional knowledge of such factors as the activities of iodide ion on the two sides of the cell membrane, the potential difference across the membrane and the interaction of iodide ion movement with the movement of particles of solvent and other ions (Stein, 1967, p. 62; Ussing, 1959).

The iodide-concentrating ability of the cultured cells was maintained at the same level for at least 1 1/2 weeks. Thereafter it decreased in magnitude, although for the next 2 1/2 weeks it was always at least twice that seen in the presence of ClO_4^-. This suggested retention of some iodide concentrating ability for at least four weeks of primary culture. The cause of diminution of activity after 1 1/2 weeks remains unclear at the present time.

The maintenance of iodide concentrating activity without diminution for the first 7-10 days of culture was of interest. This argued against any significant incidence of overgrowth during this time by non-thyroid cells (e.g., fibroblasts). Such cells constitute a small fraction of the cell population of the thyroid gland and must have been present at the time of inoculation. Occurrence of significant overgrowth by inactive cells would be expected to diminish the total iodide accumulating activity of the cell culture in a progressive manner right from the beginning. The homogenous appearance of the cells in culture throughout the growth period, where only one morphological type of cell was seen, also supports this conclusion. It is probable that the non-thyroid cells might have been eliminated from the culture system because of their relatively small initial concentration.

In contrast to their maintenance of specific iodide transport function, the cultured thyroid cells showed no demonstrable ability to iodinate protein or form the thyroid hormones as their precursors; over 99% of the cellular iodine was identified as inorganic iodide ion. The same was true for cells grown in the presence of TSH. Thus the thyroid cells in culture retained one differentiated function of such cells *in vivo*, namely active iodide transport, while the other differentiated feature of thyroidal iodine metabolism, the ability to form specific iodoproteins, was lost.

Such loss of one specialized function, with retention of a second, is infrequent for cells grown in culture, and has not been reported previously for thyroid-cell culture systems. However, a few comparable findings have been published for cultures of other hormone-producing cells (Sato and Buonassisi, 1964). Although the cause of

this partial loss of differentiated function is not understood, the following possibilities merit consideration.

1. Effect of Culture Media

The culture medium could be adequate for one aspect of cell function while containing substances *inhibitory to the differentiation* of other functions (Coon and Cahn, 1966). The conflicting results referred to earlier which were obtained by Hilfer (1962) and Spooner (1970), both using 16-day chick embryo thyroids, might be explained on this basis.

2. Effect of Spatial Arrangement

Thyroid cells in monolayers exist in a two-dimensional arrangement. However, a three-dimensional configuration as occurs *in vivo*, may be necessary for normal cell-to-cell interactions which may be essential for fully differentiated function.

3. Specific Requirements

Several tissues have been shown to require certain specific cellular products for proper differentiation. Thus normal functional development of mouse mammary gland in organ culture requires the simultaneous presence of insulin, hydrocortisone and prolactin (Topper, 1970). Proper maturation of erythroid cells in bone marrow occurs only in the presence of erythropoietin. Similar requirements, yet undefined, may exist for complete differentiation of thyroid cells.

4. Effect of Other Cell Types

The presence of additional cell types may be necessary for fully functional differentiation of thyroid cells. Instances of such heterotypic induction are fairly well documented (Grobstein, 1954). Cells of this type, if such exist in normal thyroid tissue, may have failed to survive in culture.

The inability of the cultured thyroid cells to form iodoprotein proved to be an advantage for the present investigation of thyroid iodide transport because measurement of iodide accumulation was equivalent to measurement of iodide transport. It became unnecessary to use drugs such as methimazole to block subsequent metabolism of transported iodide ion.

Since monolayer formation was extensive and essentially complete after 5-6 days of culture and because optimal iodide transport function was found to be maintained by the cells for the first 1 1/2 weeks of culture, cell cultures of 5-6 days age were chosen for future experiments on iodide transport.

In conclusion, a thyroid-cell culture system was developed which retained the ability to actively transport iodide ion. The properties of the *in vitro* transport system strongly resembled those of the thyroid gland *in vivo* including such properties as response to specific inhibitors (ClO_4^-, CNS^-), inhibitors of energy metabolism (CN^-, 2,4-dinitrophenol) and ouabain. TSH, again as *in vivo*, had a marked stimulatory action on the transport system. The experimental evidence suggests that a basic identity of mechanism may exist between the active transport systems of the cultured cells and of the gland *in vivo*. Thus, the *in vitro* system was considered a valid model with which to carry out further studies on the mechanism of iodide transport by thyroid cells.

CHAPTER IV

KINETICS OF IODIDE INFLUX

A. Introduction

With the successful development of a functional thyroid culture system, detailed studies on the process of active iodide transport became feasible. It was necessary, however, to devise a method for measuring iodide fluxes across the thyroid cell membrane. The flux, defined as the amount of the substrate which crosses the membrane per unit time, has been expressed per sq. cm. of membrane surface, per kilogram of dry cells, or in terms of other conveniently measured parameters. In the present study, it was decided to express fluxes as moles of iodide per mg of cell protein per minute.

The net flux of iodide into a thyroid cell is the difference between two opposite unidirectional fluxes, the influx, which carries iodide into the cell from the external medium, and the efflux, which leads to iodide movement in the reverse direction. Measurement of either one of these components may be done by measuring the net flux under conditions where the contribution of the opposing flux is very small and therefore can be considered negligible. Under the present experimental conditions the external medium contained an excess of ^{131}I-iodide of known specific activity. On the other hand, the cells, which had been grown in an iodide-free medium initially, had a negligible concentration of intracellular iodide. Under these conditions and the assumption that the influx and efflux were proportional to the iodide concentrations in the extracellular and intracellular compartments respectively, the rate of influx would remain constant throughout the experiment. The uptake of iodide by the cells would be insufficient

to significantly change the concentration of iodide in the external medium during the experiment. In contrast, the rate of efflux of iodide would be nearly zero during the very early period of incubation, because of the extremely low initial intracellular level of iodide. Thus by confining measurements to very early time intervals, the rate of influx of iodide by thyroid cells could be determined by measuring the net iodide accumulation by the cells, provided the steady state (or equilibrium between influx and efflux) was not reached very rapidly. These considerations led to the design of influx measurements described in this section.

Agents, such as ClO_4^-, CNS^-, cardiac glycosides and various metabolic inhibitors, were reported in the preceding chapter to markedly reduce net iodide accumulation by cultured thyroid cells. Such effects could be due either to an inhibition of iodide influx or to an increase of efflux, or perhaps by a combination of both. In view of these possibilities, a study of the effects of these reagents on the influx process was undertaken.

Finally, a study of the kinetics of thyroidal iodide influx was undertaken with the hope that knowledge of such data would provide information about the nature of the underlying reaction sequences of iodide influx. Variation of the various kinetic parameters of the influx reaction, in response to changes in experimental conditions (for example changes in Na^+ concentration), would, in particular, be useful in such analyses.

B. Materials and Methods

The methods of measurement of iodide accumulation by the cells have been described previously (Chapter III). Experiments were carried

out using 5- to 6-day old cultures of thyroid cells, grown in the presence or absence of TSH in the medium (Chapter II).

C. Results

1. Iodide Accumulation by Thyroid Cells as a Function of Time

Fig. 4.1 illustrates the accumulation of iodide by thyroid cells at various intervals of incubation up to 3 hours. The initial part of the uptake curve over the first 10-15 minutes was found to approximate a straight line. The curve then showed increasing downward curvature until after 45-60 minutes of incubation, when it became parallel to the time axis. This plateau implied that the system had attained a steady state which then was maintained for at least 3 hours. As the initial part of the uptake curve was found to approach linearity the determination of iodide accumulation at five minutes was used to calculate the initial slope of the line. This, as discussed above, was taken as the velocity of the influx process. During this 5-minute period, net iodide accumulation did not exceed 20% of the steady state value. Similar results were obtained for both control and TSH-grown cells.

2. Variation of Influx Rate with Iodide Concentration

Fig. 4.2 depicts the variation in the rate of iodide influx into cultured thyroid cells with changes in iodide concentration over the range from 1.0×10^{-5} M to 2.0×10^{-4} M. The upper curve shows the results obtained with TSH-grown cells while the lower one illustrates those obtained with control cells. Both cell populations are saturable at high iodide concentrations and as such conform to a rectangular hyperbolic type of curve obeying the relationship

$$V = \frac{V_m S}{K_m + S}$$

Figure 4.1. The accumulation of ^{131}I-iodide by thyroid cells in monolayer culture as a function of time.

Iodide accumulation by five day old thyroid cell cultures was measured over a 3 hour period under conditions similar to those described in the legend to Table 3.3. The two scales on the ordinate represent ^{131}I-iodide accumulation as counts per minute per mg cell protein. Each result shown is a mean of 5-6 determinations ± SEM. The control thyroid cells (○———○) and TSH-grown thyroid cells (●———●) represent two different cell populations.

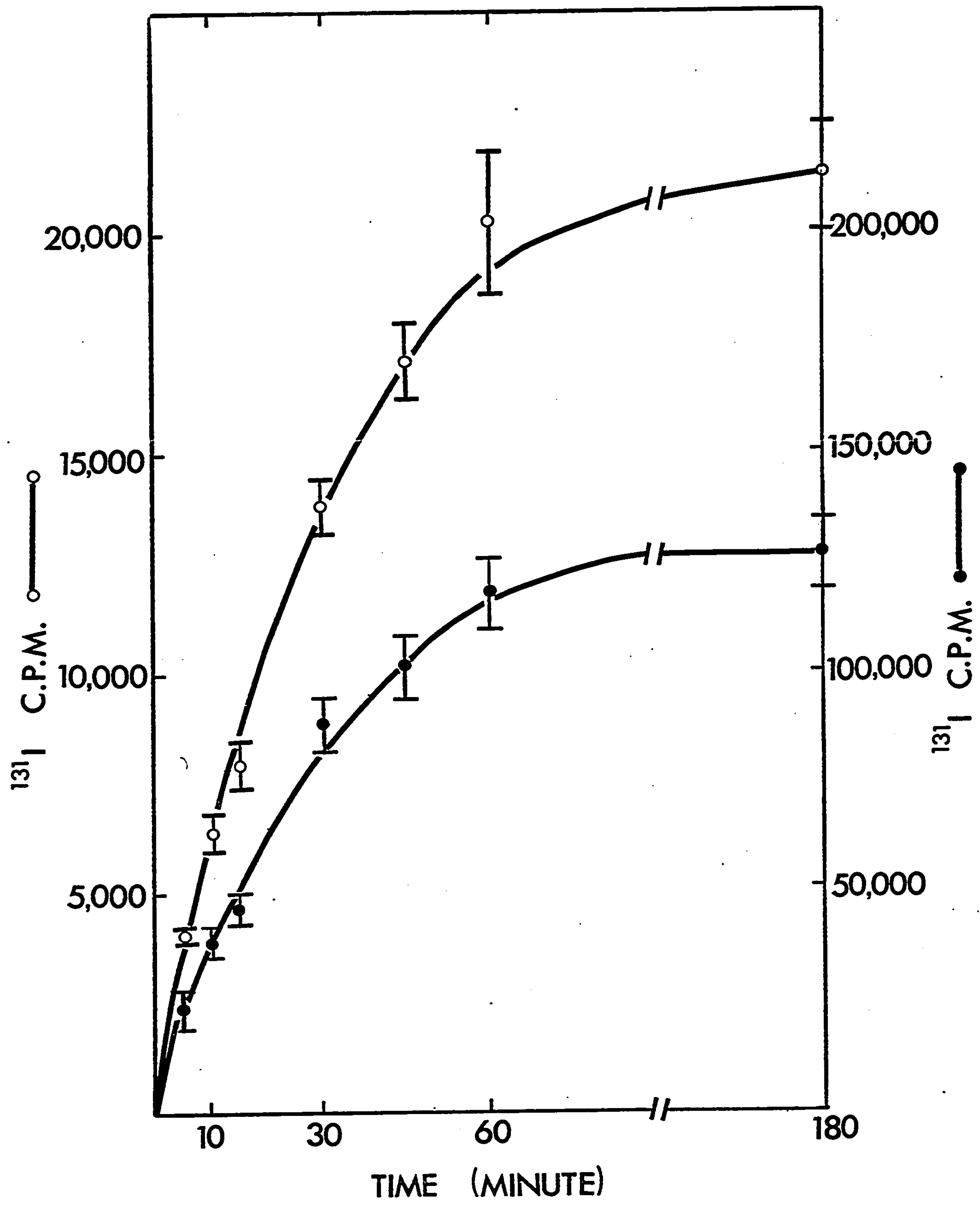

20,000
15,000
10,000
5,000
^{131}I C.P.M.
200,000
150,000
100,000
50,000
^{131}I C.P.M.
10
30
60
180
TIME (MINUTE)

Figure 4.2. Effect of the extracellular concentration of iodide on the rate of iodide influx into thyroid cells in tissue culture.

Iodide influx was determined by measuring the iodide accumulation by five day old thyroid cells during a 5 minute incubation at 37°C in KRP media containing ^{131}I of known specific activity. Each result shown represents the mean of 5-6 determinations and is expressed in units of moles of iodide/mg cell protein/minute.

Control thyroid cells: ○———○

TSH-grown thyroid cells: ●———●

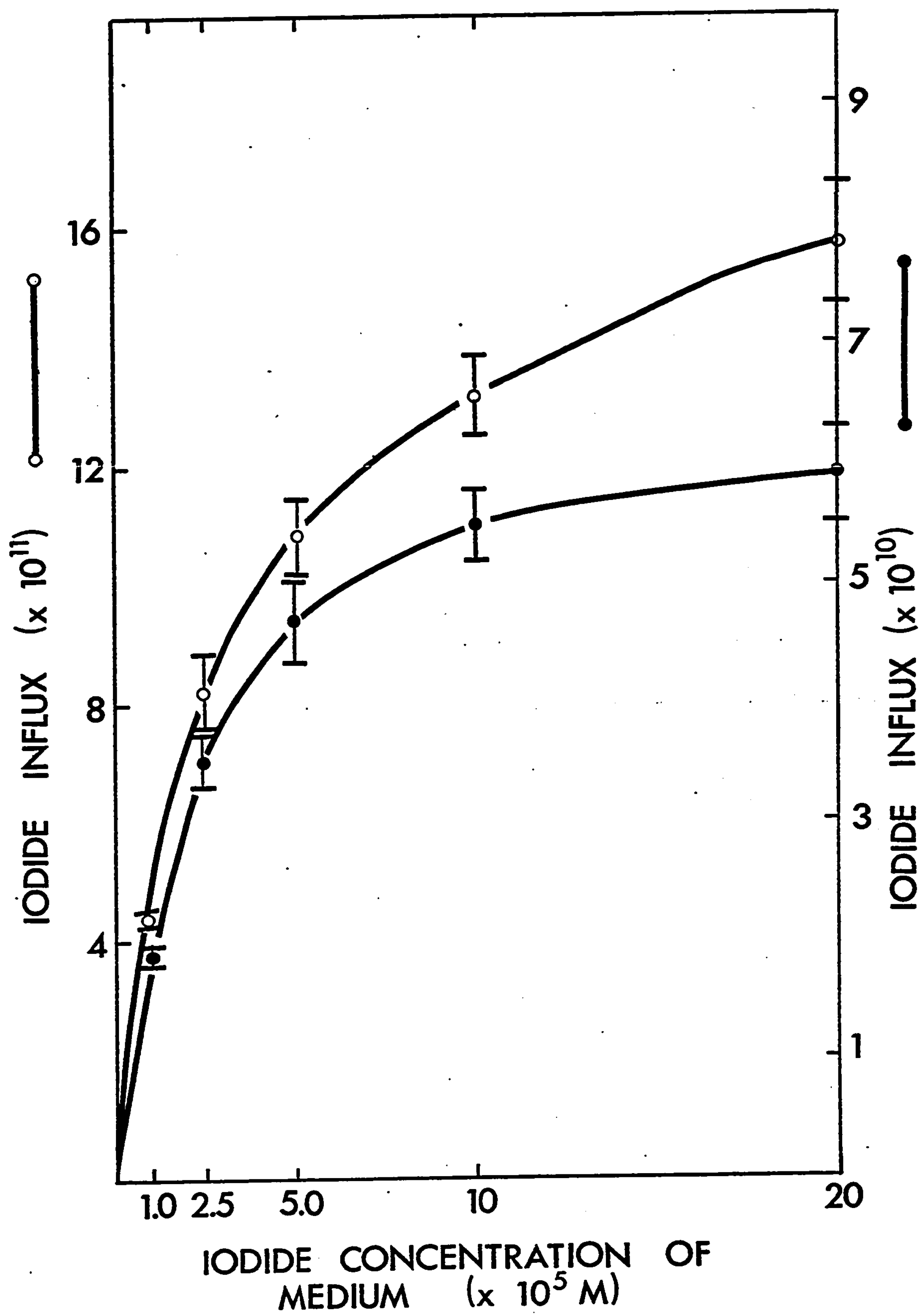

16
12
8
4
IODIDE INFLUX (x 10^11)
9
7
5
3
1
IODIDE INFLUX (x 10^10)
1.0 2.5 5.0 10 20
IODIDE CONCENTRATION OF MEDIUM (x 10^5 M)

Figure. 4.3. Lineweaver-Burk plots of iodide influx into thyroid cells as a function of the iodide concentration of the external medium.

The data from Fig. 4.2 were used for the construction of the plots shown. The velocity of influx (V) is expressed in units of moles of iodide per mg cell protein per minute. The iodide concentration of the medium (S) is expressed in units of moles per liter.

Control thyroid cells ●————●

TSH-grown thyroid cells ○————○

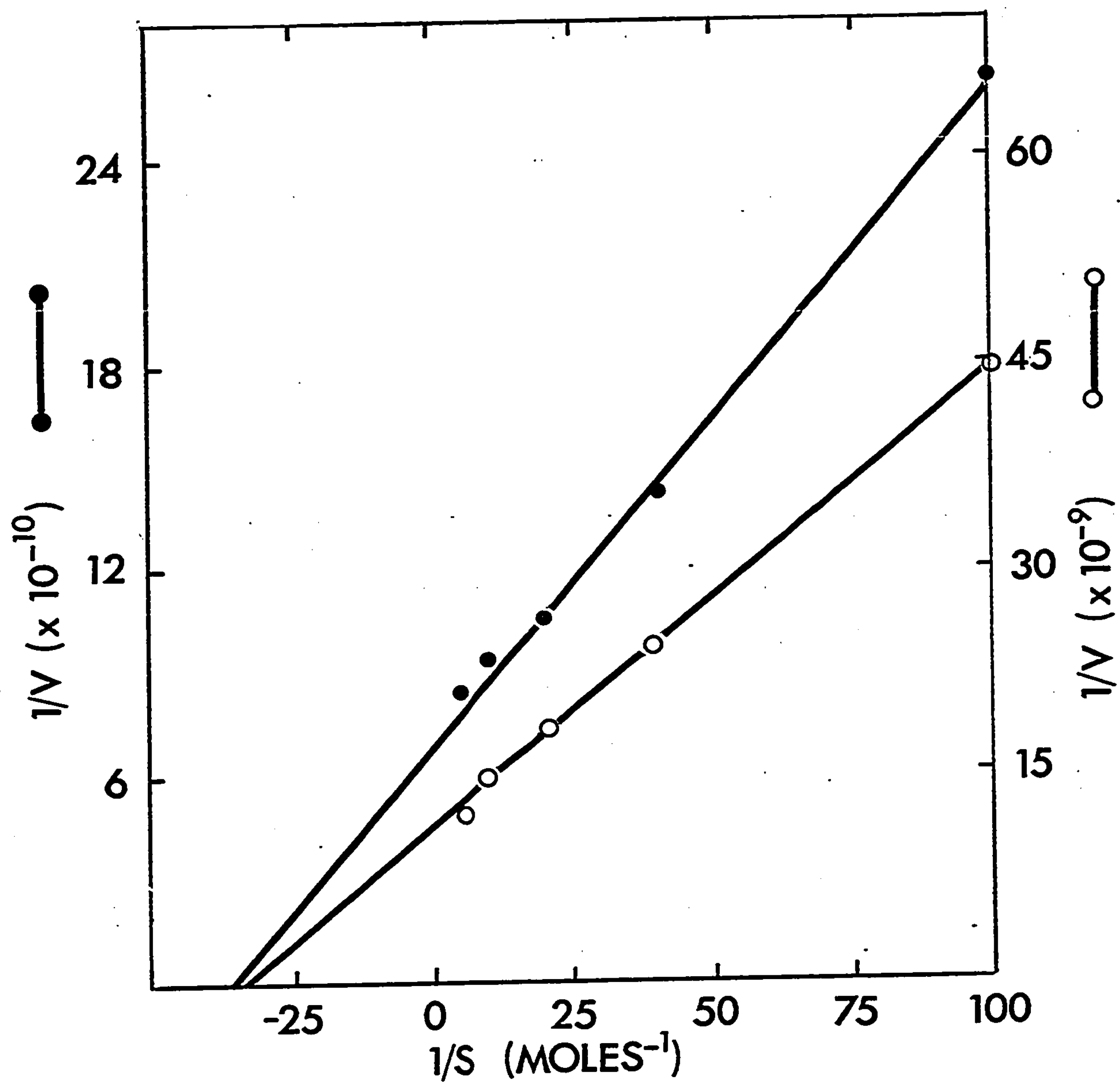

24
18
12
6
1/V (x 10⁻¹⁰)
60
45
30
15
1/V (x 10⁻⁹)
-25
0
25
50
75
100
1/S (MOLES⁻¹)

TABLE 4.1

Kinetic Parameters of Iodide Influx into Thyroid Cells in 5-Day Old Monolayer Cultures

	Type of Cells	Vm (moles/mg cell protein/min x 10^{11})	Km (moles x 10^{5})
1.	Control thyroid cells		
	1)	14.1	2.80
	2)	11.1	3.20
	3)	10.6	3.20
2.	TSH-grown thyroid cells		
	1)	87.3	2.93
	2)	66.7	3.33
	3)	93.6	3.64

Each horizontal line represents the results of one experiment.

where V = velocity of influx at iodide concentration S,

V_m = maximum velocity of influx,

K_m = a constant, operationally defined as that substrate concentration which achieves one-half the maximum velocity. (This may be derived readily from the above equation by substituting $V = V_m/2$.)

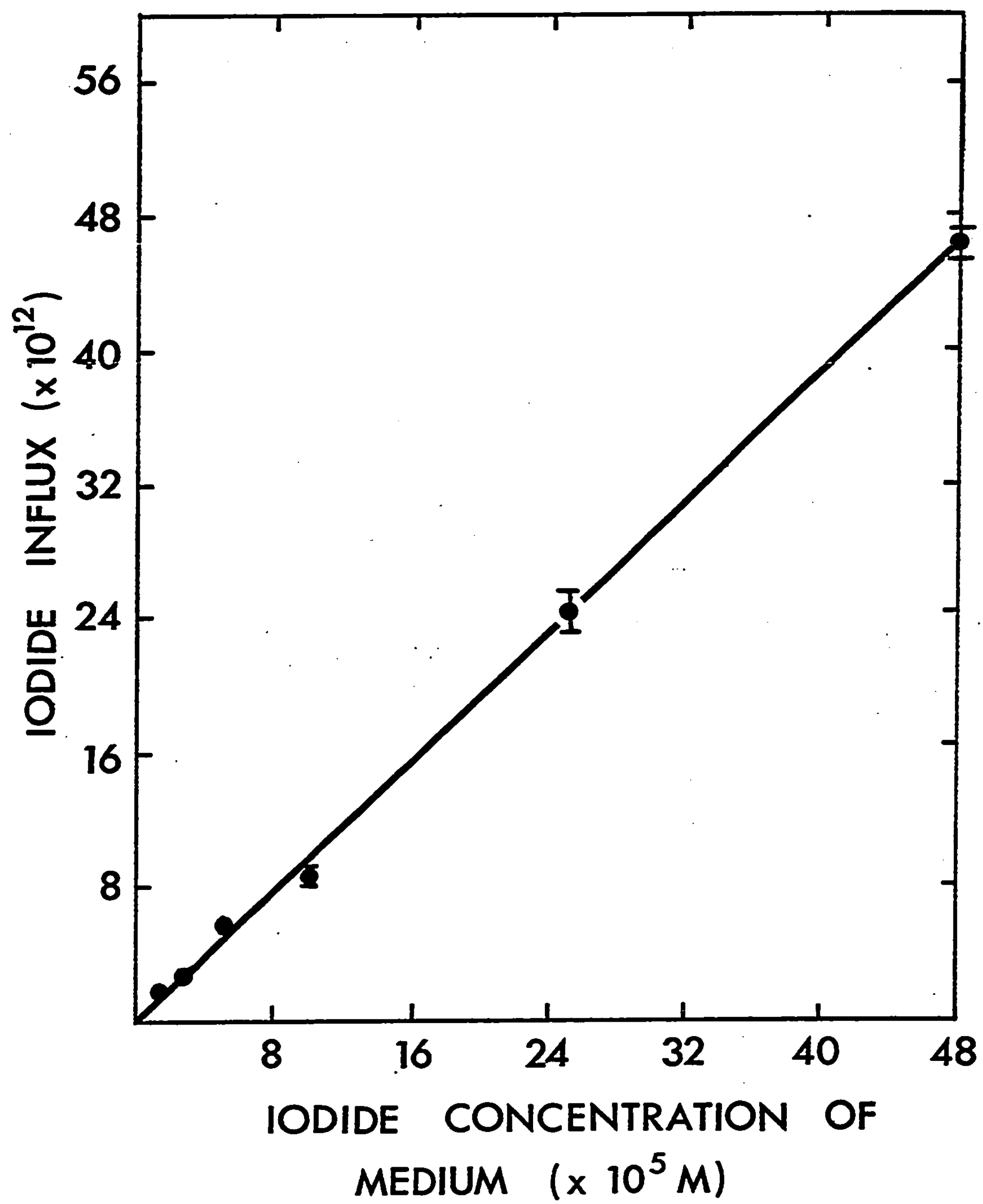
Fig. 4.3 shows the corresponding reciprocal (Lineweaver-Burk) plots, i.e., plots of $1/V$ vs $1/S$. These are found to be straight lines with intercepts on the ordinate equal to $1/V_m$ and intercepts on the abscissa equal to $-1/K_m$. From the intercepts, the values of K_m and V_m can be calculated. The values for these parameters obtained in different experiments (using different cell populations in each case) are summarized in Table 4.1. There was no significant difference in K_m value between the control and TSH-grown cells. However, the TSH-grown cells had a much higher V_m value.

3. Variation of Iodide Influx with Iodide Concentration in the Presence of Perchlorate

The experiments described in the preceding section were repeated with the addition of 1.0×10^{-4} M ClO_4^- to the incubation medium. As discussed in Chapter III, the accumulation of iodide by thyroid cells in the presence of ClO_4^- probably represents a process other than active transport. The results, shown in Fig. 4.4, are in agreement with this concept. The thyroidal iodide accumulation that occurred in perchlorate-supplemented systems was essentially a non-saturating process with respect to substrate concentration up to an iodide concentration as high as 5×10^{-4} M. In the presence of ClO_4^-, the rate of iodide accumulation increased in direct proportion to the iodide concentration of the medium. Since this study was concerned

Figure 4.4. The effect of variations in the extracellular concentration of iodide on the rate of iodide influx into thyroid cells incubated in the presence of potassium perchlorate.

Iodide influx was determined in 6-day-old TSH-grown thyroid cells under conditions similar to those described in the legend of Fig. 4.2 except that 0.1 mM $KClO_4$ was added to the incubation medium. Each result is the mean of 5-6 determinations and is expressed in units of moles of iodide/mg of cell protein/minute. The SEM corresponding to the three lowest concentrations of iodide are not shown because they were too small for the scale employed (the values were $1.09 \pm 0.08 \times 10^{-12}$, $2.89 \pm 0.34 \times 10^{-12}$ and $5.48 \pm 0.56 \times 10^{-12}$).



IODIDE INFLUX (x 10^12)
56
48
40
32
24
16
8
8
16
24
32
40
48
IODIDE CONCENTRATION OF MEDIUM (x 10^5 M)

with the active transport process only, the contribution due to "non-active processes" obtained as described for Fig. 4.4 was substracted from all influx measurements. By means of this correction a value was obtained corresponding to the amount of iodide accumulation by active transport alone.

4. Iodide Influx in the Presence of Inhibitors

Several compounds are known to inhibit the net transport of iodide by the thyroid. Table 4.2 shows the effect of such agents on the rate of iodide influx into thyroid cells when the inhibitors were added to the incubation medium. It was found that ClO_4^- (1.0×10^{-4} M) and CNS^- (1.0×10^{-4} M) inhibited the influx process almost completely. However, 2,4-dinitrophenol (1.0×10^{-4} M), CN^- (1.0×10^{-2} M) and ouabain (5.0×10^{-5} M) had no acute effect on iodide influx.

5. Effect of Intracellular Iodide on Iodide Influx

Table 4.2 also shows the effect of preloading thyroid cells with iodide on the subsequent rate of iodide influx into the cells. Control cells were preincubated with iodide-free KRP solution at 37°C for 30 minutes, while the experimental group was incubated with KRP medium containing 1.0×10^{-5} M iodide for the same duration of time. The rate of iodide influx was then measured in the usual manner for both groups of cells. The results indicated that preloading the cells with iodide had no significant effect on the initial rate of further influx of iodide into the cells.

D. Discussion

The shape of the curve which depicts the rate of accumulation of iodide by thyroid cells as a function of time (Fig. 4.1) would appear to result from the action of two opposing fluxes. At the onset

TABLE 4.2

Effect of Various Reagents on the Rate of Iodide Influx into Thyroid Cells

	Conditions**	Iodide Influx x 10^{11} (moles/mg cell protein/minute)*
1.	Control	15.9 ± 1.8
2.	Addition of 0.1 mM $KClO_4$ to the medium	0.132 ± 0.007
3.	Addition of 0.1 mM KCNS to the medium	0.120 ± 0.005
4.	Addition of 10 mM NaCN to the medium	17.0 ± 1.9
5.	Addition of 0.1 mM 2,4-dinitrophenol to the medium	16.0 ± 1.8
6.	Addition of 5 x 10^{-5} M ouabain to the medium	16.3 ± 1.5
7.	Preincubation of cells with KRP for 30 minutes	16.3 ± 1.7
8.	Preincubation of cells with KRP + 10^{-5} M NaI for 30 minutes	16.2 ± 2.2

Iodide influx was measured in six day old thyroid cells grown in the presence of TSH. Control conditions were the same as those described in the legend to Fig. 4.1. When 10 mM NaCN was added to the incubation medium, appropriate changes in the concentration of NaCl were made to maintain the same osmolality.

*Each result is the mean of 5-6 determinations ± SEM.

**All concentrations denote the final concentration of the reagent in the incubation medium.

of the experiment, only influx of iodide was significant, and this led to an initial rate of iodide accumulation that was nearly rectilinear. However, as the intracellular iodide concentration continued to increase, efflux became more significant leading to a progressive decrease in the slope of the time curve. Finally, in the steady state, achieved after 45-60 minutes of incubation, the two fluxes became equal and the net uptake of iodide by the cells did not change further. The rate at which the steady state was attained was observed to be comparatively slow confirming that the present method used to measure the rate of iodide influx was valid.

The inhibitory effects on iodide influx of ClO_4^- and CNS^- at concentrations of 1.0×10^{-4} M was very marked. This was consistent with the known properties of these ions as competitive inhibitors of iodide transport. The absence of an acute inhibitory effect of agents like ouabain, CN^- and 2,4-dinitrophenol was of considerable interest. It suggested that the influx process itself did not depend directly upon the operation of the "sodium-pump". Further, it was apparent that these drugs did not directly interfere with the influx reaction. However, since these reagents will abolish net iodide accumulation by thyroid cells after a sufficient lapse of time (Chapter III), it would appear likely that their effect was primarily to increase the rate of iodide efflux after a lag period. This aspect of their action was later investigated and is discussed in Chapter VI. The lack of effect of iodide preloading on subsequent iodide influx was also considered significant and is discussed in the final chapter in relation to the overall scheme of iodide transport.

The shape of the influx curve (Fig. 4.2) followed a Michaelis-Menten type of relationship. However, this by itself gave no

information about the particular sequence of reactions involved in influx; because a number of different schemes would satisfy such a relationship, other experimental approaches were necessary to elucidate the details of such steps. The K_m values obtained in this study were similar to those that have been reported for the thyroid glands of mice (Wollman and Scow, 1954) and rats, and for sheep thyroid slices (Wolff, 1964) obtained by different kinetic approaches. Similar values were also obtained by Knopp *et al* (1970) in their measurements of iodide influx into freshly isolated dispersed bovine-thyroid cells.

The only difference found between control and TSH-grown cells, when the kinetics of iodide influx for the two groups of cells were compared, was in their markedly different V_m parameters. Since the number of cells corresponding to 1 mg of cellular protein was not significantly different for the two cell populations (Chapter II), the observed difference in V_m data suggested that the transport function per cell was considerably increased for cells that had been grown in the presence of TSH. In contrast, the K_m values were not significantly different for the two cell populations. These data suggest that incubation of the cells with TSH may lead to the induction in the cells of extra transport sites of identical affinity for iodide. An alternate explanation would be that TSH may accelerate some rate-limiting step of influx. These possibilities regarding the mode of action of TSH will be discussed more completely in the concluding section. Similar observations on the effect of TSH on the kinetics of iodide influx have been reported by Knopp *et al* (1970) in their studies using isolated thyroid cells. Similarly, Wollman and Scow (1954) found that the K_m values for iodide influx determined on mouse thyroids were not affected

by hypophysectomy, a finding that agrees well with the present observation that TSH did not affect the K_m of iodide influx.

CHAPTER V

EFFECT OF NA^+ AND OTHER CATIONS ON IODIDE INFLUX

A. Introduction

The experiments performed to measure the rate of iodide influx into thyroid cells, as described in the last section, used Krebs Ringer phosphate solution as the external medium. This solution contained Na^+ as the principal cation. The experiments described below were designed to determine the effect of Na^+ on iodide influx. In addition, a number of different ions or solutes such as choline$^+$, tris$^+$ (tris-hydroxy-methyl-aminomethane), Li^+, K^+ and mannitol were investigated as Na^+ substitutes. In making such replacements, the total osmolality of the solution was maintained at a constant level. This was done to prevent swelling or shrinking of the thyroid cells in such substituted media and to eliminate factors such as solvent (water) flow across the cell membrane. As in previous experiments, thyroid cells belonging to the same batch were used for comparative measurements. It was decided to utilize TSH-grown cells throughout these studies because of their greater capacity for iodide transport. The differences which have been observed between TSH-grown and control thyroid cells with respect to iodide transport are discussed in a later section.

In addition to the qualitative studies outlined above, several quantitative studies were undertaken in this section of the work. These included a study of the effect of sodium ion concentration of the external medium on the kinetic parameters (K_m and V_m) of iodide influx, and a study of the influence of extracellular sodium ion concentration on the magnitude of iodide influx at a constant iodide ion concentration. Such studies were expected to provide information on the role of Na^+ in the thyroidal iodide influx process.

B. Materials and Methods

1. Cells

Five- to 6-day old monolayer cultures of bovine thyroid cells grown in the presence of TSH were used. The cells were grown under the conditions described in Chapter II.

2. Media

These were of the following types:

(i) KRP - of the usual composition described in Chapter III.

(ii) KRP in which Na^+ was completely replaced by K^+. This was achieved by replacing NaCl by equimolar KCl and by substituting $K_2H\ PO_4$ for $Na_2H\ PO_4$.

(iii) Tris-Na medium. This had the composition:

NaCl	-	130 mM
KCl	-	4.8 mM
$CaCl_2$	-	2.5 mM
$MgSO_4$	-	1.2 mM
KH_2PO_4	-	1.2 mM
Tris	-	15 mM

The pH was adjusted to 7.4 by the addition of 1N HCl.

Substitutions of Na^+ or K^+ in these media were made by using solutions of choline chloride, LiCl, NaCl, KCl, tris hydrochloride and mannitol in equimolecular amounts with the exception of mannitol where twice the molecular amount was used to yield the same number of particles in solution as the electrolytes.

C. Results

1. Iodide Accumulation by Thyroid Cells Incubated in Low-Na^+ Media

The upper curve in Fig. 5.1 shows the rate of iodide accumulation by thyroid cells incubated in KRP medium as a function of time. The lower curve shows a similar experiment done using a KRP medium in which Na^+ had been substituted with $choline^+$ to the extent that the medium had a Na^+ concentration of 31.2 mM. The results showed that there was a marked depression of iodide accumulation in cells incubated up to one hour in the low-Na^+ medium.

2. Specific Stimulation of Thyroidal Iodide Influx by Na^+

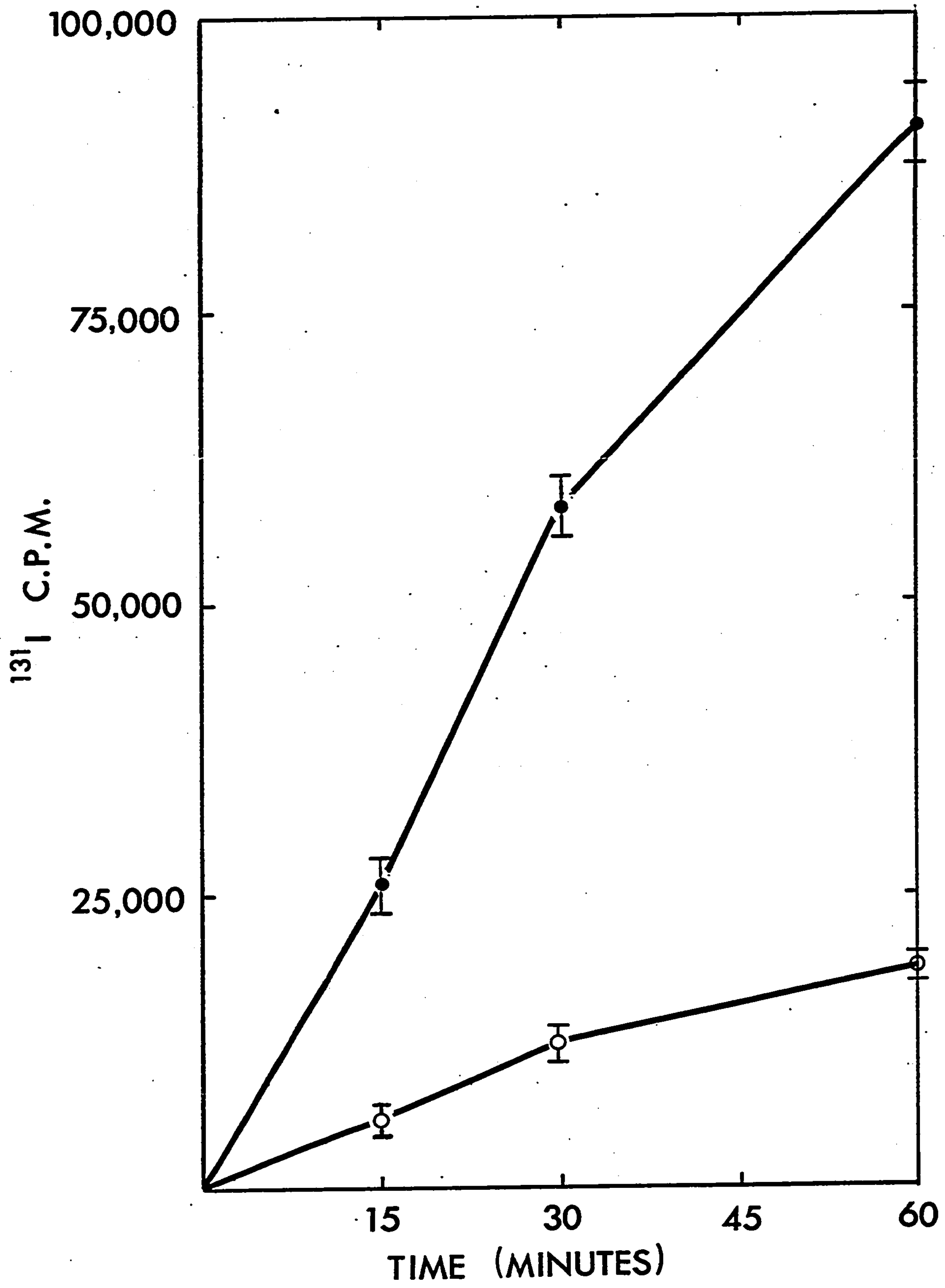
The data presented in Table 5.1 are the iodide influx measurements obtained by incubating thyroid cells in an "all-K^+" medium (KRP in which Na^+ had been totally replaced by K^+). The effect of partial substitution of the K^+ of this medium by various other solutes is also presented. The influx of iodide into the cells from the "all-K^+" medium was very low and was comparable in magnitude to that obtained in experiments where active iodide influx had been abolished (Chapter IV). Substitution of a part of the K^+ of the medium, equivalent to 70 mM, by Li^+, $choline^+$, $tris^+$ or mannitol, had no effect on iodide influx. However, substitution of K^+ by Na^+ to a similar extent was found to produce a marked stimulation of iodide influx. This suggested a specific stimulatory role of Na^+ for the process of thyroidal iodide influx.

3. Specific Depression of Thyroidal Iodide Influx by Depletion of Extracellular Na^+

The data presented in the top line of Table 5.2 show the values measured for iodide influx into thyroid cells incubated in a normal KRP medium. The table also lists the values obtained for iodide influx when the concentration of Na^+ in the medium was reduced from

Figure 5.1. Iodide accumulation by thyroid cells incubated in a low Na^+-containing medium.

Iodide influx was measured in 5-day-old TSH-grown cells for various periods of time by methods described previously (Fig. 4.2). The upper curve (●———●) represents incubation in KRP media (151.5 mM Na^+) while the lower curve (○———○) represents incubation in a modified KRP medium containing 31.2 mM Na^+ and 120.3 mM choline$^+$. The ordinate represents ^{131}I-iodide accumulation as counts per minute per mg cell protein. Each result shown is the mean of 5-6 determinations ± SEM.



100,000
75,000
50,000
25,000
^{131}I C.P.M.
15
30
45
60
TIME (MINUTES)

TABLE 5.1

The Effect of Additions of Substituents to the External Medium on the Rate of Iodide Influx into Thyroid Cells

Composition of External Medium		Iodide influx x 10^{11} (moles/mg cell protein/ minute)*
Concentration of K^+ (mM)	Potassium Replacement	
156.3	0	0.41 ± 0.04
86.3	70 mM $Choline^+$	0.51 ± 0.04
86.3	70 mM Li^+	0.40 ± 0.03
86.3	70 mM $Tris^+$	0.38 ± 0.04
86.3	140 mM Mannitol	0.53 ± 0.04
86.3	70 mM Na^+	3.82 ± 0.32

Iodide influx was measured in 6-day old TSH-grown thyroid cells by methods previously described (Fig. 4.2). The cells were incubated in "all-K" media (see text), or in similar media in which a part of the K^+ had been replaced by various substituents. The iodide concentration of the media was 1.0 x 10^{-5} M.

*Mean of 5-6 determinations ± SEM.

TABLE 5.2

The Effect of Na^+ Replacement from the External Media on the Rate of Iodide Influx into Thyroid Cells

Composition of External Medium		Iodide Influx x 10^{11} (moles/mg cell protein/ minute)*
Concentration of Na^+ (mM)	Sodium Replacement	
151.5	0	22.3 ± 2.4
81.5	70 mM K^+	5.9 ± 0.5
81.5	70 mM $Choline^+$	10.9 ± 0.8
81.5	70 mM Li^+	10.2 ± 0.9
81.5	70 mM $Tris^+$	10.8 ± 1.2
81.5	140 mM Mannitol	11.2 ± 0.7

Iodide influx was measured in 6-day old TSH-grown thyroid cells by methods described previously (Fig. 4.2). The incubation media employed were KRP modified as indicated above. The iodide concentration of the media was 1.0 x 10^{-5} M.

*Mean of 5-6 determinations ± SEM.

151.5 mM to 81.5 mM by substitution of Na^+ with other substituents. The results indicated that Li^+, Choline$^+$, tris$^+$ or mannitol could not substitute in a functional way for Na^+. The similarity of the results obtained among each of the substituents also suggested that none of them was specifically inhibitory to iodide influx. In contrast, when K^+ was used to substitute for Na^+, the measured iodide influx was lower than that obtained with the other substituents. This suggested that K^+ might have a specific inhibitory effect on iodide influx when present in high concentrations in the incubation medium.

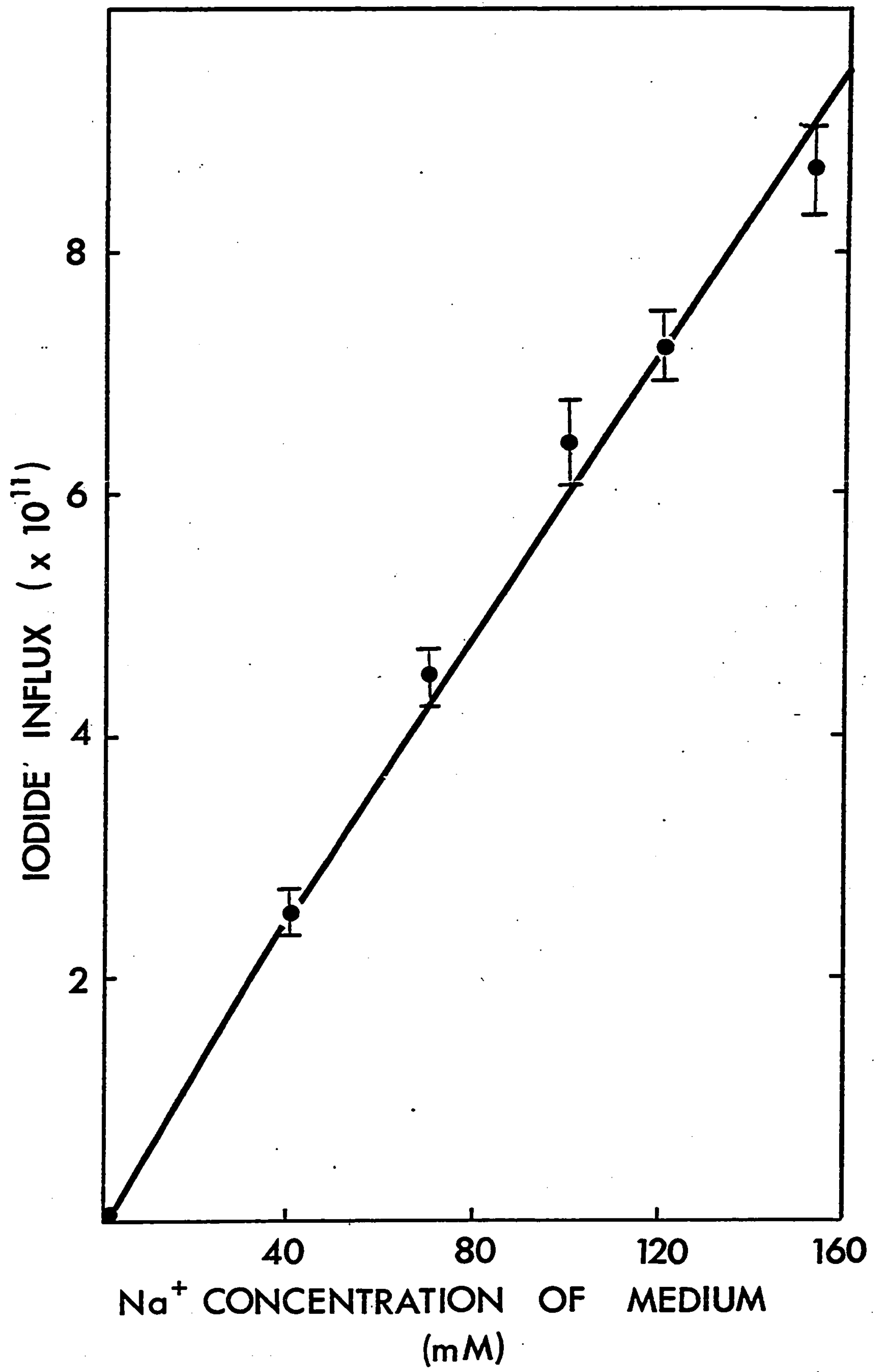
4. Variation of Thyroidal Iodide Influx with the Concentration of Na^+ in the Medium

Fig. 5.2 illustrates the values of iodide influx measured for thyroid cells which were incubated in media with Na^+ concentrations varying from 0-151.5 mM. The iodide concentration of the incubation media was constant at 1.0×10^{-5} M. The media employed were KRP solutions in which choline chloride was used to replace NaCl to the desired extent. When a Na^+-free medium was required, the tris-choline used was prepared from choline chloride instead of NaCl. Since the results described in sections (3) and (4) above showed that the use of tris-HCl resulted in no specific stimulation or inhibition of iodide influx, the use of this medium in the present section was considered justified.

As may be seen in Fig. 5.2, the absence of Na^+ resulted in the complete abolition of iodide influx into thyroid cells. As the Na^+ concentration was increased from 0 to 151.5 mM, the rate of iodide influx rose in a rectilinear fashion. Higher concentrations of Na^+ were not tested since these would have led invariably to unacceptably high osmolalities. However, it is possible that thyroidal iodide

Figure 5.2. The effect of variations in the Na^+-concentration of the external medium on iodide influx into thyroid cells.

Iodide influx was measured in 5-day-old TSH-grown thyroid cells by methods described previously (Fig. 4.2). The incubation media employed were modified KRP solutions in which a part of the Na^+ of the medium had been replaced by $choline^+$ to yield the Na^+-concentration shown. The iodide concentration of all media was 1.0×10^{-5} M. Iodide influx, expressed in units of moles per mg cell protein per minute, is represented in the ordinate. Each result is the mean of 5-6 determination + SEM.


IODIDE INFLUX (x 10^{11})
8
6
4
2
40
80
120
160
Na^{+} CONCENTRATION OF MEDIUM
(mM)

influx may attain saturation at higher Na^+ concentrations than those studied in these experiments.

5. Kinetics of Thyroidal Iodide Influx in Low-Na^+ Media

The kinetic parameters, K_m and V_m, of iodide influx were determined as described in Chapter IV using substituted KRP media with Na^+ concentrations of 40, 70 and 100 mM, respectively; choline chloride was used as the substitution for NaCl in these media. The results are summarized in Table 5.3 and are shown in a schematic form in Fig. 5.3. It was evident that the V_m values were relatively constant for each of the different solutions. However, the K_m values increased progressively as the Na^+ concentration of the medium was lowered from 151.5 mM to 40 mM.

D. Discussion

1. The Role of Na^+ in Thyroidal Iodide Influx

The results of the experiments described in this section have suggested a stimulatory role for Na^+ in thyroidal iodide influx. This stimulatory effect of sodium on iodide influx was specific, since none of the other substituents examined, whether monovalent cations or neutral solutes, could replace Na^+ effectively. The similarity in the influx values obtained when Na^+ was substituted by Li^+, choline$^+$, tris$^+$ or mannitol showed that none of these agents had a specific stimulatory or inhibitory action. The results obtained with Li^+ were of interest in view of its stimulatory role in other transport systems, where it apparently can partly mimic the role of Na^+ (Bihler and Adamic, 1967).

Theoretically, the effect of Na^+ on iodide influx might be exerted in several ways. If Na^+ participated directly in the transport of iodide across the cell membrane, this might involve Na^+ in the

TABLE 5.3

Variation of the Kinetic Parameters of Iodide Influx into Thyroid Cells with Changes in the Extracellular Na^+ Concentration

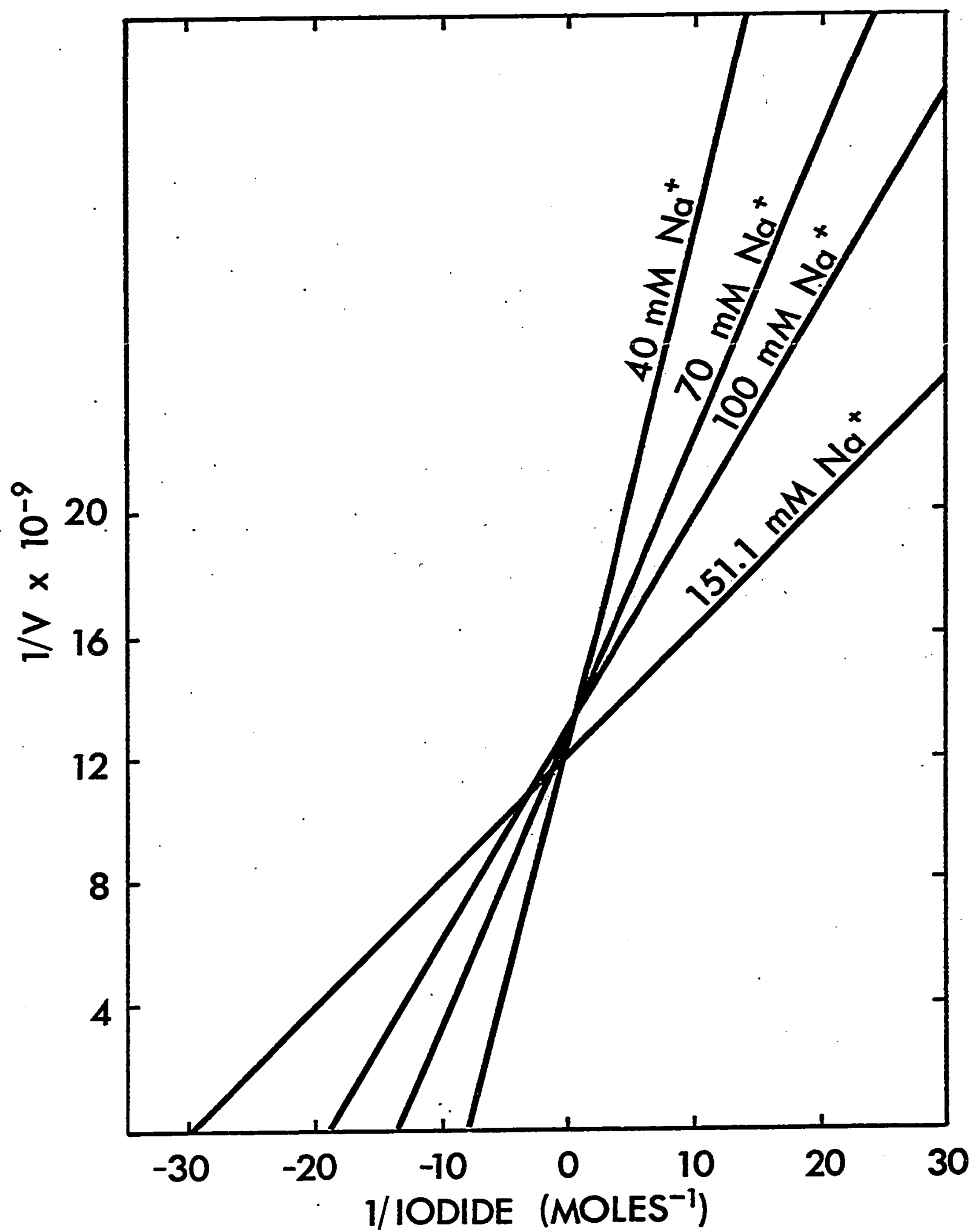
Na^+ Concentration of External Medium (mM)	Vm (moles/mg cell protein/ minute x 10^{11})	Km (moles x 10^5)
1. 40	1. 93.4	1. 14.0
	2. 70.0	2. 12.3
	3. 62.2	3. 11.2
2. 70	1. 61.8	1. 8.24
	2. 82.2	2. 7.62
	3. 88.0	3. 6.64
3. 100	1. 76.3	1. 4.84
	2. 58.0	2. 5.40
	3. 91.6	3. 5.36
4. 151.5*	1. 87.3	1. 2.93
	2. 66.7	2. 3.33
	3. 93.6	3. 3.64

Each horizontal line represents the results of a single experiment using 5-day old thyroid cell cultures grown in the presence of TSH.

*Results obtained in experiments in which thyroid cells were incubated in 151.5 mM Na^+-media (unmodified KRP) have been reproduced from Table 4.1.

Figure 5.3. Schematic Lineweaver-Burk plots of iodide influx as a function of the iodide concentration of media containing varying concentrations of Na^+.

The plots were constructed using mean K_m and corresponding mean V_m values from the data in Table 5.3. The intercept on the X-axis for each line is the negative reciprocal of the mean K_m value. The intercept on the Y-axis is the reciprocal of the corresponding mean V_m value. The four schematic lines were drawn so that each passed through the two corresponding points of interception.


40 mM Na^+
70 mM Na^+
100 mM Na^+
151.1 mM Na^+
20
16
12
8
4
$1/V \times 10^{-9}$
-30
-20
-10
0
10
20
30
1/IODIDE (MOLES^{-1})

formation of a transport intermediate in the influx step, perhaps with Na^+ as part of the complex itself. On the other hand, an indirect role of Na^+ might be of several types. One possibility is that Na^+ exerts an influence on the linking of some cellular energy yielding reaction to the influx step. An action of Na^+ on the cell membrane altering its structural characteristics so as to make the transport sites more accessible to the substrate, is another possibility.

Many of the data suggest, but do not prove, a direct participation of Na^+ in the influx process. Thus iodide influx was abolished when Na^+ was totally absent from the medium, and then increased in direct proportion to the concentration of added Na^+ over the physiological range (i.e., up to 151.5 mM). Moreover, the kinetics of iodide influx were found to be such that the effect of removing Na^+ from the medium was equivalent to that of addition of a competitive inhibitor of iodide influx to the system. All these findings strongly suggest that Na^+ is involved in the formation of an intermediate required for the influx of iodide into thyroid cells.

Increasing the Na^+ concentration in the extracellular medium has been reported to affect the kinetics of Na^+-dependent transport of various solutes in a number of different ways. These effects can be classified in a general way as follows:

(i) A decrease in K_m but no change in V_m. Such effects have been seen, for example, in alanine transport in rabbit ileum (Curran *et al*, 1967).

(ii) An increase in V_m with minimal effect on K_m, seen, for example, in 3-methyl glucose transport in rabbit ileum (Goldner *et al*, 1969).

(iii) A biphasic response, as seen in α-amino isobutyric acid transport in a marine pseudomonad (Wong *et al*, 1969). In this organism, an increase in the Na^+ concentration from 0 to 50 mM resulted in a decrease in K_m but no change in V_m. At Na^+ concentrations of 50 to 200 mM, however, the K_m remained essentially constant while the V_m continued to increase. The authors ascribed the latter phase to a modification of the porosity of the cytoplasmic membrane by Na^+ in such a way as to reduce the rate of leakage of the substrate from the cells.

(iv) A decrease in K_m as well as an increase in V_m, as seen in glycine transport in rabbit retinculocytes (Wheeler and Christensen, 1967b).

(v) No change in K_m but a decrease of V_m, as reported for lysine transport in rabbit alveolar macrophages (Tsan and Berlin, 1971). In this situation, Na^+ exerted an inhibitory role on the transport process.

It is clear the effect of Na^+ on iodide influx as observed in the present investigation falls in the first category. The implications of such changes in these kinetic parameters are discussed fully in the concluding chapter of this thesis in which a theoretical model for thyroidal iodide transport is proposed.

2. The Role of K^+ in Thyroidal Iodide Influx

The effect of external K^+ on transport processes that are Na^+-dependent is complex and as yet incompletely resolved. In low concentration, K^+ seems to exert a stimulatory effect on these processes, and in the complete absence of K^+, transport is inhibited. This effect has been reported in cases such as glucose absorption by guinea pig

small intestine (Riklis and Quastel, 1958) and amino acid accumulation by ascites cell (Riggs *et al*, 1958); in both situations the cellular accumulation of solute was depressed in a K^+-free medium. An increase in the K^+ concentration of the external medium led to an increase in net accumulation of solute which continued until an optimal K^+ concentration had been reached (generally 4-15 mM). Similar results were reported for iodide accumulation by thyroid slices (Wolff and Maurey, 1961). In that system the half maximal stimulation of net accumulation of iodide was found to occur at an external K^+ concentration of 0.9-2.9 mM.

Various explanations have been advanced to explain the effects of low external K^+ concentration on solute transport. For example, an optimal intracellular K^+ concentration may be necessary for the function of many energy-producing, enzymatic metabolic reactions (Ussing *et al*, 1960). A depletion of intracellular K^+, as a result of low external K^+, may thus have non-specific effects on transport processes that require metabolic energy. Also, a low external K^+ concentration may inhibit the cellular exchange of intracellular Na^+ for extracellular K^+ that is mediated by the "Na pump" or membrane ATPase. This in turn may lead to a rise in the intracellular concentration of Na^+ with a consequent increase in solute efflux. Eddy *et al* (1967) reported that glycine uptake by ascites cells was diminished in a K^+-free external medium and that this was due to an increase in glycine efflux, the rate of influx remaining unchanged.

On the other hand, K^+ in higher concentrations generally has been found to cause an inhibition of influx in Na^+-dependent processes, as was the case in the present investigation. This may be the result

of a direct competition between K^+ and Na^+ in the Na^+-dependent transport system as has been suggested by the data of several investigators (e.g., Eddy *et al*, 1967; Crane *et al*, 1965). An alternative explanation for the inhibitory effect of high concentrations of K^+ may be that these cause non-specific cellular changes which interfere with solute transport. For example, many cells are known to swell markedly in high-K^+ solutions. This swelling could lead to alterations in membrane function and to a non-specific inhibition of transport processes.

Finally, if solute efflux is also Na^+-dependent (see Chapter VI), it is possible that the normal high concentration of intracellular K^+ may inhibit the efflux reaction by competing with intracellular Na^+. Such lowering of efflux rate, which could also be achieved by a low intracellular Na^+ concentration, would serve to increase the net accumulation of solute. In such a way, the high intracellular K^+ concentration typical of normal cells may play a stimulatory role in the overall process of solute uptake.

CHAPTER VI

INFLUENCE OF NA^+ ON IODIDE EFFLUX FROM CULTURED THYROID CELLS

A. Introduction

Studies on the effect of Na^+ on iodide influx into cultured thyroid cells, described in the previous chapter, suggested that Na^+ may be involved in some step or steps of the influx process. However, such a conclusion could not be considered as unequivocal on the basis of the experimental evidence that had been obtained at that point. Further information on the role of Na^+ in iodide transport was then sought by means of a study of the influence of Na^+ on iodide efflux from cultured thyroid cells. These studies were considered to be of particular relevance as solute efflux has often been thought to occur by a process of retrograde influx, i.e., influx and efflux reactions that were symmetrical but oppositely directed. Such symmetrical kinetic models have been proposed for alanine transport in rabbit ileum (Curran *et al*, 1967), glycine uptake by pigeon erythrocytes (Vidaver and Shepherd, 1968), sugar uptake by rabbit ileum (Goldner *et al*, 1969) and a number of other transport systems.

The efflux measurements that are described in this section were carried out by determining the rate of loss of radioactive tracer from thyroid cells, preloaded with ^{131}I-iodide, to an appropriate external medium which contained no tracer. By keeping the volume of the external medium large in comparison to the volume of the cells, the concentration of tracer in the medium remained relatively low throughout each experiment. Under such conditions, recycling of the tracer (i.e., re-entry of ^{131}I from the medium back into the cells) should occur to a negligible extent, and the rate of loss of tracer from the cells would closely approximate the rate of iodide efflux.

The concentration of Na^+ could, in principle, be varied either on the cis (intracellular) or trans (extracellular) side of the cell membrane in order to study its effect on iodide efflux. It is quite difficult, however, to vary intracellular Na^+ concentrations in a predictable manner. In addition, the volume average intracellular Na^+ concentrations, as measured by standard analytical techniques, do not measure the effective intracellular Na^+ activities on the inner side of the cell membrane. This is due to the considerable lack of homogeneity of distribution of intracellular Na^+ as a result of factors such as intracellular binding (McLaughlin and Hinke, 1966; Cope, 1967) and higher Na^+-concentration in nuclei (Zadunaisky *et al*, 1968). A study of the effect of intracellular Na^+ concentration on iodide efflux would be difficult to interpret for these reasons and thus was not undertaken.

In contrast, studies which would determine the effect of extracellular Na^+ on iodide efflux could be carried out with media having well defined compositions. Initial studies were undertaken to investigate the trans-concentration effects of Na^+ on iodide efflux. Further experiments were then carried out to determine how such effects could be modified by various reagents known to affect iodide transport.

B. Materials and Methods

1. Cells

Five-to 6-day old thyroid cells grown on coverslips in the presence of 70 milliunits/ml of TSH as described in Chapter II were used.

2. Incubation Media

The two Tris media described in the previous chapter were used, i.e., either Tris-Na or Tris-choline (in which choline chloride

replaced NaCl completely).

3. Efflux Experiments

Monolayers of bovine thyroid cells on glass coverslips were incubated for 30 minutes at 37°C with 5×10^{-5} M NaI solution in Tris-Na medium containing 10 μCi/ml of ^{131}I. Following this first incubation each coverslip was rinsed in 0.9% NaCl solution, as described before (Chapter III), and was then transferred to a 50 ml capacity beaker containing 10 ml of an appropriate medium. This marked the beginning of the efflux experiment proper in which incubation of the thyroid cells was continued at 37°C with gentle shaking in a Dubnoff metabolic shaking incubator. One-ml aliquots of the medium were removed at intervals of 5 or 10 minutes for up to 60 minutes and counted for radioactivity in a well-type scintillation counter. The residual radioactivity of the cells at the end of the experiment was determined by counting each coverslip after it was rinsed as above. From these measurements it was possible to estimate by calculation the amount of radioactive tracer in the cells at any of the experimental time intervals as well as the total amount of tracer in the cells at the beginning of the efflux experiment. The rate of iodide efflux from the cells was calculated from these data as described below.

In some cases, the experiment was carried out as described above for an initial period of 15 to 20 minutes in a control medium to determine a baseline rate of iodide efflux. The reagent to be tested was then added to the medium in a volume of 1 ml at an appropriate concentration so as to yield the desired final concentration on dilution by the medium (1×10^{-4} M for I^-, ClO_4^-, CNS^- and 2,4-dinitrophenol; 5×10^{-5} M for ouabain, 10 mM for CN^-). The experiment was then continued to determine the efflux rate of iodide in the

presence of the added reagent.

A second modification of the procedure was followed in some instances. After initial efflux measurements had been made by incubation of thyroid cells in one medium for 20 minutes, the coverslip was transferred, after draining, to a second beaker (50 ml capacity) containing 10 ml of a medium of different composition. The remaining efflux measurements were made in the new medium in the usual manner. The efflux data were plotted to show the efflux rate both in the first medium (the initial part of the plot) and in the second (the latter part of the plot).

To demonstrate the absence of significant tracer recycling in these experiments (re-entry of ^{131}I from the medium into the cells), a coverslip bearing thyroid cells was incubated for 30 minutes with 5×10^{-5} M NaI without the addition of tracer. This control coverslip was then incubated in the same beaker in parallel with a test coverslip under the usual conditions of an efflux experiment. At the end of a 60-minute incubation period, the radioactivity of the control coverslip was found to be no more than 10% of the final radioactivity of the test coverslip, which itself was less than 0.1% of the total amount of tracer present in the control cells at the beginning of the efflux experiment. These results indicated that the re-entry of tracer from the medium into the cells did not occur to any significant extent under the present experimental conditions. An analysis of the shape of the efflux plots which will be discussed below, led to the same conclusion.

A possible complication of the experimental procedure followed would be the occurrence of significant desquamation of cells from the coverslip to the medium. Such cells would remain in suspension and

would be withdrawn as part of the aliquots taken from the incubation media for counting, thus introducing errors in the experiment. To investigate this possibility, the residual medium obtained at the end of an efflux experiment was examined microscopically after centrifugation. No cells or cellular debris were detected in such preparations. Furthermore, microscopic examination of the coverslip at the end of the experiment showed the monolayer to be generally intact, confirming that no significant desquamation of cells from the coverslip occurred during the experiment.

4. Expression of Results

If the rate of efflux of iodide from thyroid cells obeys first-order kinetics, the outward movement of labelled substrate ^{131}I from the cellular compartment to the unlabelled solution can be described, assuming negligible recycling of tracer, by the expression:

$$\frac{d\,S^*}{dt} = -k\,S^*$$

where k is the rate constant for efflux and S^* is the amount of tracer in the cells at time t.

Upon integration,

$$S^* = S_o^* \quad e^{-kt}$$

where S_o is the amount of tracer in the cells at time zero.

It follows that,

$$[S^*/S_o^*] = e^{-kt}$$

and

$$\ln\,[S^*/S_o^*] = -kt$$

Thus for a first-order process the plot of $[S^*/S_o^*]$ against time would be exponential, while the plot of $-\ln\,[S^*/S_o^*]$ against time would yield a straight line with slope equal to k.

Figures 6.1 and 6.2 show these types of plots for data obtained experimentally for the efflux of ^{131}I from thyroid cells in different media. The plot of $[I^*/I_o^*]$ against time (Fig. 6.1) was found to be exponential, while the plot of $-\ln [I^*/I_o^*]$ against time was linear. These results suggested that under the conditions of the present experiments, tracer efflux from the cells obeyed first-order kinetics. Furthermore, a rate constant for the efflux process could be calculated from the slopes of the logarithmic plots of the type shown in Fig. 6.2. Accordingly, for each efflux experiment, the values calculated for $-\ln [I^*/I_o^*]$ were plotted against time in order to evaluate the rate constant, and to determine the manner in which the rate constant varied in response to changes in experimental conditions.

C. Results

1. Rate of Iodide Efflux from Thyroid Cells

The rates of efflux of ^{131}I from thyroid cells into two iodide-free media are plotted in Fig. 6.2. Line A corresponds to Tris-Na medium and line B to a Tris- choline medium. The rates in both cases were found to be linear over the entire duration of the experiment. This suggested the absence of any compartmentalization of intracellular iodide, or alternatively, if different iodide compartments did exist, suggested that the rate of efflux of iodide into the external medium was slower than the rate of exhange of iodide among the various compartments, and was therefore rate-limiting.

From Fig. 6.1 it was apparent that virtually all of the intracellular iodide was in a diffusible form, available for efflux out of the cell. This confirmed the previous finding (Chapter III), that organic binding of transported iodide by thyroid cells occurred to a negligible extent in the present culture system.

Figure 6.1. The rate of ^{131}I efflux from thyroid cells.

Thyroid cells, adherent to a coverslip, were preloaded with ^{131}I-iodide by incubating them with 5×10^{-5} M NaI and 10 µCi/ml of ^{131}I for 30 minutes at 37°C. After they were washed, the cells were transferred to a beaker containing 10 ml of the appropriate medium at 37°C. One ml aliquots of the medium were removed at the times indicated on the abscissa. The radioactivity of these aliquots, as well as that remaining on the coverslip after 60 minutes, was measured as described in the text. The ordinate represents I^{*}/I_{O}^{*} ratios (see text).

Tris-Na medium ○———○

Tris-choline medium ●------●

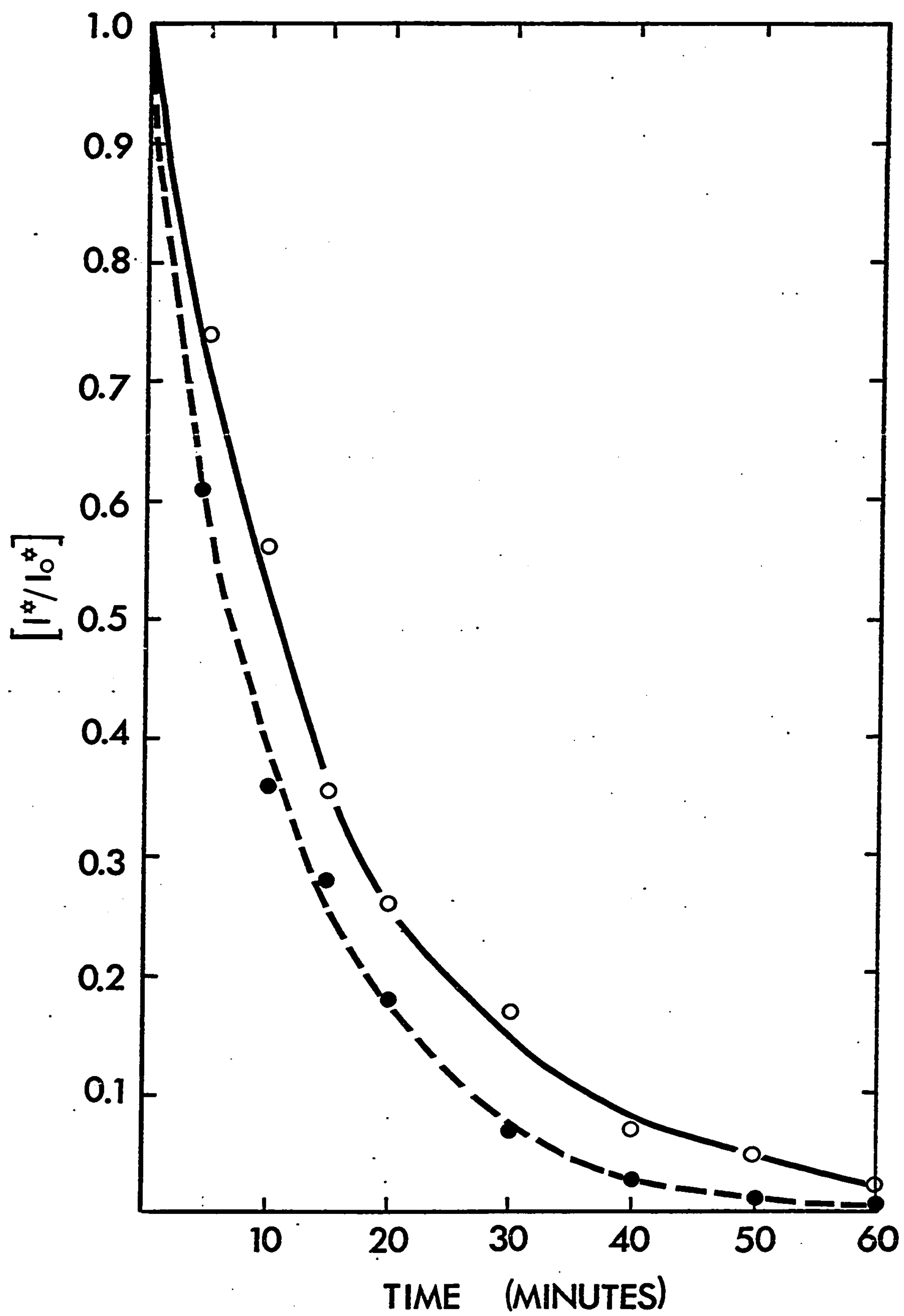
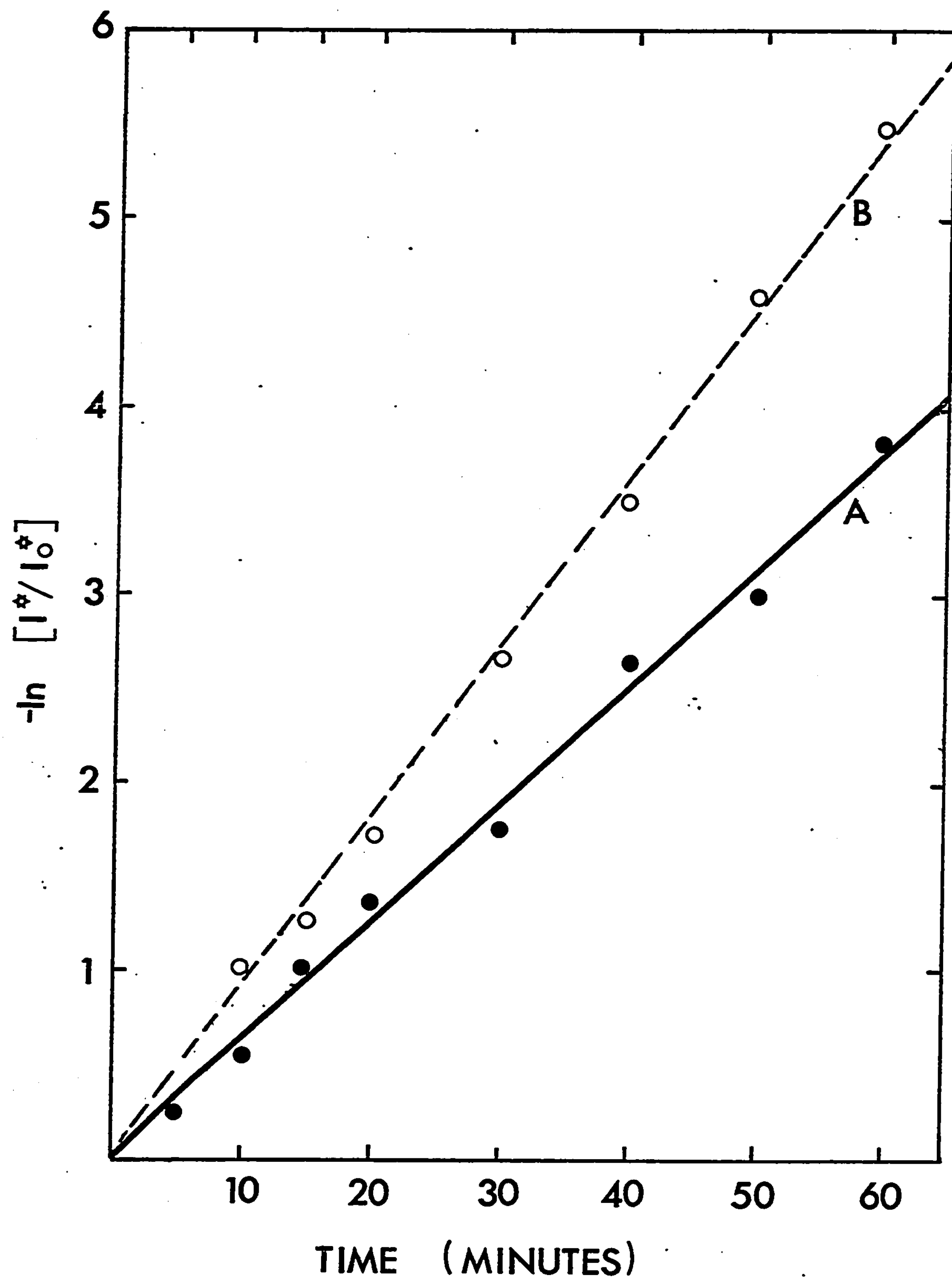

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
[I*/I$_o$*]
10
20
30
40
50
60
TIME (MINUTES)

Figure 6.2. Rate of ^{131}I efflux from thyroid cells.

The plots shown were drawn utilizing the data from the experiments described in Fig. 6.1.

Tris-Na medium ●———●

Tris-choline medium o------o


6
5
4
3
2
1
B
A
-ln [I*/I*o]
10
20
30
40
50
60
TIME (MINUTES)

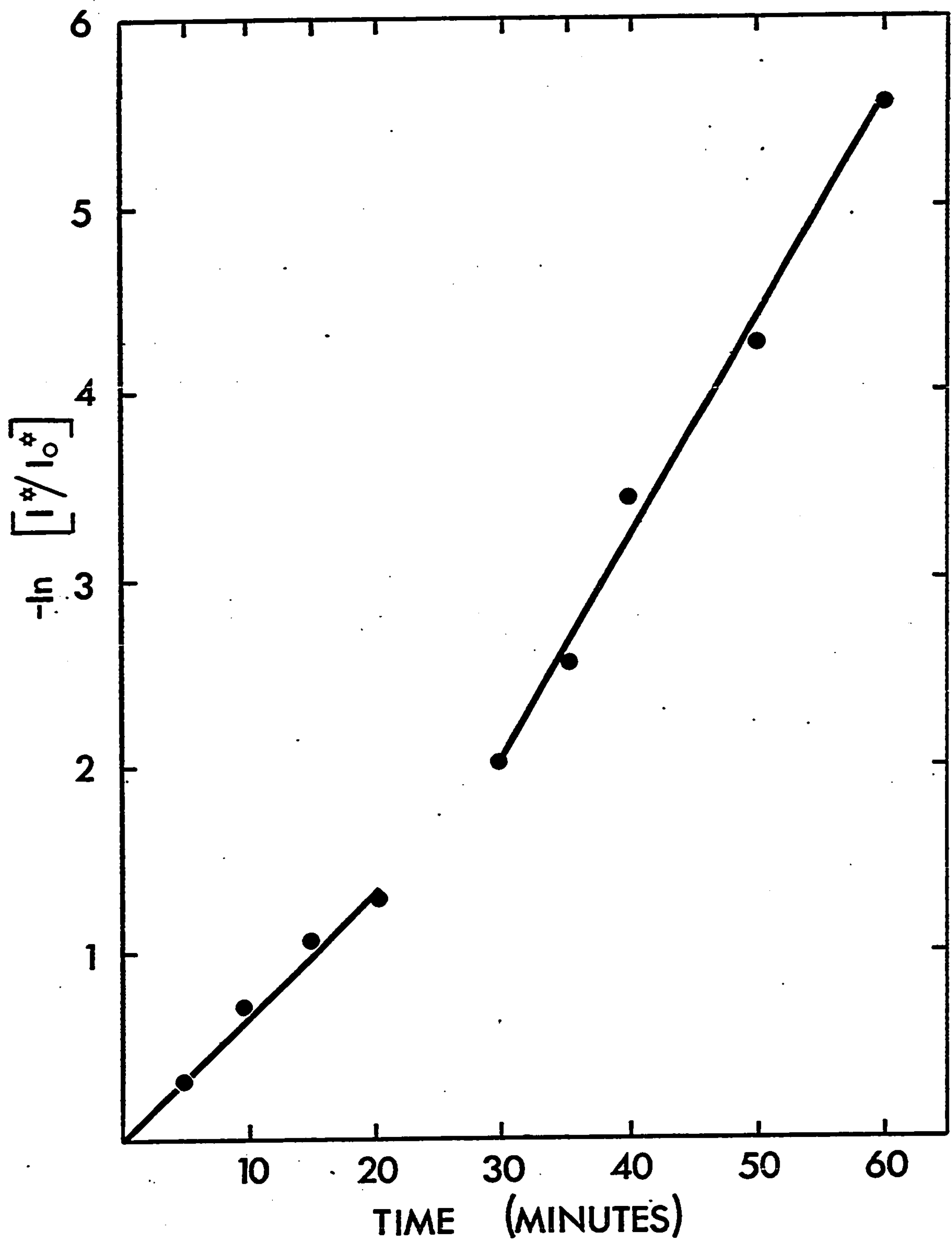
The rectilinear nature of the efflux plots in Fig. 6.2 also suggested that recycling of tracer from media back into cells occurred to a negligible extent under the present conditions. The presence of any significant recycling would have made the observed $[I^*/I_o^*]$ ratio higher than the expected value (i.e., that found in the absence of iodide re-entry into the cells) and furthermore, if significant recycling were occurring, the differences between the experimental and theoretical values would have increased with time, as the tracer concentration in the medium increased progressively. Such an effect would cause the efflux plot to deviate from a straight line and to show an increasing downward curvature with time. However, no such curvature was discernible in any of the efflux plots.

2. <u>Effect of Na^+ on Iodide Efflux from Thyroid Cells</u>

From Fig. 6.2 it was apparent that the rate of iodide efflux from thyroid cells, determined from the slope of the plot, was much higher when the external medium was Na^+-free. However, a clearer demonstration of such effects of Na^+ was obtained from another series of experiments in which the efflux medium was changed from Tris-Na to Tris-choline (or vice versa) after 20 minutes of incubation. Fig. 6.3 shows the results of one such experiment in which ^{131}I-containing thyroid cells were incubated initially in a Tris-Na medium and subsequently in a Tris-choline medium. The initial slope of the efflux plot, corresponding to efflux of iodide into a Na^+-containing medium, was 0.06 per minute. On transferring the cells to a Na^+-free medium, the slope sharply increased to 0.11 per minute. Figure 6.4 shows the results from a similar experiment in which a Tris-choline medium was used initially followed by a Tris-Na medium. The change to a Na^+-containing medium was found to result in a decrease of slope from 0.11 per minute to 0.06 per minute.

Figure 6.3. The effect of Na^+-depletion of the external medium on ^{131}I efflux from thyroid cells.

The measurement of ^{131}I efflux from thyroid cells was carried out under similar conditions to those described in Fig. 6.1 using a Tris-Na medium for the first 20 minutes of incubation. After 20 minutes, the coverslip was transferred to a second beaker containing 10 ml of Tris-choline medium and the experiment continued as before for an additional 40 minutes. Aliquots (between 30-60 minutes) were withdrawn from this second medium and counted. The radioactivity remaining on the coverslip was counted after 60 minutes of incubation. Values of $-\ln [I^{*}/I_{o}^{*}]$ are plotted on the ordinate.

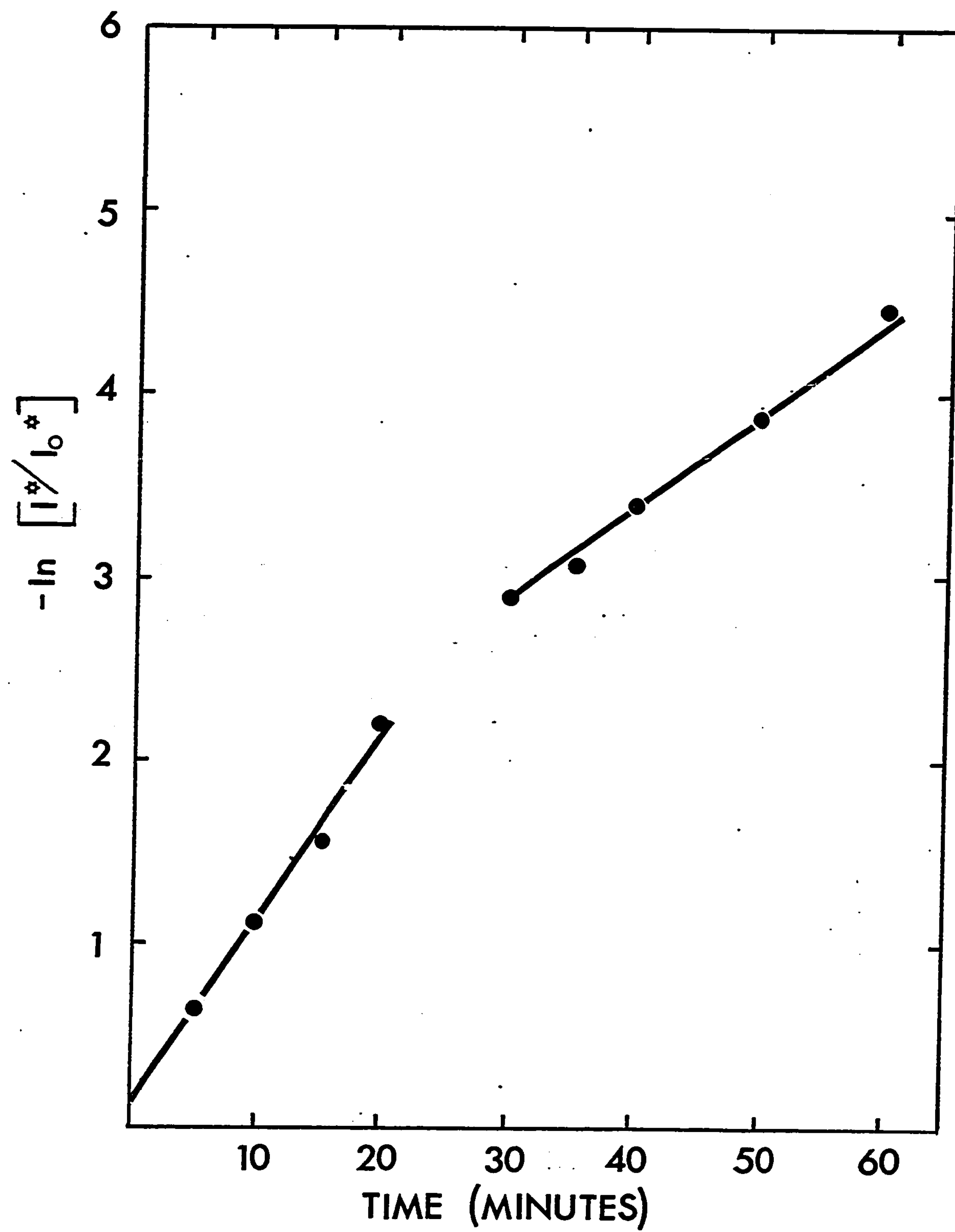


-ln $[I^*/I_o^*]$
6
5
4
3
2
1
10
20
30
40
50
60
TIME (MINUTES)

Figure 6.4. The effect of Na^{+} on ^{131}I-efflux from thyroid cells.

The experiment was carried out as described in Fig. 6.3 except that a Tris-choline medium was employed for the first 20 minutes of incubation after which the cells were transferred to a Tris-Na medium.

Ordinate: Values of $-\ln [I^{*}/I_{O}^{*}]$.



6
5
4
3
2
1
$-\ln\left[I^*/I_o^*\right]$
10
20
30
40
50
60
TIME (MINUTES)

TABLE 6.1

Rate Constants for ^{131}I Efflux from Thyroid Cells

A. Condition	B. Initial Efflux rate (minute^{-1})	C. Final Efflux rate (minute^{-1})	D. % Change from B to C
1. Change from Tris-Na to Tris-choline medium	0.066 ± 0.003	0.110 ± 0.004	+75
2. Change from Tris-choline to Tris-Na medium	0.103 ± 0.004	0.057 ± 0.003	-45
3. Addition of 0.1 mM NaI (Tris-Na medium)	0.064 ± 0.002	0.099 ± 0.044	+55
4. Addition of 0.1 mM NaI (Tris-choline medium)	0.104 ± 0.004	0.108 ± 0.005	+ 4
5. Addition of 0.1 mM $KClO_4$ (Tris-Na medium)	0.061 ± 0.004	0.103 ± 0.005	+69
6. Addition of 0.1 mM $KClO_4$ (Tris-choline medium)	0.106 ± 0.005	0.104 ± 0.004	- 2
7. Addition of 0.1 mM KCNS (Tris-Na medium)	0.061 ± 0.002	0.102 ± 0.002	+67
8. Addition of 0.1 mM KCNS (Tris-choline medium)	0.110 ± 0.004	0.106 ± 0.005	- 4

The experiments represented by the data in the top two horizontal rows were carried out as described under Fig. 6.3 and 6.4 respectively. The other experiments were carried out as described under Fig. 6.1 except that after 20 minutes, 1 ml of the appropriate reagent was added to the medium to yield, on dilution, the concentrations shown in Column A. The method by which the initial (first 20 minutes) and final efflux rates were calculated has been described in the text. Each result shown is a mean of 5-6 determinations ± SEM.

Table 6.1 (lines 1 and 2) summarizes the values of efflux rate constants obtained in replicate experiments of this type. The results clearly demonstrated that the presence of Na^+ in the external medium was inhibitory to iodide efflux from thyroid cells, and that the efflux rate in a Na^+-free medium was about 70% higher than in a Tris-Na medium (135 mM Na).

3. Effect of I^-, ClO_4 and CNS^- on Iodide Efflux from Thyroid Cells

To determine the effect of I^-, ClO_4^- or CNS^- on the rate of ^{131}I efflux from ^{131}I-containing thyroid cells, experiments similar to those described were performed with the test reagent being added to the external medium (Tris-Na) after an initial incubation period of 20 minutes. The final concentration of each test reagent was 1.0×10^{-4} M. The results of this experiment were of the type shown in Fig. 6.3. The addition of iodide to the incubation medium resulted in a sharp increase of the rate of ^{131}I efflux into the medium. Similar results were found consistently for each of the test reagents. Table 6.1 (lines 3,5,7) summarizes the values of the efflux rate constants obtained in such experiments. The results indicated that the addition of I^-, ClO_4^- or CNS^- to a Tris-Na medium promptly increased the rate of iodide efflux from the cells. Furthermore, the final rate constants calculated for the efflux of iodide occurring in the presence of the test reagents were similar in magnitude to those observed for cells incubated in Na^+-free media. However, as shown in Table 6.1 (lines 4,6,8), addition of these reagents (I^-, ClO_4^-, CNS^-) to the incubation medium produced no change in the values of the rate constants in efflux experiments in which the cells were incubated in a Na^+-free medium.

4. Effect of Cardiac Glycosides and Metabolic Inhibitors on Iodide Efflux from Thyroid Cells

Figure 6.5 (curve A) shows a plot of the results obtained during an experiment similar to those described in the previous section in which ouabain was added to a Tris-Na efflux medium to a final concentration of 5×10^{-5} M after an initial incubation period of 15 minutes. The rate of ^{131}I efflux from thyroid cells was found to increase following the addition of ouabain to the incubation medium. The increase began approximately 20 minutes after addition of ouabain, not immediately as was typical for the reagents described in the previous section. In six replicate experiments the lag period from the time of addition of ouabain to the appearance of an increased efflux rate was between 20 and 25 minutes. Similar efflux rate plots were obtained from the results of experiments in which the external medium (Tris-Na) was supplemented with 1.0×10^{-4} M NaI from the beginning of incubation (Fig. 6.5, curve B).

In contrast, when the efflux experiment was carried out using a Tris-choline medium, the addition of ouabain to the medium produced no change in the rate of iodide efflux from the cells, and the efflux plot was linear over the entire period of the experiment.

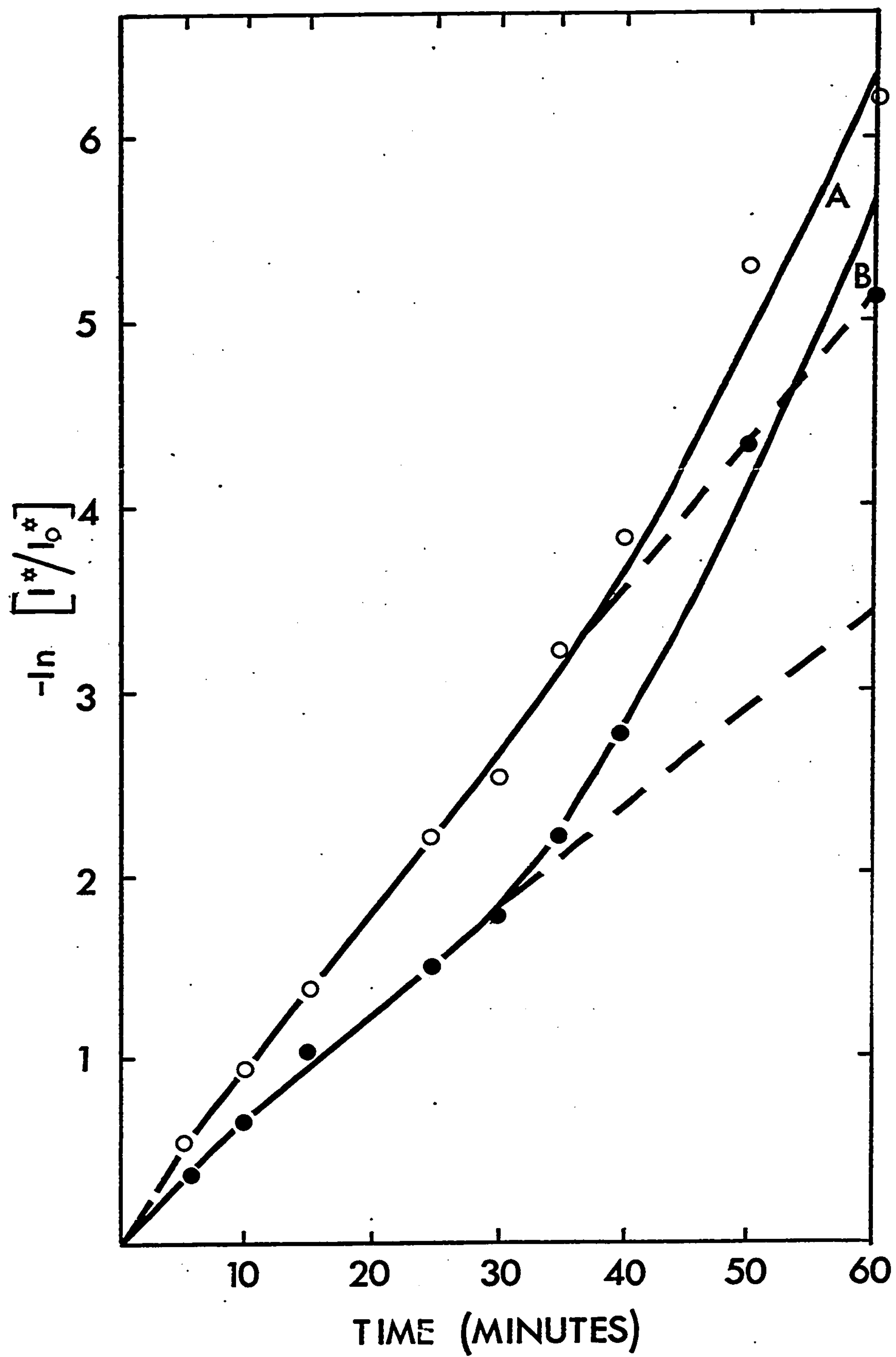
The effect on iodide efflux of the addition of NaCN or 2,4-dinitrophenol to the incubation medium was determined in experiments of the same design as those described above. The results from these studies were similar to those obtained with ouabain, both for the Tris-Na and Tris-choline media.

These results indicated that ouabain, CN^- and 2,4-dinitrophenol all were effective in producing an increase in the efflux rate of iodide from thyroid cells but only after a lag period of 20-25 minutes.

Figure 6.5. The effect of ouabain on ^{131}I-efflux from thyroid cells.

The measurement of ^{131}I-efflux from thyroid cells was carried out as described under Fig. 6.1 except that after 15 minutes 1 ml of ouabain was added to the medium to yield, on dilution, a concentration of 5×10^{-5} M. The media employed were Tris-Na with (Curve A) or without (Curve B) the addition of 1.0×10^{-4} M NaI.

Ordinate: Values of $-\ln [I^{*}/I_{O}^{*}]$.



6
5
4
3
2
1
-ln [I*/I₀*]
A
B
10
20
30
40
50
60
TIME (MINUTES)

Furthermore, their stimulatory effects on efflux were observed only if the medium contained Na^+.

D. Discussion

The kinetics of the process by which iodide exits from the thyroid gland have been studied by several investigators both *in vivo* (Wollman and Reed, 1959; Granner *et al*, 1962; Wollman, 1962a; Halmi *et al*, 1963; Granner *et al*, 1963) and *in vitro* (Iff and Willbrandt, 1963; Scranton and Halmi, 1965). Because these studies have shown that the exit process obeyed first-order kinetics, the mechanism by which iodide leaves the thyroid usually has been postulated as a simple diffusion process (Scranton and Halmi, 1965). Wollman and Reed (1959) in studies involving rats and mice found that I^- and SCN^- increased the rate constant of the exit process. The same result was also achieved by the prior treatment of the animals with TSH. Scranton and Halmi (1965) found that I^- and ClO_4^- increased the exit rate of iodide from rat thyroid lobes *in vitro*, although they interpreted the effect as being secondary to the decrease in iodide influx caused by these agents. The same authors reported that ouabain and 2,4-dinitrophenol also increased the rate of exit of iodide under similar experimental conditions. In an earlier study, Iff and Willbrandt (1963) reported the same effect for ouabain and, in addition, made the observation that the rate of iodide exit from thyroid tissue was increased when the tissue was incubated in Na^+-deficient media.

The results obtained in the present study support the findings of many of these earlier investigations. Thus it was confirmed that the rate of thyroidal iodide efflux followed first-order kinetics. The stimulation of iodide exit rate by I^-, ClO_4^-, CNS^-, ouabain, NaCN

and 2,4-dinitrophenol was also verified. However, these studies, for the first time, have demonstrated the central role played by Na^+ in the exit process. Iodide efflux from thyroid cells was found to be inhibited in the presence of Na^+ in the incubation medium. Addition of I^-, ClO_4^- or CNS^- to the medium promptly relieved the inhibition and increased the rate constant for the efflux process to the same value observed when the cells were incubated in Na^+-deficient media. The addition of ouabain or the metabolic inhibitors to Na^+-containing media also increased the rate of iodide efflux from thyroid cells but only after a lag period; it was probable that they acted by a mechanism different from I^-, ClO_4^- or CNS^-. It was of special interest that all these reagents required the presence of Na^+ in the medium in order for them to be effective stimulants of iodide efflux.

The above results cast some doubt on the generally accepted hypothesis that thyroidal iodide exit occurs by a process of thermal diffusion only. The stimulatory action on iodide efflux of agents that have a specific effect on iodide transport such as I^-, ClO_4^- and CNS^- when considered along with the observation that Na^+ is required for such effects, is difficult to explain by the diffusion hypothesis which makes the assumption that changes in the rate of efflux of iodide from thyroid cells are accompanied through appropriate structural changes in the cell membrane. The finding that the time course of the stimulatory effect of the cardiac glycosides and the metabolic inhibitors differed experimentally from that of the active anions as well as the observed Na^+-requirement of the former inhibitors again suggested that the mechanism of thyroidal iodide efflux was more complex than could be explained by a simple diffusion process. The present data are much

more compatible with a symmetrical carrier-based model of thyroidal iodide transport in which iodide efflux would occur by a reversal of the influx process. The observed first-order kinetics of the efflux process also would be compatible with this hypothesis if the assumption is made that the K_m of the efflux process is high in comparison to the intracellular concentration of iodide attained under the conditions of the experiments. The K_m of the efflux process was not determined directly in the present studies. However, if iodide efflux is assumed to occur in sequences similar to those of iodide influx (as would be postulated by a symmetrical carrier-based model) and further, if the efflux process is influenced by Na^+ and K^+ in a similar manner to that of influx, such a high K_m for efflux would be likely. The low intracellular concentration of Na^+ may, as in the case of iodide influx (Chapter V), result in an increase of K_m. Similarly, the inhibitory action of K^+, as seen for iodide influx (Chapter V), may also contribute to such an increase of K_m for the efflux reaction.

The design of the present experiments also precluded some of the explanations that have been advanced by others to explain the action of various reagents found to stimulate iodide exit from thyroid cells. For example, Wollman and Reed (1959) proposed that SCN^-, by blocking the passage of iodide from the cell to the colloid space, raised the concentration of intracellular I^- and hence secondarily increased the diffusion of iodide into the extracellular space. Such an explanation for the action of SCN^- clearly would be untenable in the present system in which the thyroid cells had no follicular arrangement. Similarly the hypothesis (Scranton and Halmi, 1965) that ClO_4^- and I^- depress the re-entry of tracer iodide into thyroid cells *in vitro*, thus artefactually increasing the measured tracer efflux, also would be

unlikely to account for the experimental results obtained in the present system where recycling of iodide has been shown to be negligible.

The results described in this section have demonstrated the trans-concentration effects of Na^+ on thyroidal iodide efflux and have provided further evidence of the importance of Na^+ to the whole process of thyroidal iodide transport. Such trans-effects of Na^+ on solute efflux also have been observed in at least one other transport system (Vidaver and Shepherd, 1968). The finding of such effects during the investigation of solute transport is significant, not only because it provides a ready explanation for the observed results on the basis of a mobile carrier hypothesis, but also because it limits the number of possible carrier models of transport that may be considered. The observed effects of Na^+ on iodide efflux, when considered with the observations made earlier on the effect of Na^+ on iodide influx, have permitted the formulation of a unique carrier model for thyroidal iodide transport which will be discussed in the next chapter. The mechanism by which the various inhibitors of iodide transport stimulate iodide efflux in the presence of Na^+ will be discussed in the light of this model.

CHAPTER VII

A KINETIC MODEL FOR THYROIDAL IODIDE TRANSPORT

A number of different models for thyroidal iodide transport have been proposed in an attempt to organize the available data on the subject (Wollman, 1962; Wollman and Reed, 1959, 1962). Most of the models have been based on the assumption that a carrier was involved in the transport process. Since many of the data had been obtained in experiments involving the intact thyroid gland, these models included the concept of various iodide compartments in the gland. Thus two-compartment (blood, thyroid cell) and three-compartment (blood, thyroid cell, colloid space) models have been proposed. The present investigation has utilized a different experimental approach to the study of thyroidal iodide transport, that of a system of thyroid cells in monolayer culture. The data obtained with this system have provided new information regarding the sodium dependence of the transport process. Therefore, a modified model for iodide transport was required which would include sodium. The question of iodide compartmentalization was clearly not relevant to the present system due to its organizational simplicity; this reduced the complexity of the model considerably.

Before a new transport model could be formulated, the experimental data, which had to be explained satisfactorily by the model, were reviewed.

Iodide Influx

1. Influx of iodide into the thyroid cells had a specific and absolute requirement for Na^+ (pp. 69,73).

2. The influx of iodide that occurred in the presence of a constant iodide concentration of the medium was directly proportional

to the first power of Na^+-concentration of the medium (p. 73).

3. When the concentration of Na^+ in the external medium was constant, iodide influx followed Michaelis-Menten kinetics as a function of iodide concentration (p. 55).

4. Na^+-depletion of the external medium resulted in an increase in the apparent K_m of the influx process but in no change in the maximum velocity of the process (p. 75).

Iodide Efflux

1. The rate of efflux of iodide from thyroid cells was highest in Na^+-free external media. Efflux was inhibited by the addition of Na^+ to the media (p. 90).

2. Iodide, perchlorate and thiocyanate ions promptly and completely abolished the inhibitory effects of Na^+ on iodide efflux. Ouabain, NaCN and 2,4-dinitrophenol also completely reversed the inhibitory effect of Na^+ but acted only after a lag period (pp. 94-95).

3. None of these agents, which reversed Na^+ inhibition, further increased the efflux rate that occurred in a Na^+-free medium (pp. 94-95).

In addition the model also must satisfy the following general criteria:

1. It must be on a molecular basis and include all the molecular species concerned in the transport.

2. It must account for the binding of substrate to carrier and be compatible with the observed specificity of binding. Particular note must be taken of the fact that the substrate, during transport, is not modified by participation in covalent linkages, i.e., the interaction between the transport system and the permeants (Na^+, I^-) must involve only hydrogen, hydrophobic and electrostatic bonds.

3. The model must be consistent with the structure of the cell membrane and be physicochemically plausible, i.e., the movements and binding abilities of the different species of molecules must be within the known capabilities of such species.

4. The model must be energetically possible, i.e., it should not violate the concepts of thermodynamics.

Some transport models described in the past have proposed that either the cell interior (Ling, 1962) or a gel-like layer beneath the membrane (Miller, 1960) was responsible for specific transport phenomena. Such models have not been considered in the present discussion. Instead, in accordance with the more prevalent view, the cell membrane has been taken as the diffusion barrier. This concept has been supported by the observation that the membrane alone (Hoffman, 1962), when properly fortified with the required energy sources, can transport many substances, and so must be the site of the transport process. Finally, the models to be considered in the subsequent discussion have all been based on the mobile-carrier hypothesis which generally has proved to be a more successful approach to explaining coupled transport systems than the various pore models (pp. 3-4).

A. Models of Iodide Transport

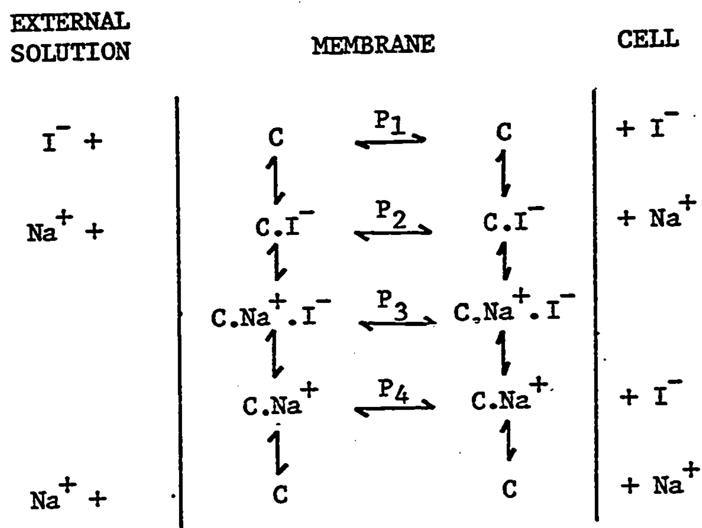
A general model, which is based on the mobile-carrier hypothesis, is shown in Fig. 7.1 for the sodium-coupled thyroidal transport of iodide. The model does not restrict the order in which the binding of the species occurs, nor does it limit the forms that can cross the cell membrane. Modified versions of this model, each with certain restrictions imposed, are shown in Fig. 7.1 A, B, C and D. However, all variations of the model assume that the translocation steps (and not the association-

dissociation reactions of the various complexes) are rate-limiting. Such an assumption would seem to be reasonable because translocation is the step in which the permeant crosses the lipid barrier.

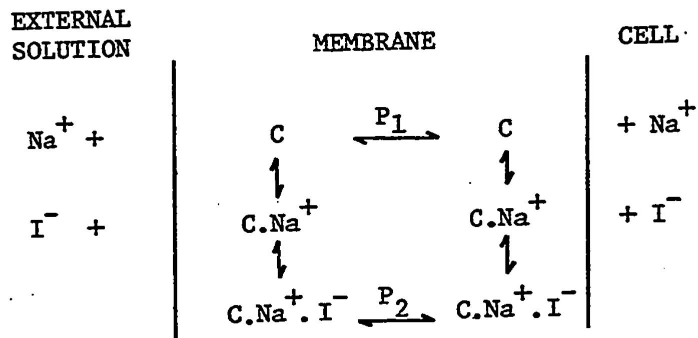
When the qualitative aspects alone of the various models A-D are considered, it is clear that only model A is consistent with all the experimental data summarized earlier in this section. For example, Model A would explain the specific and absolute Na^+ requirement of the iodide influx process, since in this model the absence of Na^+ would prevent the formation of the $C.Na^+$ complex which is the only form of the carrier capable of combining with iodide ion. Model D would fail to account for these same observations, as it permits formation of a $C.I^-$ complex which could form in total absence of Na^+. Translocation of this Na^+-free species would result in movement of iodide into the cell. Model A also would explain why the rate of influx of iodide into thyroid cells varies with the first power of the Na^+ concentration of the medium, since only one ion of sodium is postulated to bind to each molecule of the carrier. The observations that were made on the kinetics of iodide influx, and the change of these kinetic parameters that occurred with the Na^+ depletion of the medium also can be derived logically from this model, as will be discussed later (p. 111). The data from experiments in which the efflux of iodide from thyroid cells was investigated also are consistent with Model A. Thus the inhibitory effect of external Na^+ on the efflux of iodide would be explained in this model by the formation, under such conditions, of a $C.Na^+$ complex which would be unable to cross the cell membrane. Further in the absence of I^- (or its competitive inhibitors, ClO_4^- or CNS^-), the $C.Na^+$ complex would be unable to form a ternary complex $C.Na^+.I^-$. Thus a proportion

Figure 7.1. A general kinetic model for thyroidal iodide transport and four variant forms of the model (A to D).

The carrier molecule, C, can combine with extracellular Na^+ or I^- to form the binary complexes $C.Na^+$ or $C.I^-$. The $C.Na^+$ and $C.I^-$ complexes can, in turn, combine with I^- and Na^+ respectively from the external solution to form the ternary complex $C.Na^+.I^-$. The free and complexed carriers are capable of translocation across the membrane at rates P_1, P_2, etc in a rate limiting step. Dissociation of the complexed carriers, which can take place at either surface of the membrane, is assumed not to be rate limiting. Perchlorate and thiocyanate ions can substitute for I^- in the above model.

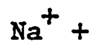


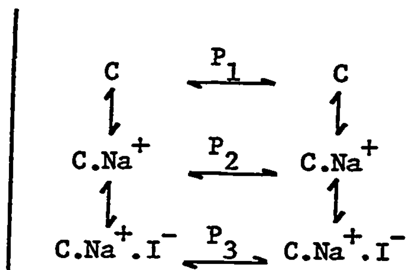
GENERAL MODEL

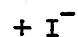


A

EXTERNAL SOLUTION | MEMBRANE | CELL

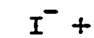


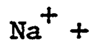


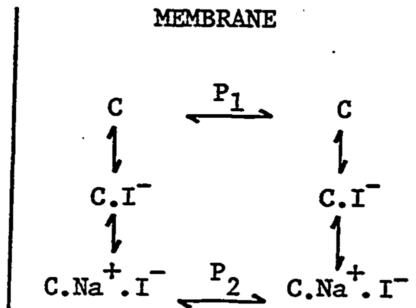


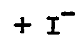
B

EXTERNAL SOLUTION | MEMBRANE | CELL



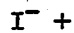


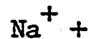


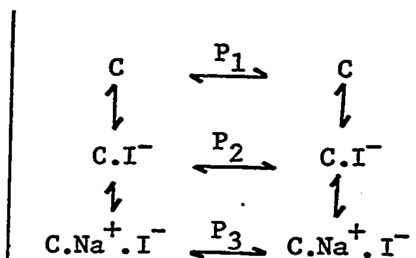


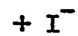
C

EXTERNAL SOLUTION | MEMBRANE | CELL









D

of the available carrier would be immobilized in a functionally inactive form. This would be equivalent to a reduction in the total amount of carrier available to the system and would thus explain the inhibitory effect of Na^+ depletion. The prompt relief of this inhibition that occurred by the addition of I^-, ClO_4^-, or CNS^- to the medium again would be consistent with Model A. In the presence of these anions, the binary complex could combine with the anion to form a ternary complex capable of translocation which would thus allow all of the carrier to become available once more to the system. None of the other models (B, C, D) can explain these observations on the effect of external Na^+ on iodide efflux. Moreover, based on similar lines of reasoning, the observation that iodide ion does not modify the rate of iodide efflux in a Na^+-free medium is incompatible with model C which would postulate such an effect due to the formation of a $C.I^-$ complex incapable of translocation.

The rate of iodide influx into the thyroid cell would be proportional to the concentration of extracellular Na^+ according to the mechanism postulated by the proposed model (model A). Similarly, the rate of efflux would be proportional to the intracellular Na^+ concentration which normally is much lower than the extracellular. The influx rate would thus exceed the efflux rate at any given iodide concentration and consequently there would be a net accumulation of iodide by the thyroid cell. Maintenance of a low intracellular concentration of Na^+, essential for the active transport of iodide by these cells, would be made possible by the function of the Na^+-K^+-dependent ouabain-sensitive ATPase enzyme. This enzyme is thus postulated to play a central role in the active transport of iodide ion by the thyroid cells. The role is, however, indirect in that no

direct link of the enzyme with the molecular process of iodide transport is proposed.

The observed action of ouabain and the metabolic inhibitors on thyroidal iodide transport can now be satisfactorily explained by the model. These agents would abolish active Na^+ extrusion from the cell, either by a direct inhibitory action on Na^+-K^+-dependent ATPase, as in the case of ouabain, or by cutting off the supply of ATP to the same enzyme, in the case of the metabolic inhibitors. The rate of influx of iodide into the cells should not be affected by such an effect since no direct link between enzyme activity and the transport process is postulated. This is in accord with the observations reported earlier (Table 4.2). In the presence of these inhibitors Na^+, from the external medium, would continue to enter the cell by passive diffusion. The concentration of intracellular Na^+ would thus gradually increase until it approached that of Na^+ in the external medium. Such a rise in the intracellular Na^+ concentration, according to the model would lead to an increase in the formation of a C.Na^+ complex intracellularly and consequently to a rise in the rate of iodide efflux. This is in accord with the experimental observations (p. 95). Further, when the Na^+ concentrations on both sides of the membrane eventually become equal, the rate of efflux would become equal to that of influx and net transport of iodide would cease, again consistent with the experimental findings (p. 45). Since the increase in intracellular Na^+ concentration produced by these inhibitors would be slow (Wheeler and Christensen, 1967a), the model would predict a lag period before efflux would increase, a lag which in fact was found in such efflux experiments. The ineffectiveness of these agents in stimulating efflux in Na^+-free media is also readily explained since

no increase in intracellular Na^+ concentration is possible under such conditions.

The action of the known competitive inhibitors of iodide transport, e.g., ClO_4^- and CNS^-, may be derived directly from the model since they would compete with iodide ion for combination with the binary complex. It must be assumed for several reasons that the resulting ternary complexes formed with the inhibitors ($C.Na^+.ClO_4^-$ or $C.Na^+.CNS^-$) are capable of translocation. In the first place, ClO_4^- ion is known to be concentrated by thyroid tissue (Anbar *et al*, 1959). Secondly, although CNS^- does not accumulate in thyroid tissue probably because the transported CNS^- is rapidly metabolized (Maloof and Soodak, 1964) it is concentrated by salivary glands and other iodide-concentrating tissues. Finally, if the proposed ternary complexes were incapable of translocation as was $C.Na^+$, the efflux of iodide observed in the presence of ClO_4^- or CNS^- would have been slowed progressively and finally halted as all the available carrier became converted to these forms.

The model does not make any assumptions about the relative rates of translocation of the free carrier and the ternary complex. However, these must be nearly equal because the rates of iodide efflux that occurred in a Na^+-free medium and in a Na^+-containing medium in the presence of I^- (or ClO_4^- or CNS^-) were approximately the same. An examination of the model shows that in the former situation, the return of the carrier from the outside of the membrane to the inside would be accomplished by translocation of the free carrier. In the latter situation, however, the return of the carrier to the inside of the cell membrane would take place largely through translocation of

the ternary complex. Since the efflux rates were not found to be appreciably different in the two situations, the translocation rates of the free carrier and the ternary complex must be nearly equal.

Mention should be made at this point of several aspects of the model which could not be verified experimentally using the present monolayer culture system. First, as a result of translocation of the ternary complex, there should be entry into the cell of an ion of Na^+ for every ion of I^-. This Na^+ would be in addition to that which enters the cell by other pathways. Thus iodide influx should increase Na^+ influx into the cell; the relative increments of the two ions should be in a 1:1 molar ratio. However, the amount of iodide transport into the thyroid cell is of an order of magnitude smaller than that associated with the basal influx of Na^+ occurring in the absence of any I^- influx. Therefore, the percentage change in Na^+ influx from the basal level that would be produced by a concomitant transport of iodide into the cell would be very small (less than 1% of the total) and would be quite difficult to detect. Nevertheless, an attempt was made to demonstrate this predicted increase in thyroidal Na^+ influx by the measurement of Na^+ influx with and without the addition of 1.0×10^{-4} M iodide to the incubation medium. ^{22}Na was added to the system as a tracer to detect accumulation of Na^+ by the thyroid cells. As anticipated, no significant difference in Na^+ accumulation was observed between the control and the iodide-supplemented cells. The demonstration of such an effect must await a different and much more sensitive experimental approach to the subject.

The second deduction from the model that could not be demonstrated experimentally was the stimulation of iodide influx that would be expected to occur following a preloading of the cells with

iodide. In the absence of intracellular iodide, a certain proportion of the carrier on the inner face of the membrane would form the C.Na^+ complex through a combination with intracellular Na^+. Since the complex is not capable of translocation, a proportion of the carrier would become unavailable to the system. However, in cells preloaded with iodide the binary complex should go on to form the ternary complex, which can cross the membrane, and permit all of the carrier to become available for the influx process. Hence, on the basis of this model, influx in cells that had been preloaded with iodide should be higher than that occurring in untreated cells. However, as the results of Table 4.2 (Chapter IV) show, no difference was found experimentally between the two groups. Since in animal cells the intracellular Na^+ concentration can be assumed to be low (about 15-20 mM) and because the dissociation constant of the binary complex is relatively high (*vide infra*), the absolute amount of the binary complex formed intracellularly in the absence of iodide preloading must be quite small. This perhaps explains the failure to detect any significant lowering of the rate of iodide influx in cells that had not been preloaded with iodide as compared to those that had.

B. The Kinetics of the Iodide Transport Model

A simple kinetic analysis is possible for carrier-based models of co-transport (Stein, 1967, p. 192). For the purpose of a discussion of the kinetics of iodide transport, the model shown in Fig. 7.2 has been considered. This diagram is the same as that for model A except that the rate constants for the various chemical reactions have been added.

For the reactions occurring at the external surface of the

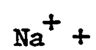
Figure 7.2.

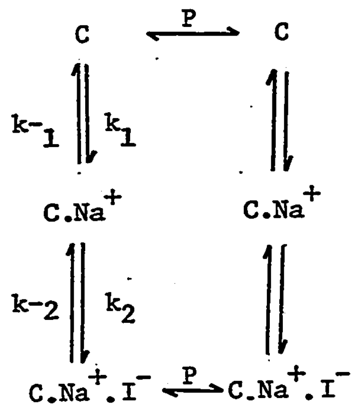
A kinetic model for thyroidal iodide transport based on an interaction of Na^+ and I^- with carrier C (during transport).

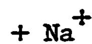
k_1, k_2 etc. = rate constants

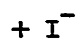
P = rate constant for translocation

EXTERNAL SOLUTION | MEMBRANE | CELL

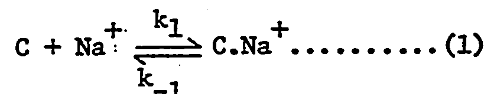




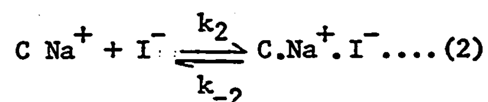




cell membrane the following equilibria may be written:



$$C + Na^{+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C.Na^{+} \ldots\ldots\ldots\ldots (1)$$



$$C\ Na^{+} + I^{-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C.Na^{+}.I^{-} \ldots\ldots (2)$$

If K_1 and K_2 are the dissociation constants of the binary and ternary complexes respectively, then $K_1 = \frac{k_{-1}}{k_1}$ and $K_2 = \frac{k_{-2}}{k_2}$

Application of the conservation equation to these equilibria yields the result:

$$\text{Tot } C = C + C.Na^{+} + C.Na^{+}.I^{-} \ldots\ldots (3)$$

where Tot C = total amount of carrier in the membrane.

Finally, if P is the rate constant for the translocation reaction (for $C.Na^{+}.\ I^{-}$), the rate of unidirectional inward flux will be $P \times [C.Na^{+}.\ I^{-}]$

From equilibria (1) and (2)

$$K_1 = \frac{[C]\ [Na^{+}]}{[C.Na^{+}]} \ldots\ldots\ldots\ldots\ldots (4)$$

$$K_2 = \frac{[C.Na^{+}]\ [I^{-}]}{[C.Na^{+}.I^{-}]} \ldots\ldots\ldots\ldots (5)$$

Substituting the expressions C and $C.Na^{+}$ derived from equations (4) and (5) in equation (3), and rearranging,

$$[C.Na^{+}.I^{-}] = \frac{[\text{Tot } C]\ [I^{-}]}{\frac{K_1 + [Na^{+}]}{[Na^{+}]} K_2 + [I^{-}]}$$

Rate of influx is then given by:

$$\frac{P \times [\text{Tot } C]\ [I^{-}]}{\frac{K_1 + [Na^{+}]}{[Na^{+}]} K_2 + [I^{-}]}$$

This expression predicts a kinetic behaviour of the Michaelis-Menten type for the iodide influx reaction with respect to variation of I^- concentration with

$$V_{max} \text{ (maximum velocity)} = P \times \text{Tot C} \quad \text{.......(6)}$$

$$\text{and } K_m = \frac{K_1 + [Na^+]}{[Na^+]} \quad \text{.......................(7)}$$

where K_m is defined as the substrate concentration at which one-half of the maximum velocity is reached.

The prediction of a saturation kinetics for the iodide influx process with respect to iodide concentration has been verified experimentally (Chapter IV). Equation (6) predicts that the maximum velocity should be independent of the Na^+ concentration of the external medium but should depend on both the total amount of carrier present in the system and the rate of translocation of the ternary complex. This is in accord with the experimental results obtained in Chapter V.

Equation (7) predicts that the apparent K_m for iodide influx should increase as the Na^+ concentration of the medium is decreased. This again is in agreement with the experimental results (Table 5.3).

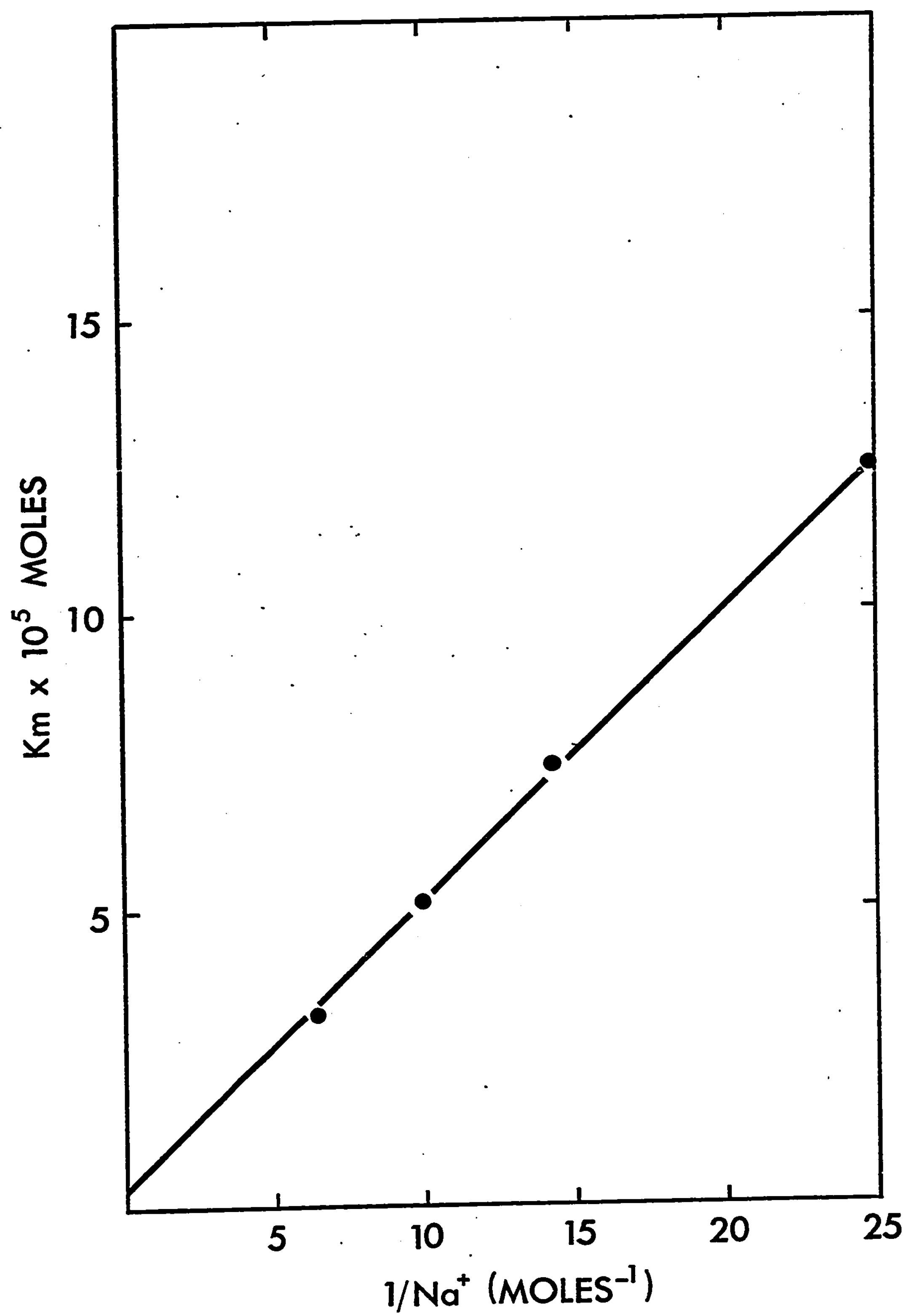
Equation (7) can be rewritten as

$$K_m = \frac{K_1 \cdot K_2}{[Na^+]} + K_2 \quad \text{.........................(8)}$$

Thus a plot of K_m vs $1/[Na^+]$ should yield a straight line. Figure 7.3 shows that the plot of the experimental data was reasonably linear. By equation (8) this plot should have a slope equal to K_1K_2 and an intercept equal to K_2. From Fig. 7.3, K_1 was found to be 1.63 moles per liter and K_2 to be 0.3×10^{-5} moles per liter. This high value of K_1 would explain why the plot of iodide influx against extracellular Na^+ concentration for a constant iodide concentration did not show evidence

Figure 7.3. The variation of Km for iodide influx into thyroid cells as a function of the concentration of Na^+ in the incubation medium.

The mean values of Km, obtained from Tables 4.1 and 5.3, are plotted on the ordinate. The reciprocals of the corresponding Na^+ concentration (in moles) of the external media are plotted on the abscissa.



Km x 10^5 MOLES
15
10
5
5
10
15
20
25
1/Na+ (MOLES-1)

of saturation over a range of Na^+ concentrations 0 - 151.5 mM. (Fig. 5.2). Again it also is apparent from the values calculated for K_1 and K_2 that Na^+-binding would result in an intermediate which could avidly bind I^-, the reaction being virtually complete even at a very low concentration of iodide. Such a system would be eminently suitable from a functional standpoint for the cellular concentration of an ion such as iodide which is distributed in nature only in trace amounts.

The effect of TSH on the kinetics of iodide transport now can be considered in the light of this kinetic model. It was observed before (Chapter IV) that the rate of iodide influx into TSH-grown cells was characterized by a higher value for V_{max} than that of control thyroid cells, but the rates observed in both systems were found to have similar K_m values. From equation (6) it is seen that an increase in V_{max} could be achieved either by an increase in the total enzyme concentration of the system or by an increase in the rate of translocation of the complex across the membrane (without any change in the enzyme content), or or perhaps by a combination of the two. Each of these possible mechanisms could be considered in relation to the mode of action of TSH. However, Knopp *et al* (1970) have shown that the acute stimulatory action of TSH on iodide influx in freshly isolated dispersed thyroid cells is abolished by actinomycin D or cycloheximide. This observation would suggest that TSH probably acts by increasing the total amount of the carrier or transport molecule in the cell membrane. Additional experimental work would be required to confirm this.

C. Further Consideration

The model shown in Fig. 7.2 depicts active iodide transport as a Na^+-coupled transport system. Contrary to the current hypothesis,

this model does not consider iodide efflux as passive diffusion, but regards it instead as a carrier-mediated process. The transport system is postulated to be bidirectional so that, if the asymmetry of Na^+ distribution across the cell membrane became abolished in some way, it would then behave as a facilitated diffusion system. In the present model, as in other systems which have been explained by a sodium gradient hypothesis, Na^+ coupling has the effect of linking iodide transport to the active Na^+ transport system. The result is net transport of iodide against an electrochemical gradient. Some (Kedem, 1961) have suggested that the term, active transport, should be reserved to describe flows which are directly coupled to chemical reactions. If this concept were followed, then all Na^+-coupled transport phenomena would be removed from the category of active transport; in that case some reclassification (e.g., "secondary active transport") would become necessary.

According to the present model, the Na^+ gradient that is maintained between the inside and outside of the cell contributes to the energy requirements of the coupled thyroidal iodide transport system. However, on the basis of the present experimental data it is not known whether the Na^+ gradient alone is sufficient to account for all the energy requirements of the process. Other asymmetries across the cell membrane also may contribute to this energy requirement. In particular, the well-known K^+ asymmetry across cell membranes should merit consideration in this respect. Thus in view of the inhibitory action of K^+ on many Na^+-stimulated processes, a high intracellular K^+ concentration may act synergistically with a low intracellular Na^+ concentration in promoting accumulation of substrate (Kipnis, 1965). Another form of cellular asymmetry, whose role in Na^+-dependent processes

is poorly understood, is the electrical potential difference across the membrane. This potential could affect the rate of translocation of charged complexes across the membrane, especially if such processes were diffusional. Further, by altering the concentration profile of Na^+ within the thickness of the membrane, the electrical potential could affect the interaction between Na^+ and the transport process.

The binding of Na^+ and I^- proposed by the model under discussion is consistent with a carrier which possesses two separate sites for binding to these ions. It also follows from the model that when Na^+ combines with the carrier, the resulting complex has the ability to bind I^-, a property not possessed by the free carrier. This raises the possibility that the binding of Na^+ to carrier brings about conformational changes in the carrier molecule which changes its iodide-binding properties, a phenomenon reminiscent of "allosteric" changes in an enzyme molecule (Monod *et al*, 1965). The Na^+-binding also could involve coulombic interaction of Na^+ with an anionic group in the carrier molecule, which, by neutralizing the negative group, would make I^--binding at a nearby site possible. Such coulombic interactions have been postulated to explain the transport of cationic amino acids. In these systems, which show a relative lack of Na^+-dependence, a relatively stable binary complex is believed to form with the positively charged group on the amino acid serving the role postulated above for Na^+. On the other hand, the transport of anionic amino acids which displays considerable Na^+-dependence is thought to involve formation of an unstable binary complex with a relatively high dissociation constant (Schultz *et al*, 1970).

The ultimate goal of the investigation of any membrane transport system must be to elucidate the molecular mechanisms underlying

the process. Kinetic approaches have great usefulness but they are essentially speculative in nature. However, by serving to organize the available knowledge in a meaningful way, they stimulate further investigation at a molecular level. The aim of such research would be to identify and characterize the components of the transport system in order to achieve an understanding of their binding properties and mobilities. In the case of the thyroidal transport system the knowledge of the specific substrate binding properties already has been utilized to isolate certain phospholipids that bind iodide ion preferentially (Vilkki, 1962; Schneider and Wolff, 1965). Utilization of the Na^+ binding abilities of such transport molecules, in addition to their I^- binding properties, should prove beneficial when new attempts to isolate such "carrier" components are made. Again, even before complete purification of the transport species is achieved, an investigation of the chemical components of the "active centers" of these molecules might be possible by means of a detailed study of the inhibitory action of chemical reagents possessing defined specificities. This type of approach has been frequently successful for defining the nature of active centers of enzymes. For example, studies on the inactivation of ribonuclease by dinitrofluorobenzene have identified a specific lysine residue near the active center of the enzyme (Hirs, 1962). To date no such studies have been attempted on the iodide transport system.

Finally, it must be emphasized that no single transport model that is proposed can ever be considered final. It exists only as long as it remains compatible with the available data. When new experimental observations are made which can no longer be accommodated within the existing model, the latter must be discarded in favour of a new one. A successful model serves a useful function in that it provides

a clear conceptional framework on which future research is based.

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