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The electrogenic, Na^+-dependent I^- transport system in plasma membrane vesicles from thyroid glands *

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Using vesicles from the plasma membrane of hog thyroid, we have characterized its Na^+-dependent I^- transport system. We have found it to be totally Na^+ dependent; K^+ cannot substitute and Li^+ can partially substitute for Na^+; the $Na^+ : I^-$ flux ratio is larger than one; the system is electrogenic, being stimulated by a $\Delta\psi$ negative inside the vesicles. A number of large, lipophilic anions are fully-competitive inhibitors of Na^+-dependent I^- uptake; the closer their atomic radii are to that of iodine, the smaller their K_i values.

Introduction

I^- transport across biological membranes in mammalian tissues is Na^+ dependent in the thyroid gland [1] and in the choroid plexus [2]. Particularly the former transport system has been subjected to a great number of investigations and as early as 1963 Iff and Wilbrandt [1], working with intact thyroid gland, demonstrated, in addition to its total dependency from extracellular Na^+, that it is stimulated by low concentrations of K^+_{out} (< 5 mM) and is inhibited by preincubation in K^+-free or ouabain-containing buffers. Thus, I^- transport across the plasma membrane of the thyroid gland was suggested to be driven by an inwardly directed Na^+ gradient, which in turn is brought about by the ouabain-sensitive K^+_{out}-activated Na^+/K^+ pump. This conclusion was reinforced in subsequent years by work on cultured thyroid cells or intact thyroidal tissue (see, for example, Refs. 3–7).

To the best of our knowledge relatively little work has been done using membrane vesicles – in spite of the obvious advantage which such a simplified non 'living' biological preparation would have in singling out the transport system from the rest of the cell and allowing great freedom in the composition of the media at both sides of the membranes (reviewed, for example, in Refs. 8–10). One paper using plasma membrane vesicles [11] has indicated that a K^+ out-in gradient can substitute for a Na^+ gradient – which, however, has not been confirmed by any other group (and is ruled out by the data to be presented below).

Most vesicles used in this field are the reconstituted proteoliposomes of the group of Saito [12–16]. While this system is undoubtedly of value,

* This paper is dedicated to Professor J. Mauron for his contribution to the study of human nutrition and for his support of fundamental research.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MMI, 2-mercapto-1-methylimidazole.

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it does not yield itself to a kinetic characterization of the transport system as occurring in the original membrane: in addition to the well known pitfalls inherent to reconstitution, to the unknown effects, if any, of lipids in the original membrane, the possible (partial) denaturation of the transport protein etc., it has never been possible to make reliable measurements of initial transport rates, which casts doubts on the reliability of the K_m values calculated therefrom.

We have now used a preparation of plasma membrane vesicles from hog thyroid gland, which allowed us to better characterize this Na^+-dependent I^- transport system. We find that this system is completely dependent on the presence of extravesicular Na^+, the Na^+-dependence being strongly sigmoidal ($\bar{n} = 1.61$). This Na^+-dependent I^- transport is electrogenic, being stimulated by $\Delta\psi$ negative inside the vesicles. K^+ does not substitute for Na^+, whereas Li^+ can, albeit with low affinity. Large lipophilic anions, which have been known since very long to inhibit I^- uptake (for review, see Ref. 17), such as ClO_4^-, SCN^- and N_3^-, are strong inhibitors of the fully competitive type (at least ClO_4^- and SCN^-, K_i 11 and 15.3 μM, respectively.)

Some of these results have been presented in a preliminary form [37].

Materials and Methods

Preparation of plasma membrane vesicles

Plasma membrane vesicles were prepared from frozen hog thyroid glands by a method based on that of Aronson and Touster for liver plasma membranes [18] and on that of Saito et al. [12] for thyroid membranes. The procedure consists of tissue fractionation by differential centrifugation of the homogenate in 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0). All operations were carried out at 4°C.

Solutions:

A: 250 mM sucrose in 10 mM Tris-HCl (pH 8.0)
B: 34% (w/w) sucrose in 10 mM Tris-HCl (pH 8.0)
C: 57% (w/w) sucrose in 10 mM Tris-HCl (pH 8.0)
D: 300 mM D-mannitol in 10 mM Hepes-Tris (pH 7.5)

100 g thyroid glands kept frozen at −70°C up to one month, were cut in small pieces, thawed in 300 ml solution A and then homogenized in a Waring blender four times for 15 s. After filtration through a Büchner funnel, the suspension was homogenized with one up-and-down stroke of the pestle of a Potter-Elvehjem glass-Teflon homogenizer mounted in a drill press. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was poured off and saved. The pellet was washed twice with 175 ml solution A, each time pottered and centrifuged as above and the supernatant saved. The pellet was discarded. The three supernatants were combined, filtered through two layers of cheesecloth and centrifuged at $20\,000 \times g$ for 5 min. The supernatant was removed by aspiration and saved. The pellet was resuspended in 120 ml solution A, homogenized with one up-and-down stroke of the homogenizer and centrifuged as above. The pellet was discarded, the two supernatants were combined and centrifuged at $78\,000 \times g$ for 100 min. The supernatant was carefully removed by aspiration and discarded.

The pellet and the loosely packed layer on top were suspended in 120 ml solution C and homogenized with two up-and-down strokes of the pestle.

20 ml of the suspension were placed in the bottom of centrifuge tubes, overlaid with 30 ml solution B and 8 ml solution A. The gradient was centrifuged at $75\,500 \times g$ for 16 h.

After centrifugation, the tubes were placed on ice. The top layer was removed by aspiration and discarded. The following band, the plasma membrane fraction, between solution A and B, was carefully collected with a pipette, washed with two volumes of cold water to remove sucrose and centrifuged at $105\,000 \times g$ for 1 h. The supernatant was removed by aspiration and discarded. The vesicle pellet was resuspended in solution D. Aliquots at protein concentration of 15–25 mg/ml were stored in liquid nitrogen up to one month. By this method the yield of membrane protein was about 0.4 mg per g of thyroid tissue.

At freeze etching (Fig. 1) vesicle population had a fairly homogeneous size distribution, centering around 0.15–0.2 μm; the fracture surfaces showed particles, presumably of proteinaceous nature.

We did not attempt to establish how much of

Fig. 1. Freeze-etching of the thyroid plasma membrane vesicles. The bar corresponds to 100 nm.

the vesicles in our preparation were right side out, inside out, or in the form of membrane fragments. Whatever the heterogeneity in the state of our vesicles, it does not, however, lead to heterogeneity in the kinetic characteristics of I^- uptake, as deduced from quasi-initial rates: their dependence on the extravesicular I^- concentrations and their inhibitions by fully-competitive inhibitors do not deviate in the least from a Michaelian behaviour (see under Results).

Transport measurements

For transport experiments, vesicles were quickly thawed at 37°C, washed once by diluting the vesicles with six times the volume of buffer and centrifuged. The vesicles were resuspended to the desired amount of mg protein per ml in 300 mM mannitol, 10 mM Hepes-Tris (pH 7.5) and kept on ice.

All incubation and preincubation media contained (unless explicitely stated otherwise) 300 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 100 mM NaCl, 10 μM NaI and 1 mM 2-mercapto-1-methylimidazole. Further additions as well as incubation procedures different from the one described here are detailed in the legends.

Incubations were done at room temperature with an automated procedure according to Kessler et al. [19]. The standard procedure was as follows: 10 μl incubation medium containing the radioac-

tively labeled substrate (approx. 400 000 cpm) were placed onto the bottom of a clear polystyrene tube fitted into a vibration device controlled by an electric timer. 10 μl vesicles (about 100 μg protein) were placed approximately 1 mm apart from the incubation medium. At the start of the timer, the shaking of the vibrator rapidly mixed the two drops together. At the chosen incubation time, 2.5 ml of 'stop-solution' (250 mM KCl/1 mM Tris-HCl (pH 7.5), cooled to 2°C) were automatically injected into the test tube. The sample was then quickly filtered through a wet Sartorius filter (diameter 0.45 μm) and washed twice with 5 ml of the ice-cold stop-solution. The efflux of I^- during this washing step must have been minimal, because clean Michaelian kinetics were observed (see below), the uptakes were satisfactorily reproducible and the S.D. values were small.

The uptake values measured were corrected for the 'zero-time blank', i.e. for the radioactive I^- remaining associated with the filter plus membrane vesicles from samples subjected to the 'stopping, washing and filtering procedure' immediately after mixing, i.e. without incubation. This correction was always very small.

Protein

Protein was determined by the method of Lowry et al. [20] with bovine serum albumin as standard.

Materials

All reagents were of highest purity available, purchased from Fluka, Merck and Sigma. $Na^{125}I$ was supplied by EIR, Würenlingen, Switzerland. [*Me*-^{3}H]Methionine was from Amersham International, U.K.; D-[^{3}H]glucose, L-[^{3}H]glucose and L-[1-^{3}H]alanine from NEN, Research Products, Du Pont, Boston, MA. Isoflavone and saponin extracts of soybean flour were supplied from Laboratoires de Recherche Nestlé, La Tour-de-Peilz, Switzerland. The apparatus for the automated short-time incubations is produced by Innovativ-Labor AG., CH 8134 Adliswil, Switzerland.

Results

1. Characteristics of the preparation of vesicles from plasma membrane of hog thyroid glands

The procedure worked out is based essentially on that to prepare liver plasma membranes [18] (see under Methods). The thyroid plasma membrane vesicles used in the present work are far from being 'totally' pure; the preparation routinely used was a compromise between the necessity of limiting the working time to a reasonable length on the one hand, and a reasonable degree of purification on the other. As Table I shows, the markers of plasma membrane (5'-nucleotidase, $(Na^+ + K^+)$-ATPase) are enriched to approximately the same extent (ranging in the various batches from approximately 23 to 27 times, i.e., 1.5 more than in other comparable plasma membrane vesicles (see for example, Ref. 12); markers of mitochondrial (cytochrome *c* oxidase) and of lysosomes (acid phosphatase) are enriched to a lower and variable extent. Glucose-6-phosphatase activity is enriched to the same extent as the markers of plasma membrane; we did not investigate whether this indicates that this activity is

TABLE I

MARKER ENZYMES DISTRIBUTION IN THYROID HOMOGENATE AND VESICULAR FRACTION

The data in the table (expressed as 10^{-3} I.U. per mg protein; the figures in brackets are the enrichment factors referred to the original homogenate) are taken from two representative experiments. The experimental variation was within 10% of the data reported.

Fraction	5'-Nucleotidase	Glucose-6-phosphatase	$(Na^+ + K^+)$-ATPase	Cyt. *c* oxidase	Acid phosphatase
Homogenate	4.0	1.2	1.66	7.26	8.00
	3.6	1.3		4.73	10.20
Vesicles	104.0 (26×)	32.5 (27×)	37.8 (23×)	35.42 (5×)	103.0 (13×)
	98.3 (27×)	30.3 (23×)		55.60 (12×)	59.0 (6×)

associated with plasma membranes or (more probably) that the vesicle preparation used here was rich in microsomal membranes – indeed, from the separation scheme used (see Methods) also enrichment of microsomal membranes in the final preparation should be expected.

A number of transport systems other than that for I^- studied here are present in the vesicles preparation used. Under the conditions of our measurements of I^- uptake (in particular, in the presence of an initial NaCl out → in gradient), Na^+-dependent transport of both L-alanine and L-methionine was noted, producing overshoot of either substrate (data not shown). Possibly, transport systems for these aminoacids may have been associated with other membrane vesicles in this preparation, because larger amounts of amino acids were associated with the vesicles at equilibrium than either D- or L-glucose or I^-. D-Glucose uptake was neither concentrative nor Na^+ dependent. The vesicular space available to D-glucose was approx. 0.404 ± 0.014 (S.D.) $\mu l \cdot mg^{-1}$ protein; that to L-glucose, 0.355 ± 0.036 (S.D.) $\mu l \cdot mg^{-1}$ protein. In comparison the vesicular space of brush border membrane vesicles (Me^{2+} method) is approx. 1 $\mu l \cdot mg^{-1}$ (see, for example, Refs. 21, 22).

2. The 'uptake' of I^- in thyroid plasma membrane vesicles

In principle, the I^- associated with the vesicle preparation after incubation can be bound to (positive) charges of the membrane(s); be dissolved in their lipid phase; be in solution in the vesicular space; and be in a combination of these compartments. To differentiate among these possibilities, attempts were carried out to reduce the vesicular space by osmotic shrinkage using either sucrose or mannitol, but no unequivocal results were obtained. However, from the amount of I^- remaining associated with the vesicles (in the presence of 2-mercapto-1-methylimidazole) at long incubation times (e.g., 2 h) the 'space' available to this anion was calculated to be approx. 0.602 ± 0.007 (S.D.) $\mu l \cdot mg^{-1}$ protein, i.e., larger, but still similar to the vesicular space available to D- or L-glucose (see previous paragraph).

Furthermore, in the presence of an inwardly directed initial NaCl gradient, a temporary accumulation of I^- (an 'overshoot') was observed (see, for example, Fig. 2, ●). We conclude that, at least during the first minutes of incubation, the I^- found to be associated with the membrane vesicles represents transported, rather than 'bound' I^-, and that our vesicle preparation allows the investigation of the major properties of I^- transport under the conditions used in this paper. Among these conditions, the presence of 2-mercapto-1-methylimidazole (already used by Ref. 11 and shown in Refs. 6, 12 not to inhibit Na^+-dependent I^- uptake) must be particularly emphasized. In fact, in the absence of this compound, the amount of I^- which associated with the vesicles was much larger, by a factor 5–6 (data not shown), probably reflecting its oxidation to the more lipophilic I_2 which is then taken up into the lipid phase of the membranes.

The contribution of the so-called 'passive diffusion' to the uptake of I^- into our vesicles must have been negligibly small, because no deviation from Michaelian behaviour was observed (see below), and because the I^- uptake in the absence of Na^+ was minimal (see below).

We surmise that most or all the I^- transport in our vesicle preparation occurs across the plasma, rather than other, membranes, because of its characteristics similar to those previously reported for I^- transport in the whole thyroid gland (in particular, its Na^+ dependency, see below), and because the specific activity of I^- transport is increased in the vesicle preparation roughly by the same degree (and occasionally more) than the markers of plasma membranes.

3. Time course and K_m of Na^+-dependent I^- uptake

Fig. 2 (●) shows that the uptake deviates from the linearity fairly early; at 10 s (the earliest practical time) some deviation is already evident. The apparent K_m of 4.8 μM (Table II) was estimated from uptake values at 10 s. Also, the conditions were those of a dissipating initial NaCl gradient, which may not have provided the maximum $\Delta\psi$ which we have recommended elsewhere in connection with another, also rheogenic, transport system [23]. However, it proved difficult to produce optimally high $\Delta\psi$ due to a number of reasons to be mentioned in later sections. Thus,

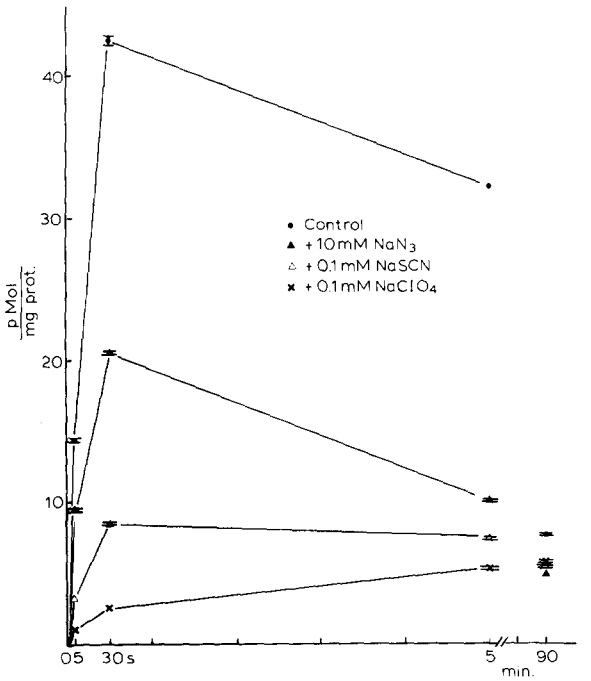


Fig. 2. Time course of I^- uptake into thyroid plasma membrane vesicles, in the absence of inhibitors (●), or in the presence of 10 mM NaN_3 (▲), or of 0.1 mM NaSCN (△), or of 0.1 mM $NaClO_4$ (×). At the beginning of the incubation the extravesicular concentration of $Na^{125}I$ was 10 μM, with an initial out → in NaCl (100 mM → 0) gradient. For other details, see under 'Methods'. In this and in the following figures each point is the means of at least three determinations; the bars indicate the S.D. values; when they are not given, the S.D. values were smaller than the symbol used.

the K_m values given in this paper should be regarded as operational values only, referring to the experimental conditions employed, i.e., in 10 mM Hepes-Tris (pH 7.5), 300 mM mannitol, with an initial NaCl gradient (100 mM out, zero in). With a smaller initial gradient of NaCl (50 mM out, zero in) an apparent K_m of 6 μM was measured.

In whole, cultured rat thyroid cells the apparent K_m for Na^+-dependent I^- uptake is approximately 31 μM [4,5]. The difference with our figures may be related to difference in species and/or in experimental conditions.

4. Cation-dependency of I^--uptake

The quasi-initial uptake of I^- (i.e., at 10 s incubation) was investigated as a function of extravesicular Na^+, at two concentrations of I^-, 3 μM (i.e., close to the K_m for I^-, about 5 μM, see above) and 0.5 μM (i.e., about 10-times smaller than the K_m) (Figs. 3A, B ● and C). The initial Cl^- gradient was kept constant (300 mM out, zero in), and so was that of the sum of Na^+ + choline (or $Na^+ + Li^+$ + choline) (300 mM out, zero in) *. Clearly, the Na^+ dependence is sigmoidal, the $\bar{n}$ being at both I^- concentrations 1.61.

In principle, this sigmoidicity can be interpreted in one of three ways, or in a combination thereof: it may indicate (i) that Na^+ acts as an allosteric activator of I^- uptake: (ii) that, if Na^+ and I^- are obligatory cosubstrates in a non-rapid equilibrium Random (Iso) Bi-Bi mechanism, a Na^+ first, I^- second sequence of events prevails at low Na^+ out concentrations [24] (see also Ref. 23 for discussion); (iii) and/or that the Na^+ and I^- are cosubstrates with a flux ratio $Na^+ : I^-$ is larger than one.

Possibility (i) is unlikely since no sigmoidicity is detected in the dependence of uptake from the outer initial concentration of I^-, at 100 mM initial NaCl out (Fig. 4) or at 50 mM NaCl out (data not shown). Likewise, possibility (ii) above is unlikely because no opposite deviation from a Michaelian behaviour is detected, either (Fig. 4) (see Refs. 23, 24).

On the other hand, possibility (iii), i.e., a $Na^+ : I^-$ flux ratio larger than one, is rendered very likely by the electrogenicity of the transport system (see below).

If an initial KCl gradient is substituted for the

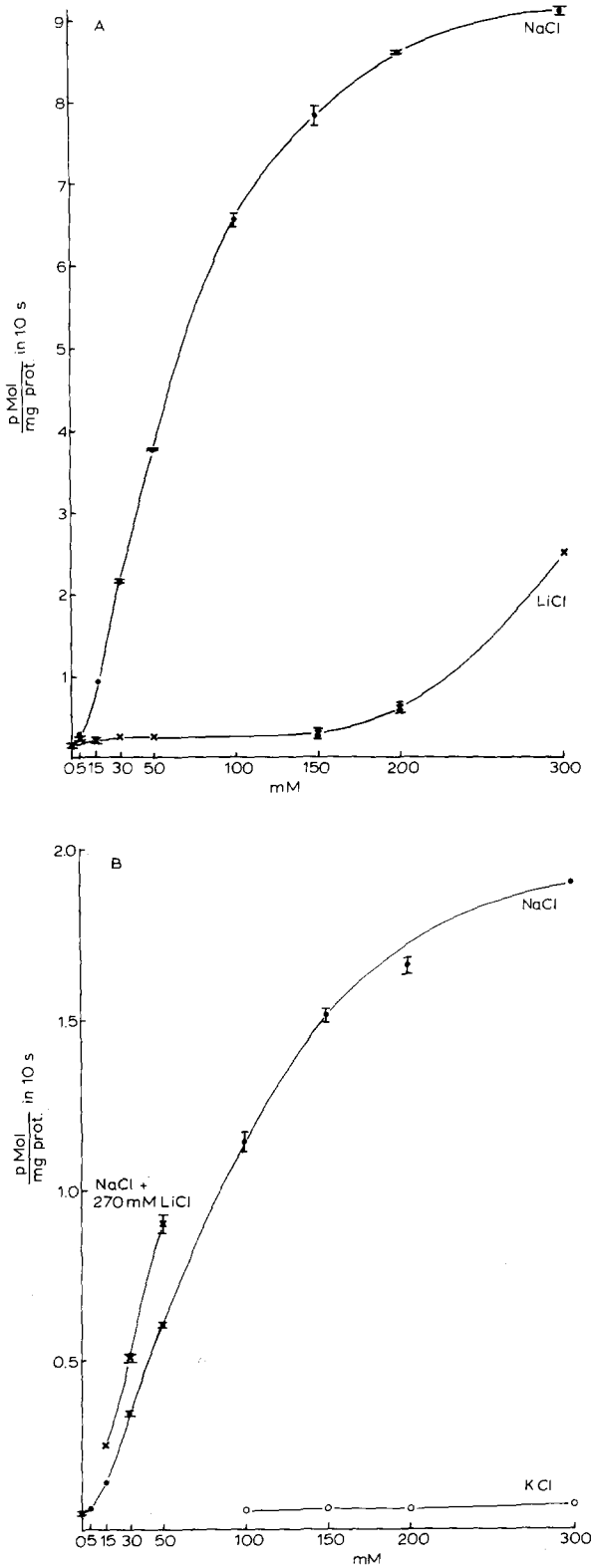
TABLE II

IONIC RADII AND K_i (or K_m) VALUES OF SOME LIPOPHILIC ANIONS

	Ionic radius (Å)	K_i for fully-competitive inhibition (K_m for transport, for I^-), $\bar{x} \pm$ S.D. (μM)
ClO_4^-	2.36	11.0 ± 0.4
I^-	2.20	4.8 ± 0.37
SCN^-	1.95	15.3 ± 1.38
N_3^-	≈1.59 [a]	> 5000

[a] Estimated, by analogy with NCO^-.

* Except for the highest concentration of Na^+ in Fig. 3B, where the initial outer concentrations were 270 mM LiCl + 50 mM NaCl.



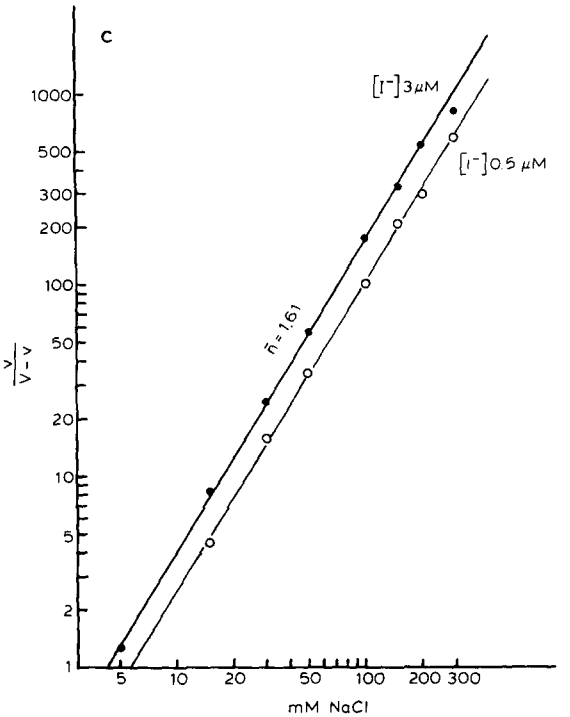


Fig. 3. Effect of Na^+, or K^+, or Li^+ on I^- uptake. At the beginning of the incubation these cations (all as chlorides) were present in the extravesicular fluid only, at varying concentrations. Choline chloride was used to balance the ionic strength. (A) ^{125}I was 3 μM; ●, Na^+ alone; ×, Li^+ alone. (B) ^{125}I was 0.5 μM; ●, Na^+ alone; ×, 270 mM Li^+ plus varying concentrations of Na^+; ○, K^+ alone. (C) Hill plot of the data of I^- uptake in Na^+ alone (+ choline), from Figs. 3A and 3B, at the I^- concentrations given.

NaCl gradient (Fig. 3B, ○) no activation of I^- uptake is detected. Thus K^+ cannot substitute for Na^+ as a cosubstrate, which fully agrees with reports in the literature using intact thyroid or intact thyroid cells [1,3–7], or proteoliposomes [12] and sharply contrasts with a report [11] using another type of vesicle preparation.

A LiCl gradient can substitute for NaCl gradient, albeit much less efficiently (Fig. 3A, x) and, if superposed to the NaCl gradient produces an additive activation (Fig. 3B, x). This observation may or may not have clinical relevance (see Discussion).

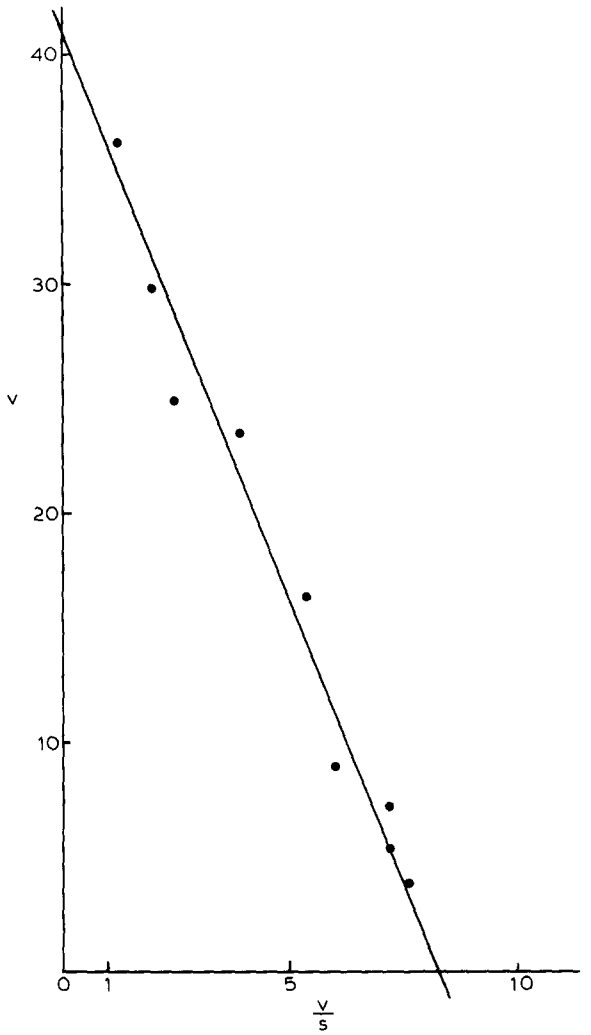


Fig. 4. Uptake of I^- in the presence of an initial NaCl gradient (100 mM out, 0 in), as a function of the I^- concentration (Eadie-Hofstee plot). v, expressed as pmol of I^- taken up in 10 s, per mg vesicle protein; s, μM. The K_m was 4.70 ± 0.31 μM; the V_{max} 41 pmol·(10 s)$^{-1}$·mg^{-1} protein.

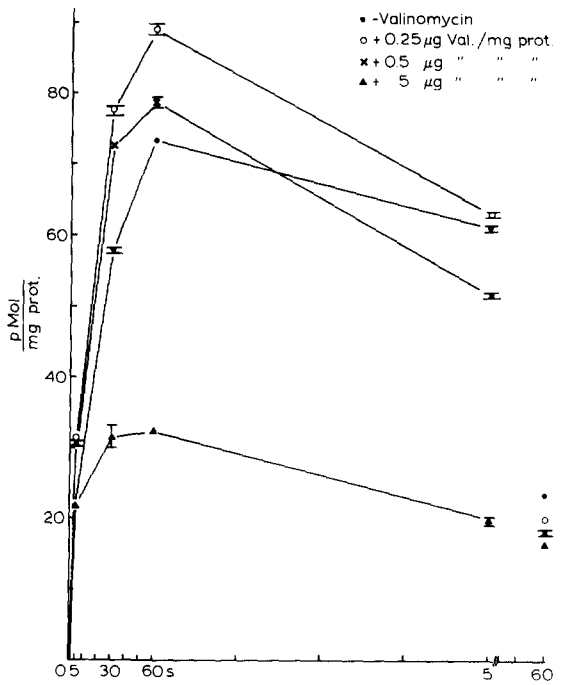


Fig. 5. Effect of valinomycin on I^- uptake into K^+-preloaded vesicles. The vesicle suspension was diluted with six times its volume with 'vesicle buffer' (see Methods) enriched with 50 mM K_2SO_4, spun down, resuspended in the same buffer (again, with K_2SO_4) to a concentration of approx. 17 mg protein·ml^{-1}, and left to equilibrate for 1 h at 20°C. Valinomycin was added in ethanol at the amounts given below, and I^- uptake was initiated by diluting the suspension 1 to 9 into a medium yielding the following final composition: 11 μM $Na^{125}I$, 50 mM Na_2SO_4, 5.6 mM K_2SO_4, 1.1 mM MMI, 10 mM Hepes-Tris (pH 7.5) and 300 mM D-mannitol. ●, no valinomycin; ○, 0.25 μg, ×, 0.5 μg and ▲, 5 μg valinomycin·mg^{-1} protein.

5. Electrogenicity of Na^+-dependent I^- transport

Large lipophilic anions, e.g., SCN^-, ClO_4^- or N_3^-, could not be used to generate diffusion potentials in this system, because they strongly inhibit I^- transport (see below), probably all of them fully-competitively. H^+ gradients plus proton carriers could not be used either, because of the strong pH-dependence of I^- transport (not shown).

K^+ gradients (in > out) plus valinomycin were thus used in order to generate a diffusion potential, negative inside the vesicles; but, again, only after it was made certain that, contrary to a report in the literature [11] K^+ cannot substitute for Na^+ (Fig. 3B, ○). Fig. 5 (compare the faster uptake in the presence of 0.25 or 0.5 μg valinomycin per mg protein, ○, ×, with the control in the absence of valinomycin, ●) clearly shows that a negative membrane potential inside the vesicles accelerates Na^+-dependent I^- transport, which is thus identified as being electrogenic. This agrees with the $Na^+ : I^-$ flux ratio being larger than one, as suggested in one of the previous paragraphs.

Larger amounts of valinomycin, however, inhibit the (net) uptake of I^- (see, e.g., in Fig. 5 ▲

the experiment with 5 μg valinomycin per mg protein). This observation can be rationalized by suggesting that the large and lipophilic anion I^-, once taken up, can soon easily diffuse out of the vesicle together with the large, lipophilic cationic valinomycin-K^+ complex, by forming with it a lipophilic, easily diffusable ion pair. That is, the decreased net uptake of I^- in the presence of large amounts of valinomycin is the result of an increased passive efflux of I^-, rather than of a decreased influx. This is most probably the mechanism whereby massive doses of valinomycin elicit an accelerated efflux of I^- from thyroid tissue slices (see, for example, Ref. 25).

6. Inhibitors

The inhibition of I^- uptake in the thyroid by large lipophilic anions, such as SCN^- or ClO_4^- has been known for a long time, from both in vivo and in vitro studies. However, the exact kinetic type of the inhibition and also quantitative comparison among these inhibitors had been previously inaccessible to experimentation, due to the unavailability of a suitable vesicle preparation (see Introduction). The vesicles developed in the present paper allowed a fairly reliable kinetic investigation, since it made accessible quasi-initial uptake rates most likely unbiased by the unstirred layers which plague most kinetic work using tissue preparations.

Fig. 2 shows the effects of 0.1 mM SCN, 0.1 mM ClO_4^- or 10 mM N_3^- on the time course of I^- uptake. Already from this semi-quantitative comparison it is clear that the two former anions are the stronger inhibitors. Figs. 6A, B report Dixon plots for these two. The kinetic type of the inhibition is fully-competitive, the K_i values being 15.3 μM and 11.0 μM for SCN^- and ClO_4^-, respectively.

For some years it has been known that a number of (mostly unidentified) soya fractions inhibit the in vivo uptake of I^- by the thyroid gland [26,27] and may indeed lead to hypothyroidism. It was of interest, therefore, to test the potential inhibition by water or methanol extracts, of saponins and isoflavones on the I^- uptake activity of our vesicle preparation. None of the fractions and none of the isolated compounds displayed any inhibitory activity.

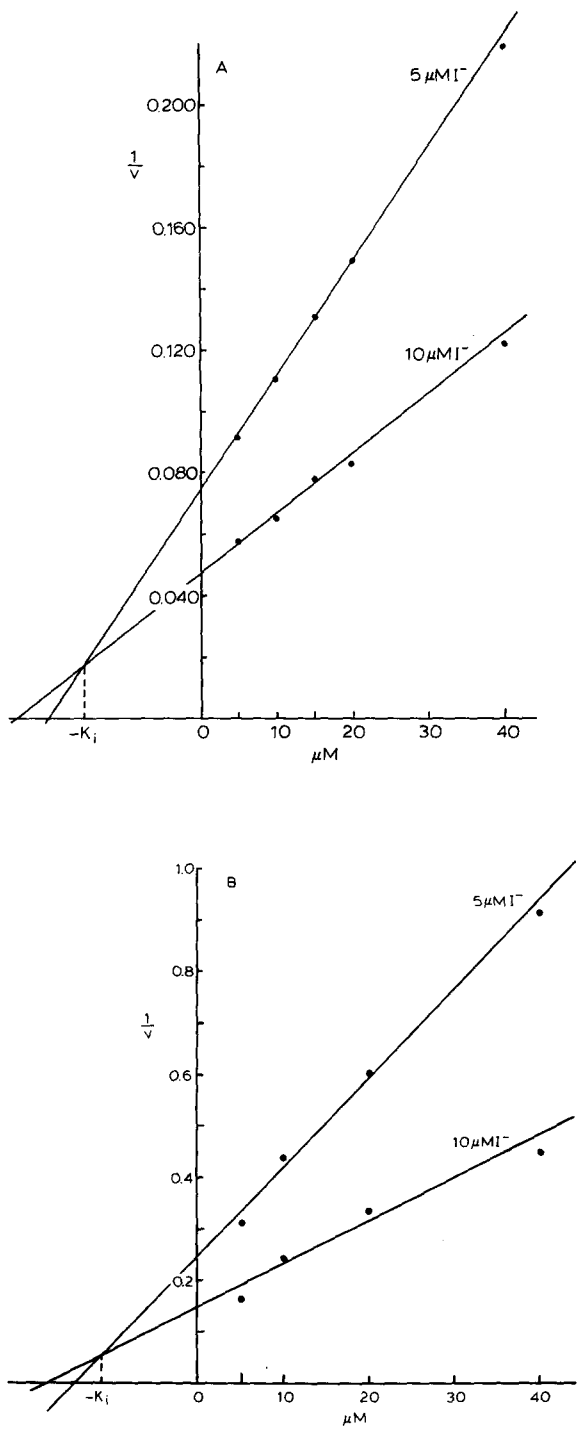


Fig. 6. Dixon plots of I^- uptake inhibition by NaSCN (A) or $NaClO_4$ (B). v is expressed as pmol I^- taken up in 10 s per mg protein. The calculated K_i values were: 15.3 ± 1.38 μM and 11.0 ± 0.4 μM for SCN^- and ClO_4^-, respectively.

Discussion

The preparation of thyroid gland plasma vesicles developed and used here is admittedly not quite 'pure' (e.g., it still contains sizeable glucose-6-phosphatase activity, contrary to another [28]), but proved to be adequate for the purpose of our investigation. Like other vesicle preparations from other sources, they offer various advantages over surviving tissues or even cells (see, for example, Refs. 8–10). For example, it was important, in the case of I^-, to measure its transport in the presence of a compound (i.e., 2-mercapto-1-methylimidazole) preventing this anion from oxidizing to I_2, which would be taken up by the lipid phase (see under 'Results', section 2); to carry out the uptake experiments under well-defined initial ion gradients; not to have to take into consideration the operation of the $(Na^+ + K^+)$-ATPase (no ATP in the media); to use a 'simpler' biological preparation when employing valinomycin (which perforce leads to equivocal conclusions in whole cells, e.g. Ref. 25).

As pointed out under 'Results' the apparent K_m values measured for I^- uptake (approx. 5 μM) is to be taken as an operational value related to the particular experimental conditions used (neutral pH, out > in NaCl gradient, 20°C, etc.), but it should not be too dissimilar from the actual K_m in vivo, since our conditions were 'nearly' physiological. The K_m for I^- uptake in intact, cultured thyroid cells is approx. 31 μM [4,5], which may reflect the difference in species (hog vesicles, vs. rat cells), or differences in conditions, or both. Either K_m-value, however, is far larger than the concentration of free I^- normally present in the blood (between 0.006 and 0.047 μM [29]). Clearly, some factors must operate in vivo, in addition to the ones investigated by us, to allow, and perhaps to regulate, the entry of I^- into the thyroid cells in large quantities. As we show below, I^- uptake into the thyroid is electrogenic; but we doubt that $\Delta\psi$ could play a key role in this postulated regulation, since the apparent K_m is little or not affected by a more negative $\Delta\psi$ in the vesicles (O'Neill, unpublished results).

The transport system is (almost) totally dependent on the presence of Na^+_{cis} (Figs. 3 A, B, C) and K^+ cannot substitute for this cation. This is in excellent agreement with the original observations and interpretation of Iff and Wilbrandt [1] on intact thyroid glands and with some later ones, on cells (see, for example, Ref. 5), who attributed the small and slow activation of I^- uptake by low concentrations of K^+_{cis} to an indirect mechanism, i.e., to activation of $(Na^+ + K^+)$-ATPase, which is of course not operating in our vesicles.

The effects of K^-_{out} and/or valinomycin need some comment, since quite a confusion is apparent in the literature. Endo et al. [11], for example, using a preparation of membrane vesicles different from ours, suggested that a K^+ out → in gradient can substitute for the Na^+ gradient. Our own observations show unequivocally that this is not the case (Fig. 3B, ○) and thus agree (in being actually even more clear-cut) with other reports of the literature, utilizing intact cells (see, for example, Ref. 4) or reconstituted proteoliposomes [12].

As to valinomycin (plus a K^+ in → out gradient), one must clearly distinguish between the two effects. Whereas small amounts of valinomycin (Fig. 5, 0 and x) stimulate Na^+-dependent I^- uptake (which shows its electrogenicity, see below), large amounts of this ionophore lead to a decrease in the net uptake of I^- (Fig. 5, ▲). The simplest explanation of this 'inhibition' (also in view of the lack of any interaction between K^+ and the Na^+/I^- cotransporter, e.g. in Fig. 3 B, ○) is that it arizes independently of the cotransporter itself, i.e., that the positively charged, lipophilic valinomycin-K^+ complex facilitates the efflux of the lipophilic, negatively charged I^-, by forming uncharged, highly lipophilic ion pairs which would be highly mobile across the lipid barrier (see also the experiments in Figs. 1 and 3 of Ref. 13). It is thus unnecessary to postulate that valinomycin severely disturbs the lipid bilayer structure in the membrane, or that it acts by interacting with hypothetic (indeed highly improbable [12]) lipidic I^- carriers [30–32]. Valinomycin has been shown to induce efflux of I^- also from intact thyroid tissue [e.g. 25, 30], to which again the same simple explanation above can be given.

A word of warning seems to be needed at this point against the uncritical use of valinomycin in the investigation of the mode of action of TSH (e.g., in Ref. 25): if this ionophore produces a dual effect in a system as simple as membrane vesicles

(Fig. 5), an inexticable complexity, and thus ambiguity, must be expected with valinomycin acting on intact tissue. A partial list of effects to be expected includes a transient hyperpolarization (and thus stimulation of I^- uptake); increased I^- efflux; decreased K^+_{in}; inhibition of oxidative phosphorylation, etc.

Li^+ can substitute for Na^+, albeit rather ineffectively (Fig. 3A, x) and has an additive effect over Na^+ (Fig. 3B, x). There are a number of reports on a variety of effects of Li^+ on thyroid function in man; for example Li^+ may produce hypothyroidism [33,34]. Whether this effect of Li^+ is related to its action on Na^+-dependent I^- transport, it is premature to say.

The $K_{1/2}$ for Na^+ is decreased by increasing concentrations of I^- (Fig. 3C), and the apparent K_m for I^- is somewhat decreased by reducing the initial NaCl out $\rightarrow$ in gradient from $100 \rightarrow 0$ to $50 \rightarrow 0$ mM, which reinforces the notion of I^- and Na^+ cotransport. While these observations agree with those made in intact cells [4], they are not sufficient to identify the kinetic mechanism of Na^+, I^- transport (which our data above indicate to belong at least to an (Iso)Ter Ter group). In view of the complexity of the kinetic analysis of a system having a Ter Ter mechanism, we did not try to identify the order of substrate binding.

The Na^+ dependence of I^- uptake is clearly sigmoid, with an $\bar{n}$ value of 1.61 at both I^- concentrations tested (Fig. 3 C). For the reasons mentioned under Results, section 4, this sigmoidicity is interpreted as indicating that the Na^+ to I^- flux ratio is larger than one. In agreement with this $Na^+ : I^-$ flux ratio, we find that Na^+-dependent I^- uptake into thyroid membrane vesicles is electrogenic (compare in Fig. 5, ● with ○ or x). That is, the thermodynamic driving force producing accumulation of I^- into the cells and into the vesicles (see e.g., Figs. 2, 5) in addition to the I^- gradient across the membrane is the $\Delta\tilde{\mu}_{Na^+}$, i.e., the gradient of Na^+ across the membrane plus $\Delta\psi$, because the substrates, taken together (I^- plus more than one Na^+), carry at least one positive charge. Some of the earlier interpretations (e.g., of some experiments in Ref. 13) may now have to be revised. Also this characteristic of this transport system seems not to have been observed by earlier workers, probably due to the number of experimental difficulties inherent to the system (see under Results). This failure to recognize the electrogenic nature of I^-/Na^+ cotransport is nevertheless surprizing, since TSH has been reported by some authors to produce a hyperpolarization, by others a depolarization of the thyroid plasma membrane (see literature quoted in Ref. 25).

The electrogenic nature of Na^+/I^- cotransport may well have physiological significance in securing a more efficient trapping of this anion from the very low concentrations in the blood, into thyroid cells, for whose hormonal activity it is essential. Indeed, it is not exceptional that the Na^+ : anion flux ratio of the membrane transport of an important anion may be larger than one, thus making it electrogenic and capable to 'ride' also on the electrical component of $\Delta\tilde{\mu}_{Na^+}$ (e.g., see the ascorbate transport in renal brush border vesicles [35]).

As shown in Figs. 2 and 6, large lipophilic anions inhibit I^- uptake in vesicles of thyroid plasma membrane. The inhibition is fully-competitive, which is not surprizing, in view of their close similarity to I^-. Table II compares their K_i values (or K_m value, in the case of I^-) with their ionic radii (calculated from the hydration heat and the lattice energy, Ref. 36). Clearly, the closer the ionic radius is to that of I^- (2.20 Å), the smaller the K_i. The small K_i values of SCN^- and of ClO_4^- account for the well-known efficiency of these compounds in the management of hyperthyroidism.

Other inhibitors, of as yet unidentified nature, have been reported to occur in soya bean extracts (see, for example, Ref. 26, 27). We have thus tested, in addition to the anions of Fig. 2, several water or methanol extracts of soya flower, as well as a number of saponins and isoflavones, without identifying, however, any component capable of inhibiting Na^+-dependent I^- uptake in our vesicles.

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