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Characteristics of Thyroxine Swelling in Skeletal Muscle Mitochondria: Relationship to Valinomycin Swelling and Swelling in the Absence of Mg^{2+}

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Introduction

Very extensive swelling of mitochondria both *in vivo* and *in vitro* has been observed following thyroxine treatment.[1,2] However, the mechanism by which thyroxine initiates such swelling is still unknown. It is well established that changes at energy conservation sites in the electron transport system are intimately related to both permeability and structural changes in mitochondrial membranes.[3–7] Indeed, evidence has been presented for an energy requirement for thyroxine-activated swelling of liver mitochondria.[2] However, contrary to conditions in the presence of other swelling agents, thyroxine or triiodothyronine at 5×10^{-5} M or lower concentration does not significantly uncouple oxidative phosphorylation in isolated liver mitochondria.[8] The hormone does not uncouple the phosphorylation observed in an isolated enzyme complex.[9] When a change in basal metabolic rate was effected by doses of thyroxine small enough to have no effect on growth rate, isolated liver or muscle mitochondria failed to show any loosening of respiratory control.[10] Indeed, muscle mitochondria from thyrotoxic animals, isolated in the presence of 10 mM EDTA, also failed to show any uncoupling effect.[11] Staehelin[12] has suggested that an observed uncoupling action of thyroxine depends upon the presence of Ca^{2+}; Lehninger[13] has emphasized a possible functional relationship between thyroxine and Ca^{2+}. Indeed the swelling action of thyroxine is strikingly potentiated in the presence of Ca^{2+} or Zn^{2+}.[14] Thyroxine is known to form chelates with several divalent cations which cause swelling and uncoupling of oxidative phosphorylation, e.g. Ca^{2+}, Cu^{2+}, Zn^{2+}.[15] The hormone also forms a chelate with Mg^{2+}, which prevents the energized swelling which occurs in the absence of Mg^{2+} and which imposes control of respiration by phosphorylation.[16] Although the uncoupling action of thyroxine is counteracted by EDTA[17] or by Mg^{2+},[9] thyroxine swelling is not inhibited by Mg^{2+} even at molar ratios of this metal ion to hormone of 10:1.[14]

It has been concluded that thyroxine induces swelling by a mechanism which is distinctly different from that of gramicidin, valinomycin and the non-actin homologs since it does not release the oligomycin inhibition of tightly coupled respiration.[18] However, Lardy *et al.*[8] on the basis of inhibitor studies with aurovertin, have postulated that the induction of swelling with thyroxine, as with several other substances, is related

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to a high-energy intermediate off the main pathway for oxidative phosphorylation; they have suggested that a category of high-energy compounds for ion transport and swelling-contraction phenomena may be generated irreversibly either from ATP or from intermediates produced during respiration. Insensitivity of ATPase to aurovertin puts valinomycin in the same category as thyroxine although swelling by these agents can still be differentiated by their different concentration requirements for alkali metal cations.

The following study was designed to clarify the role of thyroxine in mitochondrial swelling in circumstances which preclude a significant involvement with metal ions. The findings show that thyroxine does not promote swelling simply because it chelates Mg^{2+}. They also show that thyroxine in the presence of excess Mg^{2+} acts at the first coupling site to inhibit oxygen uptake and energy coupling and to promote an electron by-pass. They suggest that, as with the lipid-soluble K^+ carrier, valinomycin in the presence of Mg^{2+}, a change in the status of electrical gradients in the membrane can account for the osmotic swelling observed.

Experimental Procedures

Reagents

All reagents employed in these studies were prepared with deionized water and gave a negative test for heavy metal contamination, as tested with 8-hydroxyquinoline.[19]

The Na-L-thyroxine solution used was prepared as a thyroxine-albumin complex, as follows: Na-L-thyroxine (Sigma) was dissolved in 0·05 N NaOH, complexed with albumin by mixing for 30 min, buffered with Tris and neutralized to pH 7·4 with HCl. The final concentration was 7·5 mM thyroxine, 26 mM Na^+, 33 mM Tris and 2·7% albumin. Other thyroxine analogue-albumin complexes were prepared in a similar manner. The albumin concentration of all media used in the present studies was maintained at 0·2%. Where thyroxine analogue-albumin complex was employed, it was substituted so as to maintain the total albumin concentration at 0·2%. The thyroxine concentration of these media was 5×10^{-4} M.

Treatment of Animals

Young male Hooded rats received a diet of Purina rat cubes and water *ad lib.*

Isolation of Mitochondria

Skeletal muscle mitochondria were isolated in the cold from the hind legs of 150–200 g rats. Heparin 0·5% (330 units/ml medium, crystalline sodium heparin, General Biochemicals) was employed throughout in the isolation medium because this had been shown to preserve the functional integrity of the organelles. Divalent cation-depleted mitochondria were isolated and suspended finally in a solution containing mannitol 0·21 M, sucrose 0·07 M, Na_2 . K_2 . EDTA 0·01 M, Tris . HCl, pH 7·4, 0·010 M, and heparin 0·5%. The isolation procedure and the high respiratory and energy-coupling activities observed for such mitochondria are outlined in a previous publication.[20] These activities deteriorated very little over a period of 6–8 hours.

Determination of Protein Content of Mitochondria

The protein content of the mitochondrial suspension was determined by the method of Gornall *et al.*[21]

Polarographic Determination of Respiratory Control

Oxidation rates and respiratory control indices were measured at 37° C in a Gilson Polarograph, Model KM, employing a Clark oxygen electrode. Polarographic determinations were performed on all mitochondrial samples used for swelling studies to ensure that all samples showed high respiratory activity and tight respiratory control. Oxygen utilization was measured routinely as outlined in a previous publication.[20] Oxygen utilization was also measured in media identical to those used for the swelling studies to test cation involvement in uncoupling of respiratory control.

Measurement of Change in Mitochondrial Volume

Measurement of change in the absorption of light in the visible region by suspensions of mitochondria was used as a qualitative measure of mitochondrial volume. Absorbance measurements at 520 mμ in a Beckman Model B spectrophotometer were made at room temperature in isotonic salt media at pH 7·4, as outlined under the various tables and figures. Approximately 0·2 mg of mitochondrial protein was used to give a contracted state absorbance of 0·5 to 0·6. At zero time the mitochondria were added to the salt medium containing substrate or ATP as the energy source and absorbance readings were taken at quarter or half-minute intervals. The swelling agent, valinomycin or thyroxine, was added after a specific time interval or was present initially. Absorbance readings were continued at half-minute intervals up to 20 min. The effects of various metabolic inhibitors were tested by adding them to the media prior to the addition of the mitochondria.

A previously-reported correlation between absorbance change and Coulter Counter volume change in Mg^{2+}-free circumstances[16] has been further substantiated by the demonstration under the same conditions of a correlation between absorbance change and inner membrane volume change (electron micrographic evidence).[22] Consequently, direct measurement of change in mitochondrial volume was made employing the Coulter Electronic Particle Counter. Coulter Counter measurements were made at room temperature employing a 30 μ aperture. At zero time the mitochondria were added to the salt medium containing substrate or ATP, with or without a swelling agent, and counts were taken as quickly as possible at different threshold levels across the spectrum of particle sizes. Spectra were obtained at several time intervals until the mitochondria showed no further significant swelling (steady state volume). The isotonic media, as outlined below the various figures involved, were the same as those used for the absorbance studies; the concentration of mitochondria used, however, was considerably less, in order to avoid significant coincidence counting. The effects of various inhibitors were tested by adding them to the media prior to the addition of the mitochondria. Oligomycin and atebrin were preincubated with the mitochondria.

As was observed by other workers,[23,24] electronic noise prevented the accurate counting of the smaller mitochondria in the Coulter Counter. Consequently, for particle volume measurements, the heavy mitochondria only were recovered as an additional isolation step from 0·88 M sucrose + 0·5 mM EDTA centrifuged at 12,000 × *g* for 15 minutes in a Servall refrigerated centrifuge. The pellet was resuspended finally in the original suspension medium.

All media used for the Coulter Counter were filtered twice through a washed 0·1 μ

Sartorius membrane filter just prior to their use in order to reduce the background count as much as possible. The Sartorius filters do not have the high detergent content reported for other membrane filters.[25] Standard size polystyrene spheres of uniform diameter (0·796 μ, Dow Chemical Company) were employed for standardization of the instrument.

Measurement of DPN Reduction by Dual Wavelength Spectrophotometry

DPN reduction in the presence of NaCN with and without triiodothyronine-albumin complex was measured at room temperature using the Aminco-Chance Dual Wavelength Spectrophotometer at 340 mμ with reference wavelength at 374 mμ. The K^+ medium used was the same as that used for substrate-supported swelling in the presence of triiodothyronine-albumin complex [Fig. 5(B)]. The protein concentration was 1·5–1·75 mg/ml.

Measurement of Na^+- and K^+-activated ATPase Activity

The Na^+- or K^+-activated ATPase activity induced by the various agents in the presence or in the absence of Mg^{2+} was measured at 23° C in a buffered 40 mM tris acetate swelling medium containing either 2·2 mM or 90 mM cation as NaCl or KCl. The composition of the media, which is outlined beneath Fig. 9, was the same as that used in the swelling studies. The incubations were terminated after 5 or 15 min by the addition of an equal volume of 10% TCA and the tubes were chilled immediately in an ice-bath. ATPase activity was measured as inorganic phosphate released, according to the method of Sumner.[26]

ATPase activity was also measured by the rate of H^+ production, as detected by the pH electrode in conjunction with the Beckman Expandomatic pH meter and Expandomatic range selector. The composition of the media, as outlined in Table II, was the same as that used in the various swelling studies.

Measurement of Na^+- and K^+-activated Pyrophosphatase Activity

The Na^+- or K^+-activated pyrophosphatase activity induced by thyroxine in the presence and absence of Mg^{2+} was measured under the same conditions as those employed for Na^+- and K^+-activated ATPase activity (see description beneath Fig. 8). The incubations were terminated after 5 or 15 min by the addition of an equal volume of cold 10% TCA and the free pyrophosphate in the resulting supernatant fraction was separated from other phosphate compounds employing ascending paper chromatography.[27] The chromatographic spots were eluted with 0·04 N HCl + 0·04 M NaCl and the eluate was digested with H_2SO_4. The resulting inorganic phosphate was determined according to the method of Bartlett.[28]

Results

Thyroxine-albumin Complex

The extraction of a cellular thyroxine-binding protein fraction from rat skeletal muscle made possible the observation of a direct competition between serum- and intracellular-thyroxine-binding protein for the binding of the hormone.[29] It is thus

apparent that the actual concentrations of thyroxine available for intracellular processes depend on the relative magnitudes of the affinity constants of the intracellular proteins competing for the hormone.[30] In an attempt to duplicate such physiological conditions, thyroxine was employed in the present trials as a neutral solution, bound to albumin.

ATP-Supported Swelling

ATP-supported swelling of skeletal muscle mitochondria in a neutrally-buffered isotonic medium in the presence of neutral, solubilized thyroxine-albumin complex (T_4) and Mg^{2+} is illustrated as an observed decrease in steady-state absorbance (Fig. 1). As with ATP-supported swelling induced by the absence of Mg^{2+} [16] or by the presence of valinomycin (see Fig. 2 and refs. 31, 32), such swelling shows a specific cation and a permeant anion dependency. However, unlike ATP-supported swelling in the absence of Mg^{2+} but like valinomycin-induced swelling, Na^+ supports very little T_4-induced swelling with phosphate as permeant anion. The findings were similar with acetate. On the other hand, K^+ supports ATP-activated swelling with both anions. In contrast to valinomycin-activated swelling (Fig. 2), swelling in the presence of T_4 shows a high K^+ concentration requirement. In contrast to both of the other swelling conditions (see Fig. 2 and ref. 16), the more-ionized phosphate is more effective than acetate at the same concentration.

A correlation between absorbance change, Coulter Counter volume change and inner membrane volume change (electron micrographic evidence) was demonstrated in earlier trials involving swelling changes in the absence and presence of Mg^{2+}.[16,22] Nomograms of mitochondrial count plotted against mitochondrial volume, as measured in the Coulter Counter, are presented here for ATP-supported swelling in the presence of $T_4 + Mg^{2+}$ (Fig. 3) and in the presence of valinomycin (Fig. 4). Swelling in both circumstances is confirmed as an observed increase in particle steady-state volume. As shown previously for swelling in the absence of Mg^{2+}, the measurements show no swelling in the presence of oligomycin, confirming the energy-dependency of the swelling induced by these agents. Oligomycin sensitivity was demonstrated also in the absorbancy studies [see Fig. 5(A)]. Azide, atebrin and PCMB were inhibitory also to ATP-dependent swelling under all three conditions and DNP largely prevented swelling. In agreement with the reported reversible equilibrium between extracellular and intracellular binding of thyroid hormones,[29] the addition of excess albumin to mitochondria which had been actively swollen in the presence of T_4 promoted rapid contraction (Fig. 3). Presumably the K^+ and PO_4^{2-} which have been entering by energy-dependent changes are released again when the energy for ion transport is no longer made available by thyroxine. Such a contraction effect has been demonstrated for the addition of Mg^{2+} to mitochondria swelled in the presence of ATP without Mg^{2+}.[16,22] Swelling which occurs in the absence of Mg^{2+} or in the presence of valinomycin is not reversed by albumin.

Figure 5(A) shows that an immediate absorbance decrease of large amplitude occurs when T_4 is added to a medium containing ATP + Mg^{2+} in the presence of acetate and a high concentration of K^+. The observed change in absorbance is of similar magnitude to that presented in Fig. 6 for Na^+-activated ATP-supported swelling in a Mg^{2+}-free medium and for K^+-activated ATP-supported swelling in the presence of valinomycin. However, the rate of swelling is somewhat slower. The use of triiodothyronine-albumin

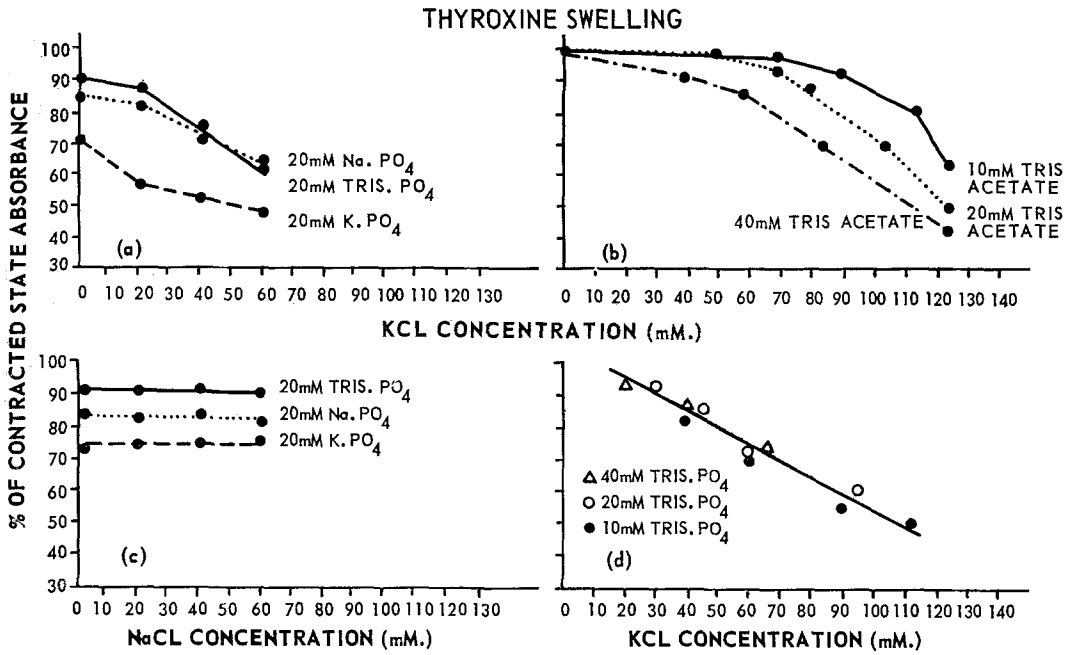


Figure 1. The specifics of thyroxine-induced swelling. The composition of the media was as follows: Na^+, K^+, or Tris phosphate, pH 7·4, levels as recorded on graph; K^+ or Na^+, added as KCl or NaCl except that K^+ salt or Na^+ salt was substituted for Tris salt at the highest levels to preserve isotonicity, levels as recorded on graph; Tris HCl, pH 7·4, 2·0 mM; $MgCl_2$, 5·0 mM; Tris ATP, 5·0 mM; bovine serum albumin 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·17–0·18 mg protein/ml; Na-L-thyroxine (albumin complex), 0·5 mM.

complex (T_3) instead of T_4 caused a faster rate of swelling, approaching that observed in a Mg^{2+}-free medium. The use of phosphate, in place of acetate, caused an even greater degree of swelling. Again, Na^+ failed to support ATP-activated swelling in the presence of $T_4 + Mg^{2+}$. Neither denatured albumin, diiodothyronine-albumin complex (T_2) nor KI promoted ATP-activated swelling.

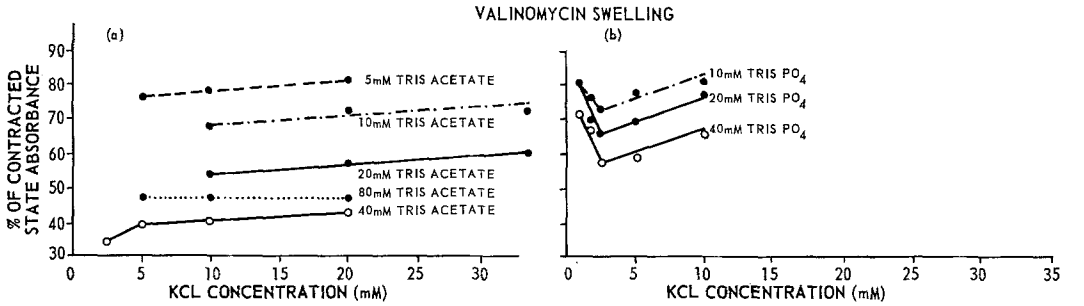


Figure 2. Ion specificity of valinomycin-induced swelling. The composition of the media was as follows: Tris PO_4, pH 7·4, 10–40 mM, as recorded on graph, or Tris acetate, 5–40 mM, as recorded on graph; KCl, 2·5–20·0 mM, as recorded on graph; Tris HCl, pH 7·4, 2·0 mM; Na_2.ATP (neut. with Tris), 2·5 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolated medium), 0·01%; mitochondria, 0·17 mg protein/ml (phosphate medium), 0·20 mg protein/ml (acetate medium); valinomycin 83 ng/ml.

Substrate-Supported Swelling

Figure 5(B) shows that pyruvate + malate, in the presence of malonate and oligomycin, supports pronounced swelling in the presence of T_3 + Mg^{2+}. The same figure shows that this swelling is of similar rate and magnitude to that produced by the same substrate in a Mg^{2+}-free medium in the absence of hormone. It is apparent that both conditions support swelling equally in a Na^+ or a K^+ medium and both show a linear relationship between cation concentration and swelling (see 16). By contrast, valinomycin-activated

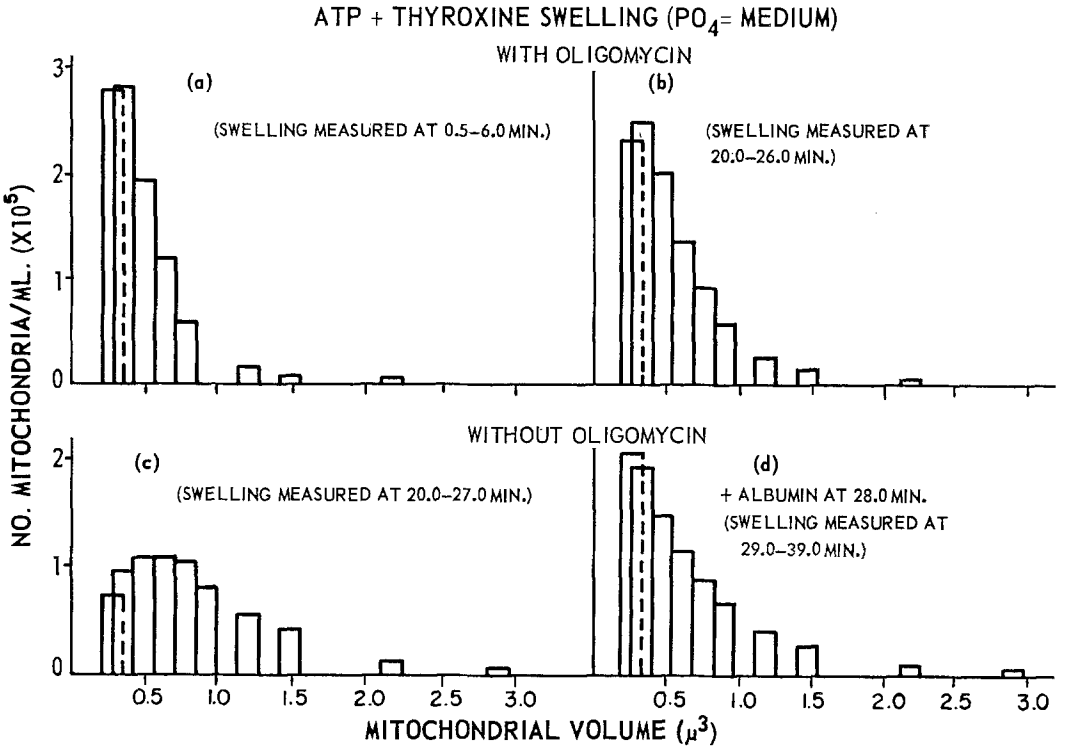
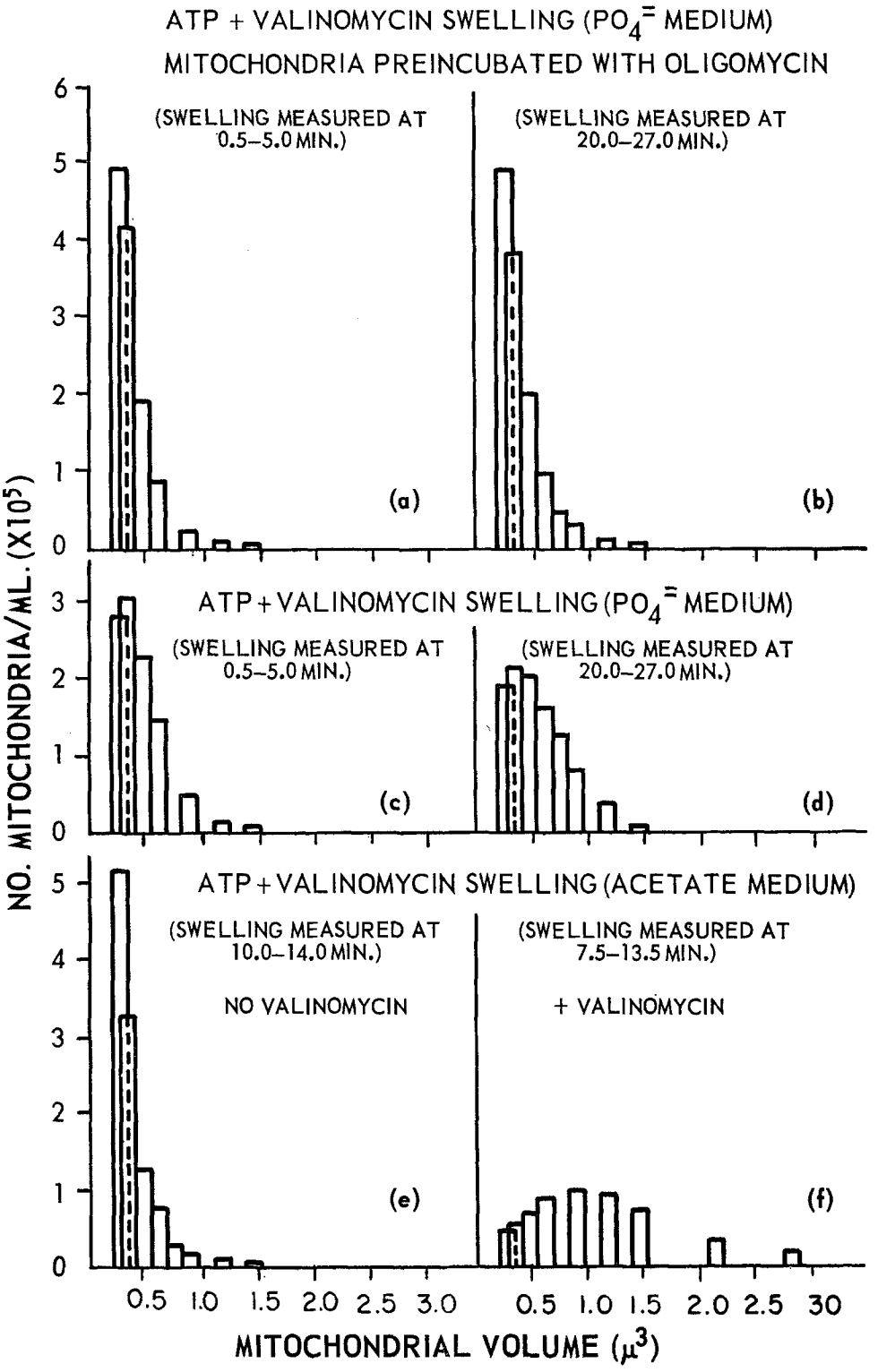


Figure 3. Coulter counter measurement of energy-dependent thyroxine-induced change in mitochondrial volume. The composition of the media was as follows: Tris PO_4, pH 7·4, 10 mM; KCl, 110 mM; Tris HCl, pH 7·4, 2·0 mM; $MgCl_2$, 5·0 mM; Na_2, ATP neut. with Tris, 5·0 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 6·4 μg protein/ml; Na-L-thyroxine (as albumin complex), 0·5 mM; ± oligomycin, 6 μg/ml; albumin, added later, →1·5%.

swelling in the presence of substrate shows the same low K^+ requirement as for such swelling supported by ATP and again swelling does not occur with Na^+ (Figs. 6 and 7). All three swelling conditions show a permeant anion requirement. The K^+-equilibrating antibiotic, Nigericin, largely inhibited the swelling observed in the presence of T_3 + Mg^{2+} in a K^+ acetate medium. Substrate-supported swelling in the presence of T_3 + Mg^{2+} showed the same pronounced inhibitory effect of PCMB [Fig. 5(B)] as was shown for such swelling in the absence of Mg^{2+} [16] or in the presence of valinomycin. By contrast, as shown previously,[16] 5 mM Mg^{2+} inhibited markedly the swelling which occurred in a Mg^{2+}-free medium [Fig. 5(B)], but it did not inhibit the swelling which occurred in

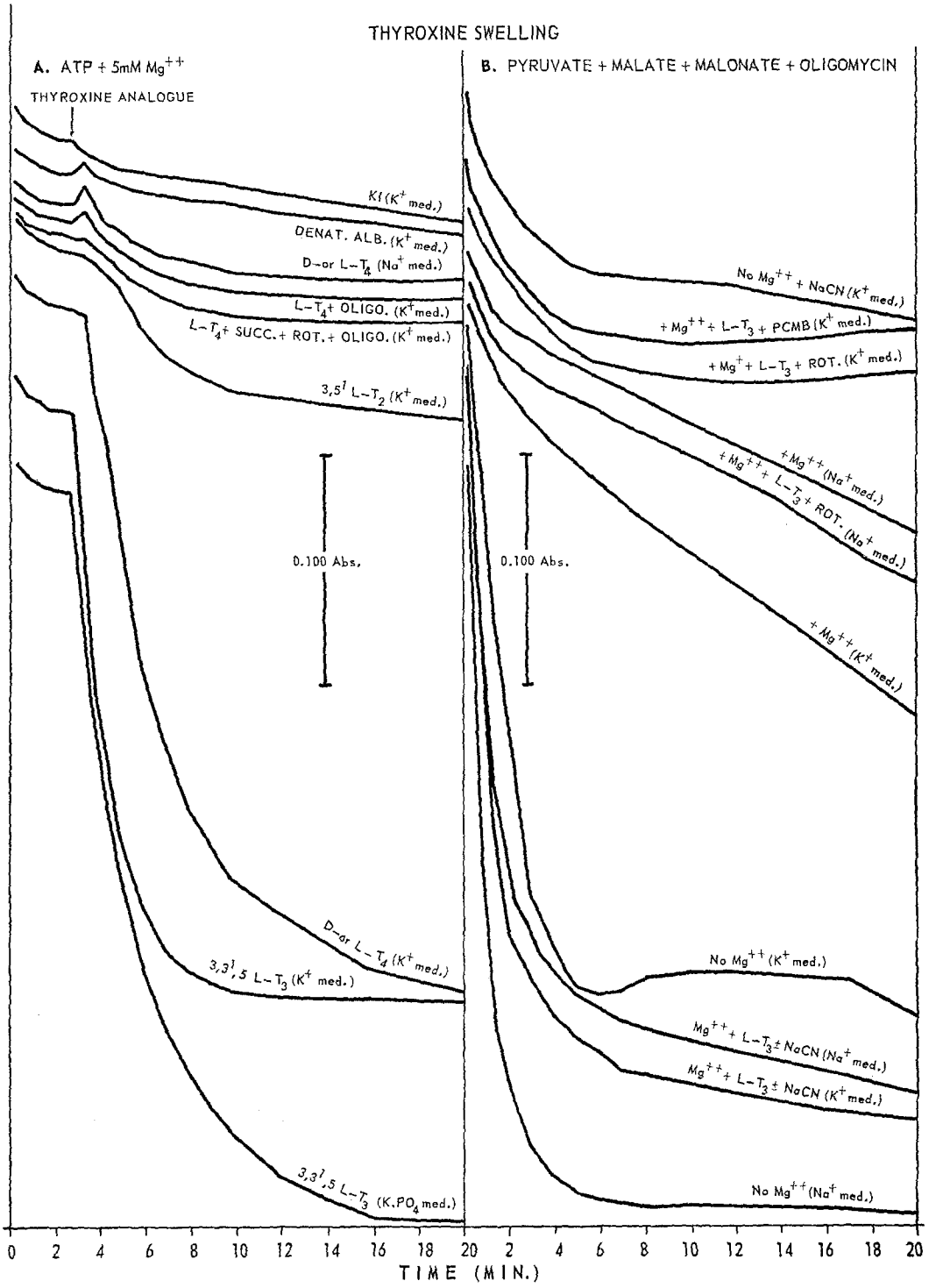

ATP + VALINOMYCIN SWELLING ($PO_4^=$ MEDIUM)
MITOCHONDRIA PREINCUBATED WITH OLIGOMYCIN
(SWELLING MEASURED AT 0.5–5.0 MIN.)
(SWELLING MEASURED AT 20.0–27.0 MIN.)
(a)
(b)
ATP + VALINOMYCIN SWELLING ($PO_4^=$ MEDIUM)
(SWELLING MEASURED AT 0.5–5.0 MIN.)
(SWELLING MEASURED AT 20.0–27.0 MIN.)
(c)
(d)
ATP + VALINOMYCIN SWELLING (ACETATE MEDIUM)
(SWELLING MEASURED AT 10.0–14.0 MIN.)
NO VALINOMYCIN
(SWELLING MEASURED AT 7.5–13.5 MIN.)
+ VALINOMYCIN
(e)
(f)
NO. MITOCHONDRIA/ML. (X10^5)
0 1 2 3 4 5 6
0.5 1.0 1.5 2.0 2.5 3.0
0.5 1.0 1.5 2.0 2.5 30
MITOCHONDRIAL VOLUME (μ^3)

the presence of T_3 [Fig. 5(B)] or valinomycin (Fig. 7). Also, the substrate-supported swelling which occurred in a Mg^{2+}-free medium was markedly inhibited in the presence of cyanide, but the same solution of this respiratory inhibitor failed to affect substrate-supported swelling in the presence of the hormone [Fig. 5(B)]. On the other hand, rotenone markedly inhibited all three kinds of swelling in the presence of DPN-linked substrates. An apparent lack of involvement of electron transfer through the terminal end of the respiratory chain in the swelling process in the presence of thyroid hormone was substantiated by studies employing succinate as respiratory substrate. Although succinate oxidation in the presence of rotenone is largely uncoupled in these skeletal muscle mitochondria, this substrate combination failed to support swelling in the presence of hormone with either alkali metal cation. By contrast, the cyanide-sensitivity observed for substrate-supported swelling in the absence of Mg^{2+} and in the presence of valinomycin was confirmed by the observance of support for swelling by succinate in the presence of rotenone.

Respiratory Activity in Swelling Circumstances

Figure 8 shows the polarographic measurement of respiratory activity in the three swelling circumstances. Respiratory activity in the presence and absence of thyroxine analogues is shown in Fig. 8(B). Trace 1 shows the tight control of respiration by phosphorylation which is characteristic of these EDTA-treated skeletal muscle mitochondria in the presence of DPN-linked substrates and Mg^{2+}. It also shows that the addition of T_4 prevents the activation of respiration which normally occurs upon the addition of ADP as energy acceptor to such a system. Trace 2 shows that both T_4 and T_3 inhibit not only the basal respiratory rate observed in a similar Mg^{2+}-containing medium, but also the uncoupled respiration regularly observed in a Mg^{2+}-free medium containing oligomycin.[16] T_4 was also found to inhibit valinomycin-activated uncoupling of respiration. Denatured albumin failed to duplicate the findings observed for the thyroxine analogue-albumin complexes. Trace 3 shows that although the initial inclusion of T_3 in a medium lacking Mg^{2+} prevents the uncoupling of respiration which normally occurs in such a medium, the inclusion of diiodothyronine-albumin complex (T_2) in a similar medium fails to prevent the uncoupling. Trace 4 shows that T_4 also inhibits the uncoupled respiration that normally occurs in the presence of succinate plus rotenone. The findings are in agreement with those of Graven *et al.*[18] that thyroxine does not release the oligomycin inhibition of tightly coupled respiration. They indicate also that thyroxine, like Mg^{2+},[16] acts to inhibit such uncoupling of respiration. The findings indicate that thyroxine acts to inhibit oxygen uptake. Indeed, findings with the dual wavelength spectrophotometer in swelling circumstances (Table I) confirmed the findings of Chance and Hollunger[33] and of Hess and Brand[34] and showed an inhibitory effect of thyroxine on both the rate and the extent of DPN reduction in the presence of DPN-linked substrates and NaCN. An effect of thyroxine on extent of reduction is also indicated

Figure 4. Coulter counter measurements of energy-dependent valinomycin-induced change in mitochondrial volume. The composition of the media was as follows: Tris PO_4, pH 7·4, 20 mM or Tris acetate, pH 7·4, 40 mM; KCl, 2·0 mM; Tris HCl, pH 7·4, 2·0 mM; Tris ATP, 5·0 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 6·2 μg protein/ml (phosphate medium), 5·4 μg protein/ml (acetate medium); valinomycin, 83 ng/ml; ± oligomycin, 6 μg/ml.


THYROXINE SWELLING
A. ATP + 5mM Mg^{++}
THYROXINE ANALOGUE
KI (K^+ med.)
DENAT. ALB. (K^+ med.)
D-or L-T_4 (Na^+ med.)
L-T_4 + OLIGO. (K^+ med.)
L-T_4 + SUCC. + ROT. + OLIGO. (K^+ med.)
3,5' L-T_2 (K^+ med.)
0.100 Abs.
D-or L-T_4 (K^+ med.)
3,3',5 L-T_3 (K^+ med.)
3,3',5 L-T_3 ($K.PO_4$ med.)
B. PYRUVATE + MALATE + MALONATE + OLIGOMYCIN
No Mg^{++} + NaCN (K^+ med.)
+ Mg^{++} + L-T_3 + PCMB (K^+ med.)
+ Mg^+ + L-T_3 + ROT. (K^+ med.)
+ Mg^{++} (Na^+ med.)
+ Mg^{++} + L-T_3 + ROT. (Na^+ med.)
+ Mg^{++} (K^+ med.)
0.100 Abs.
No Mg^{++} (K^+ med.)
Mg^{++} + L-T_3 ± NaCN (Na^+ med.)
Mg^{++} + L-T_3 ± NaCN (K^+ med.)
No Mg^{++} (Na^+ med.)
0 2 4 6 8 10 12 14 16 18 20
2 4 6 8 10 12 14 16 18 20
TIME (MIN.)

in the respiratory studies where a smaller protein concentration and thus a larger thyroxine: protein ratio showed complete inhibition of oxygen uptake. The findings suggest that thyroxine acts as an electron acceptor.

Respiratory activity in the presence and absence of valinomycin and/or Mg^{2+} is shown in Fig. 8(A). Traces 1 and 3 show a pronounced activation of respiration by the addition of valinomycin to a Mg^{2+}-free swelling medium containing acetate and K^+, with pyruvate plus malate, in the presence of malonate and oligomycin, as substrate. Contrary to the findings for swelling activation (Fig. 7) the enhancement of respiration was greater at 150 mM K^+ than at 5 mM K^+ concentration. These findings of an uncoupling action of valinomycin on skeletal muscle mitochondria are in agreement with the early findings of Moore and Pressman[35] who reported an uncoupling action of valinomycin in rat liver mitochondria. But Mg^{2+} restored the valinomycin-activated respiratory activity to its basal rate or prevented the activation of respiration when valinomycin was subsequently added to the medium (traces 2 and 3). In agreement with earlier findings,[16,22] Mg^{2+} also imposed respiratory control in the absence of valinomycin (traces 2 and 3). By contrast, the uncoupled respiration observed with succinate + rotenone failed to show activation by valinomycin or inhibition by Mg^{2+}. These findings of Mg^{2+} control of respiration, even in the presence of valinomycin, support the earlier suggestion[16] that the presence of such a principle in the mitochondrial structure may play the role of a regulator of the extent to which generated high-energy bonds are exposed to hydrolysis and may thus constitute a basis for metabolic control. But, the extent of swelling which occurs under conditions which permit valinomycin uncoupling is not significantly different from that which occurs when the presence of Mg^{2+} prevents uncoupling (see Fig. 7), suggesting that uncoupling is not accounted for by swelling. Similarly, ATP-supported swelling changes in the presence of valinomycin are accompanied by very low rates of K^+-specific ATP hydrolysis (Fig. 9). Although higher levels of K^+ support higher rates of valinomycin-activated ATPase activity, they do not support a higher rate or degree of swelling (see Fig. 2). Mg^{2+} failed to inhibit the low rate of K^+-activated ATPase which is supported by valinomycin in swelling circumstances (Fig. 9); it also failed to inhibit valinomycin-induced swelling in these circumstances. Apparently swelling in the presence of valinomycin is supported by the basal respiratory rate or by an equally low rate of K^+-specific ATPase activity. The findings support the contention of Pressman[31] that the energy required to initiate cation influx is small.

Figure 5. A comparison of specificities involved in absorbance change in the absence of Mg^{2+} and in the presence of thyroxine + Mg^{2+}. The composition of the media were as follows:

(*a*) *ATP-supported swelling:* Acetate, as the Na^+ or K^+ salt, 20 mM, with NaCl or KCl, to give a total monovalent cation concentration of 130 mM (or Tris phosphate, 10 mM, with KCl, 110 mM); Tris HCl, pH 7·4, 2·0 mM; ± $MgCl_2$, 5·0 mM; ATP, 5·0 mM (succinate, 20 mM + rotenone, 5×10^{-7} M + oligomycin, 6 μg/ml, + cytochrome *c*, 5 μM, + DPN, 400 μM, was substituted for ATP where cited); bovine serum albumin, 0·20%; mannitol, to bring to isotonicity; Na_2EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·20 mg protein/ml. KI, diiodothyronine (L-T_2), triiodothyronine (L-T_3), and thyroxine (L-T_4) were added at the times indicated, to give a final concentration of 0·5 mM; all were added as neutral solutions complexed with albumin (except T_2 which was neutralized to pH 8·2). The various thyroxine-analogue solutions had varying degrees of stability at neutral pH.

(*b*) *Substrate-supported swelling:* Acetate, as the Na^+ or K^+ salt, pH 7·4, 20 mM; NaCl or KCl, to give a total monovalent cation concentration of 150 mM; Tris HCl, pH 7·4, 2·0 mM; pyruvate + malate + malonate, 10 mM each, + oligomycin, 6 μg/ml; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; cytochrome *c*, 5 μM; DPN, 400 mM; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·20 mg protein/ml; ± triiodothyronine (L-T_3)-albumin complex, 0·5 mM; ± $MgCl_2$, 5·0 mM; ± PCMB, 0·25 mM; ± rotenone, 5×10^{-7} M; ± NaCN, 0·8 mM.

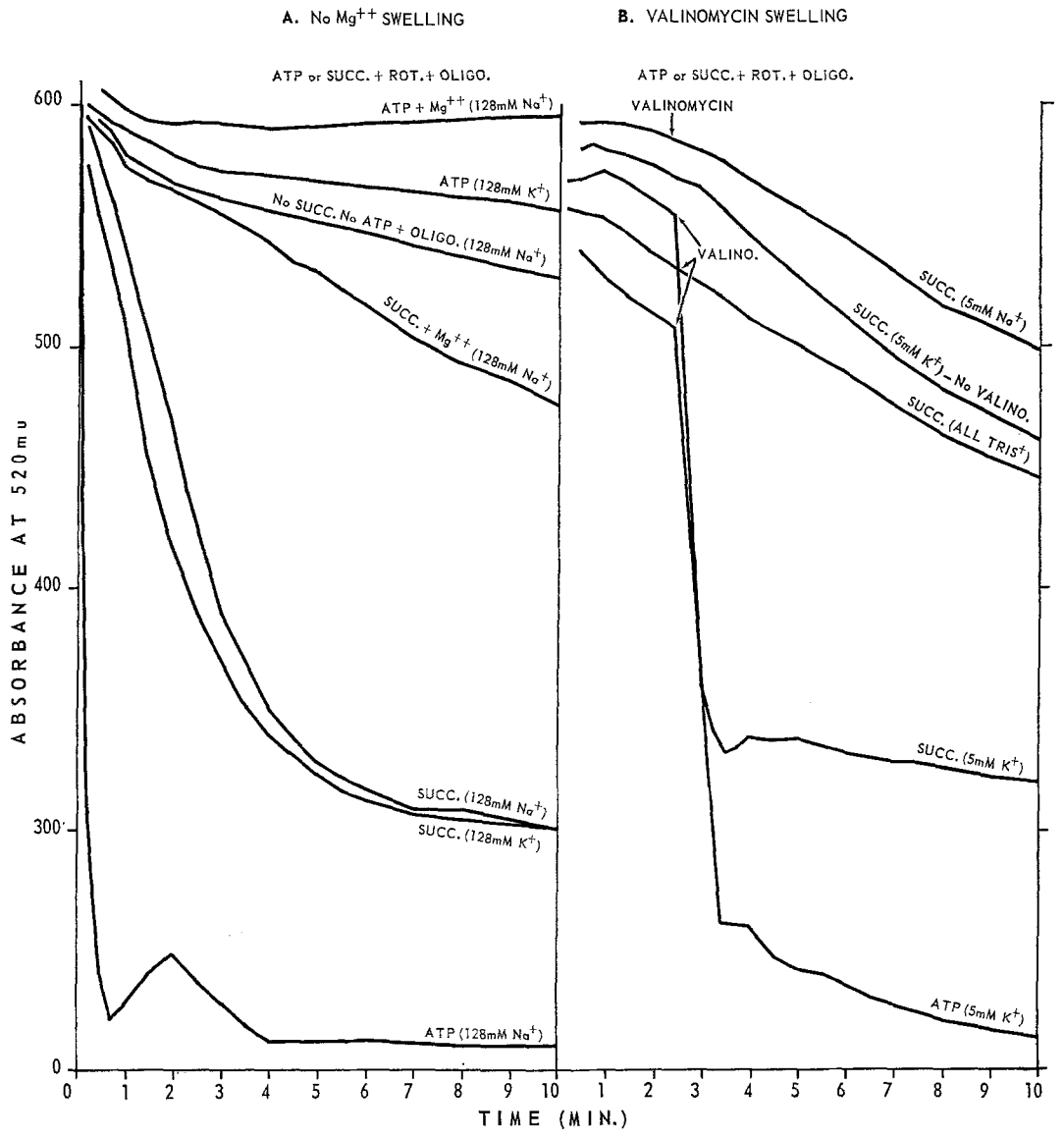


Figure 6. A comparison of specificities involved in absorbance change in the absence of Mg^{2+} and in the presence of valinomycin. The composition of the media was as follows:

(*a*) *Swelling in the absence of* Mg^{2+}: Acetate, as the Na^+ or K^+ salt, pH 7·4, 20 mM; NaCl or KCl, to give a total monovalent cation concentration of 128 mM; Tris HCl, pH 7·4, 2·0 mM; Tris ATP, 5·0 mM, or succinate, 20 mM + rotenone, 5×10^{-7} M, + oligomycin, 6 μg/ml, + cytochrome *c*, 5 μM, + DPN, 400 μM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0.5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·20 mg protein/ml.

(*b*) *Swelling induced by valinomycin:* Tris acetate, pH 7·4, 20 mM; KCl, NaCl or Tris HCl, 5·0 mM; Tris HCl, pH 7·4, 2·0 mM; Tris ATP, 5·0 mM or succinate, 20 mM + rotenone, 5×10^{-7} M, + oligomycin, 6 μg/ml, + cytochrome *c*, 5 μM, + DPN, 400 μM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·20 mg protein/ml; valinomycin 83 ng/ml.

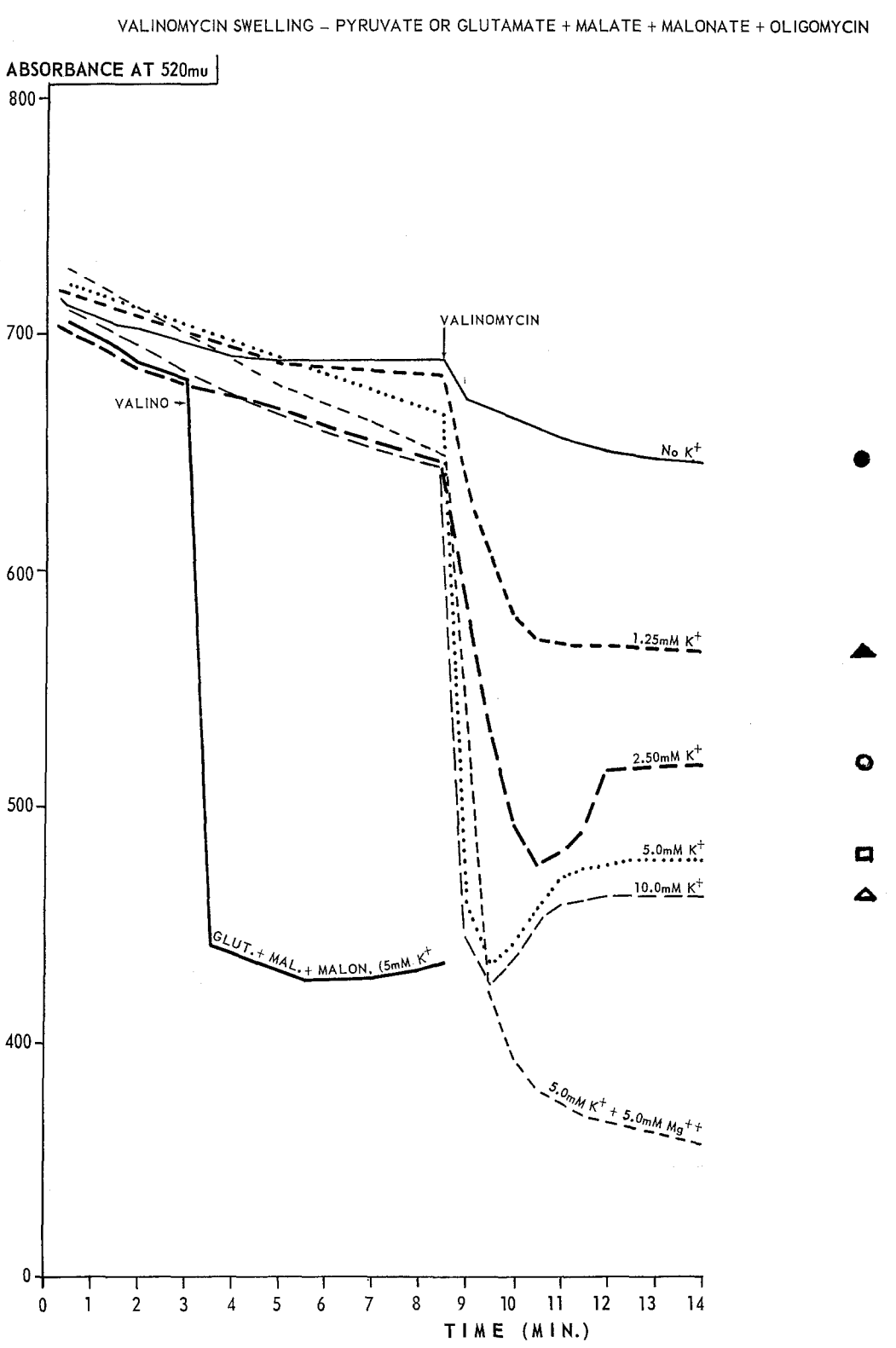
ATPase Activity in Swelling Circumstances

Figure 9 shows that, contrary to the situation for the other two kinds of swelling, thyroxine fails to promote measurable P_i release under swelling conditions with ATP in an acetate medium. Indeed, ATPase activity was the same for a K^+ medium which supports measurable swelling as for a Na^+ medium which does not. Thyroxine also failed to promote pyrophosphatase activity under these conditions. But the preferential transport of phosphate which occurs with ATP as energy source in the presence of thyroxine could obscure measurement of P_i release. Consequently, ATP-dependent pH changes under swelling and non-swelling circumstances were measured (see Table II). It is apparent here that although large pH changes occur under swelling circumstances in the absence of Mg^{2+} (Na^+ acetate medium) and in the presence of valinomycin without Mg^{2+} (K^+ acetate medium), nevertheless pH changes in swelling circumstances in the presence of thyroxine $\pm$ Mg^{2+} (K^+ phosphate medium) are scarcely different from changes observed in non-swelling circumstances (Na^+ phosphate + thyroxine; Na^+ acetate + oligomycin or K^+ acetate in Mg^{2+}-free circumstances). However, since ATP-supported swelling changes in the presence of valinomycin + Mg^{2+} were accompanied by a very small pH change, as well as by very low rates of K^+-specific ATPase activity, it is apparent that the energy requirement for swelling is small. Further, it is apparent in Table II that a pH change of minor proportions could be obscured by the buffering capacity of the permeable anion required for swelling. The findings obtain support from those of Van Dam and Ter Welle[36] who showed that relatively little energy is needed to drive the energy-linked transhydrogenase.

It is apparent in Fig. 9 that addition of T_4 even inhibits both the Mg^{2+}- and Na^+-activated ATPases normally present in these skeletal muscle mitochondria. The findings are at variance with those of Chance and Hollunger[33] and of Bronk[37] who reported activation of the Mg^{2+}-ATPase of liver mitochondria by thyroxine *in vitro*. However, they are in agreement with those of Lindberg *et al.*[38] who reported thyroxine inhibition of both DNP- and Mg^{2+}-activated ATPases, as well as P_i-ATP exchange.

Discussion

The present findings show many similarities between energy-dependent swelling promoted by thyroxine and that promoted by the lipid-soluble K^+ carrier, valinomycin. Graven *et al.*[18] had concluded that thyroxine induces osmotic swelling by a mechanism which is distincly different from that of lipid-soluble antibiotics because it does not release the oligomycin inhibition of tightly coupled respiration. However, the present findings of Mg^{2+} inhibition of valinomycin-activated uncoupling without inhibition of valinomycin swelling show that uncoupling is not accounted for by swelling and that the substrate or ATP energy required to initiate and maintain swelling is small. Lardy *et al.*[8] had already put thyroxine in the same category as valinomycin on the basis of insensitivity to aurovertin, although these agents still show a different concentration requirement for alkali-metal cations. Now evidence has been presented that, as suggested by Mitchell,[39] pH or membrane electrostatic gradients in respiring mitochondria may limit the penetration of ions. Agents such as valinomycin, which alter the permeability of the membrane, alter the status of these gradients and therefore can cause large volume changes.[40] The findings suggest the possibility that, as with the lipid-soluble K^+


VALINOMYCIN SWELLING – PYRUVATE OR GLUTAMATE + MALATE + MALONATE + OLIGOMYCIN
ABSORBANCE AT 520mμ
800
700
600
500
400
0
VALINOMYCIN
VALINO
No K+
1.25mM K+
2.50mM K+
5.0mM K+
10.0mM K+
GLUT.+ MAL.+ MALON. (5mM K+
5.0mM K+ + 5.0mM Mg++
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME (MIN.)

carrier, valinomycin, energy-linked changes in membrane electrostatic gradients could account for the osmotic swelling observed when thyroxine promotes a change in permeability to cations.

The present findings show many similarities between energy-dependent swelling promoted by the presence of thyroxine and that promoted by the absence of Mg^{2+}. Removal of Mg^{2+} from the membrane permitted alkali metal cation permeability and energy-dependent swelling. However, Mg^{2+} removal also caused respiratory uncoupling, even in the absence of alkali metal cation.[16,22] The addition of Mg^{2+} prevented the swelling which occurred in its absence and also imposed control of respiration by phosphorylation. However, Mg^{2+} did not inhibit thyroxine-activated swelling. Indeed, thyroxine at a concentration of 5×10^{-4} M promoted active swelling in the presence of 5 mM Mg^{2+}. Further, thyroxine in the presence of Mg^{2+} acted very specifically to promote a K^+-specific ATP-dependent swelling without exposing the Na^+-specific ATPase which becomes functional in Mg^{2+}-depleted mitochondria when albumin is added to prevent spontaneous ATP hydrolysis.[16,20,22] Although binding of Mg^{2+} has been suggested as the mechanism of action of thyroxine,[41] it is apparent that thyroxine does not promote swelling simply because it chelates Mg^{2+}. Rather, the findings suggest that thyroxine promotes a selective change in accessibility of cations, possibly by effecting a change in membrane tertiary structure.

Scott and Hunter[2] have concluded that any one of the three energy conservation sites associated with electron transfer can, by itself, support swelling induced by thyroxine. However, in contrast to the present findings, these workers failed to demonstrate a permeable anion requirement for such swelling. Permeability to Cl^- can be induced following modification of the mitochondrial membrane by interaction with Zn^{2+} or other divalent cations.[42] Also swelling has been shown to be strikingly potentiated when thyroxine and micromolar concentrations of Ca^{2+} or Zn^{2+} are tested in the presence of each other and such swelling does not show a requirement for added permeant anion.[14] The latter results suggested that attempts to demonstrate specific swelling with the hormone would be futile unless rigorous precautions were taken to assure a very low metal contamination. The present data were obtained under circumstances which precluded significant involvement with such divalent cations. The findings indicate that electron transfer through the terminal end of the respiratory chain is not involved in support of thyroxine-activated swelling. Indeed, an effect of thyroxine on DPNH oxidation in the presence of cyanide has been demonstrated in swelling circumstances. The findings suggest that thyroxine acts at the first coupling site as an electron acceptor. The finding that the energy requirement for swelling is small, even with ATP as energy source, supports such a concept. Indeed, the concept finds support in other work. An inhibitory effect of triiodothyronine on electron transfer between F_p and cytochrome *b* has been demonstrated.[43] Also, mitochondria from thyroxine-injected rats behaved towards amytal as if an agent capable of effecting energy transfer (thyroid hormone?) were

Figure 7. The dependency of valinomycin-activated swelling on monovalent cation concentration. The composition of the media was as follows: Tris acetate, pH 7·4, 20 mM; Tris HCl, pH 7·4, 12·0 mM, replaced by KCl to give the levels of K^+ indicated; Tris pyruvate + Tris malate + Tris malonate, 10 mM each, + oligomycin, 6 μg/ml, or Tris glutamate + Tris malate + Tris malonate, 10 mM each, + oligomycin, 6 μg/ml; cytochrome *c*, 5 μM; DPN, 400 μM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·21 mg protein/ml; valinomycin, 83 ng/ml.

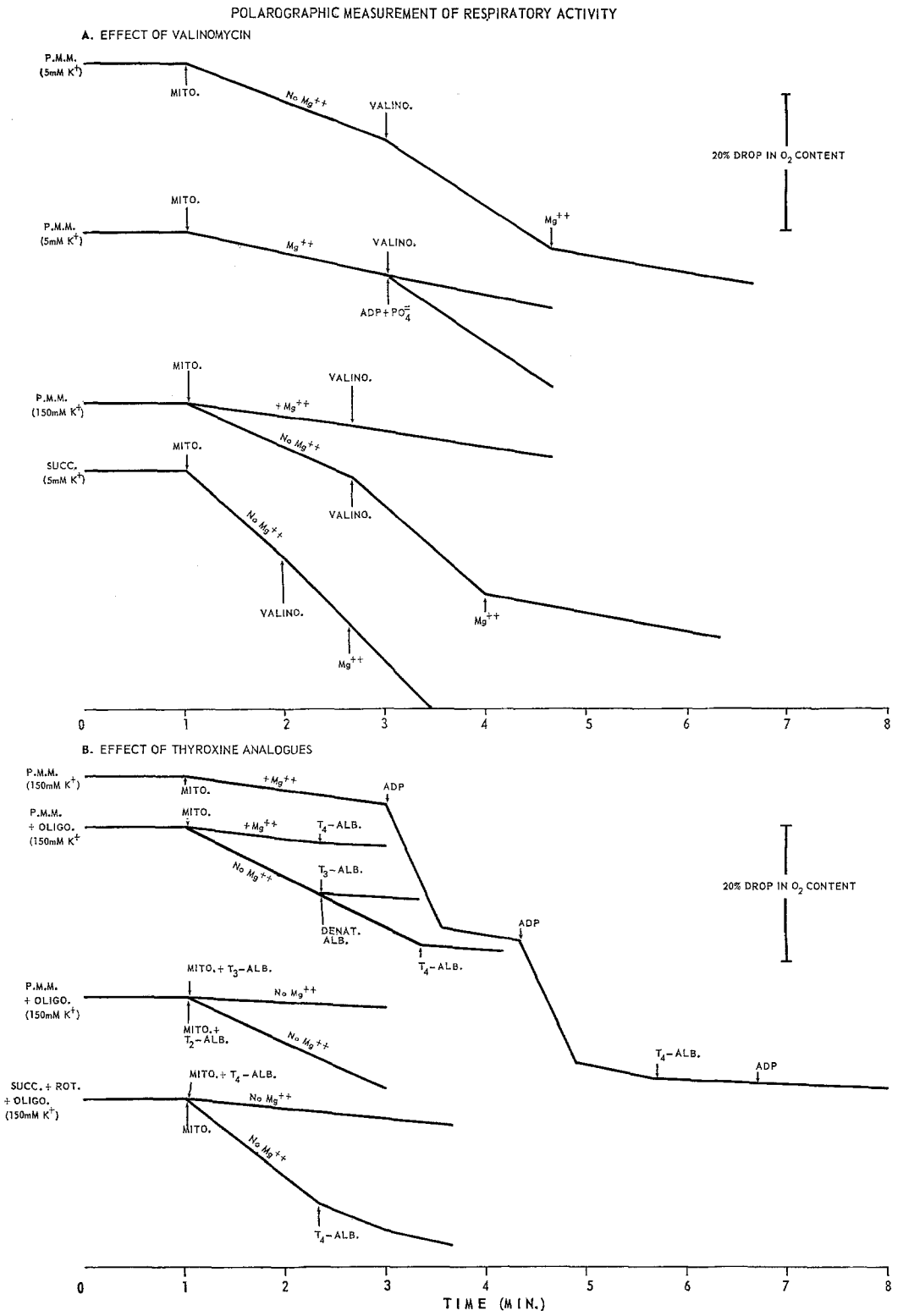

POLAROGRAPHIC MEASUREMENT OF RESPIRATORY ACTIVITY
A. EFFECT OF VALINOMYCIN
P.M.M. (5mM K+)
MITO.
No Mg++
VALINO.
20% DROP IN O2 CONTENT
Mg++
P.M.M. (5mM K+)
MITO.
Mg++
VALINO.
ADP + PO4--
P.M.M. (150mM K+)
MITO.
+ Mg++
VALINO.
No Mg++
SUCC. (5mM K+)
MITO.
No Mg++
VALINO.
Mg++
B. EFFECT OF THYROXINE ANALOGUES
P.M.M. (150mM K+)
MITO.
+ Mg++
ADP
P.M.M. + OLIGO. (150mM K+)
MITO.
+ Mg++
T4 - ALB.
No Mg++
T3 - ALB.
DENAT. ALB.
T4 - ALB.
ADP
20% DROP IN O2 CONTENT
P.M.M. + OLIGO. (150mM K+)
MITO. + T3 - ALB.
No Mg++
MITO. + T2 - ALB.
T4 - ALB.
ADP
SUCC. + ROT. + OLIGO. (150mM K+)
MITO. + T4 - ALB.
No Mg++
MITO.
T4 - ALB.
0 1 2 3 4 5 6 7 8
TIME (MIN.)

TABLE I. Effect of thyroxine on DPN reduction (340–374 mμ absorbance)

Incubation conditions	Final absorbance	Extended absorbance
P.M.M.* + oligo. + Mg^{2+} + mito. + NaCN	(a) 0·354 (0·8 min)	(a) 0·354 (6·0 min)
	(b) 0·330 (0·5 min)	(b) 0·332 (5·0 min)
P.M.M.* + oligo. + Mg^{2+} + T_3 + mito. + NaCN	(a) 0·280 (6·0 min)	(a) 0·275 (9·0 min)
	(b) 0·260 (4·0 min)	(b) 0·255 (8·0 min)

* P.M.M.= pyruvate + malate + malonate.
The composition of the media was the same as that used for substrate-supported swelling in Fig. 5(B) except that the protein concentration was 1·5–1·75 mg/ml.

present.[44] Thyroxine acted in the vicinity of the first coupling site to inhibit the energy-linked transhydrogenase.[45] Also, as confirmed under the present swelling conditions, thyroxine was shown to depress energy-dependent reduction of pyridine nucleotide.[33,44]

Thyroxine analogues have been shown to form charge-transfer complexes and to be good electron donors under physiological conditions; however, there is no simple relationship between electron donor properties of various substituted phenols and thyromimetic activity of the corresponding thyroxine analogues.[46] On the other hand, Mulliken[47] has concluded from a variety of physical and chemical evidence that halogens in organic halides may serve both as donors and also as acceptors of electrons. The present findings suggest an electron acceptor function for thyroxine analogues and this role is apparently specific for biologically-active compounds. Indeed the findings suggest that thyroxine in higher concentration acts to inhibit oxygen-uptake by acting as an electron sink.

The chemiosmotic hypothesis of Mitchell[39] couples the flow of electrons against the flow of hydrodehydration particles and such an energy-dependent separation of charges across the mitochondrial membrane has been used to explain energy-dependent osmotic swelling in a variety of circumstances.[40] Since thyroxine appears to promote a flow of

Figure 8. Polarographic measurement of respiration. The composition of the media were as follows:
(*a*) *Effects of valinomycin:* Tris or K^+ acetate, pH 7·4, 20 mM; KCl, to give a total alkali metal cation concentration of 5 or 150 mM; Tris HCl, pH 7·4, 2·0 mM; pyruvate + malate + malonate, 10 mM each, or Tris succinate, 20 mM + rotenone, 5×10^{-7} M; ± oligomycin, 6 μg/ml; ± $MgCl_2$, 5·0 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0.20 mg protein/ml; ± valinomycin, 83 ng/ml. Where indicated, the following additions were made: ADP, 0·4 μmole, + Tris PO_4 → 5·0 mM.
(*b*) *Effects of thyroxine analogues:* K^+ phosphate, pH 7·4, 20 mM; KCl, to give a total alkali metal cation concentration of 150 mM; Tris HCl, pH 7·4, 2·0 mM; pyruvate + malate + malonate, 10 mM each, or Tris succinate, 20 mM + rotenone, 5×10^{-7} M; oligomycin, 6 μg/ml; ± $MgCl_2$, 5·0 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·20 mg protein/ml; ± thyroxine (T_4), tri-iodothyronine (T_3) or diiodothyronine (T_2), as the thyroxine analogue-albumin complexes, 0·5 mM. Where indicated, 0·4 μmole aliquots of ADP were added.

electrons and since it promotes osmotic swelling under the same circumstances, it would appear that hormone-induced swelling might also be explained on the basis of energy-dependent separation of charges across the membrane. On such a basis osmotic swelling could be explained either by an energy-dependent change in permeability to monovalent cations or by an electrogenic buildup of ionized acid when unionized permeant acid is used for neutralization of the negative potential of the internal compartment. But equilibration of H^+ across the membrane as the result of an anionic electrogenic

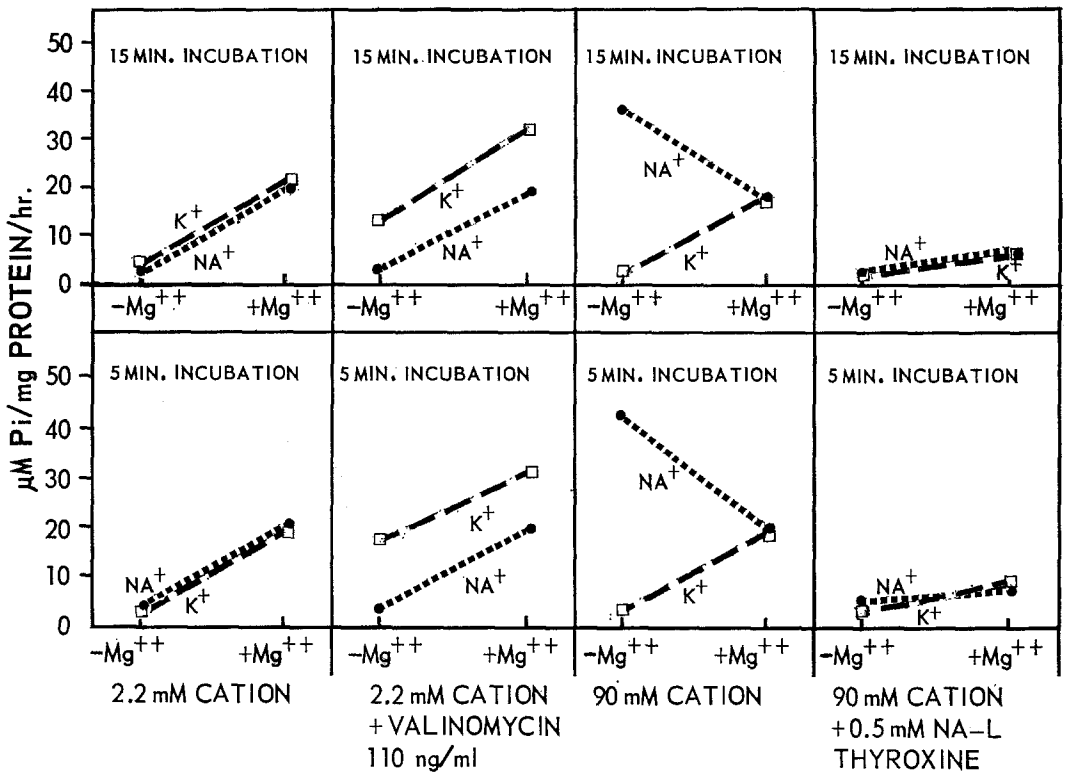


Figure 9. ATPase activity in swelling circumstances. The composition of the media was as follows: Tris acetate, pH 7·4, 40 mM; Na^+ or K^+, added as NaCl or KCl, as recorded on graph; Tris HCl, pH 7·4, 2·0 mM; Tris ATP, 5·0 mM; ± $MgCl_2$, 5·0 mM; bovine serum albumin, 0·2%; mannitol, to bring to isotonicity; Na_2.EDTA (neut. with K^+), 0·5 mM; heparin (from isolation medium), 0·01%; mitochondria, 0·075 mg protein/ml; ± valinomycin, 110 ng/ml; ± Na-L-thyroxine (albumin complex), 0·5 mM; incubation time, 5 or 15 min; incubation temp. 23° C.

buildup would promote further respiration, even in the absence of monovalent cation. Indeed, such an uncoupling has been reported with Na^+, K^+ or $Tris^+$ salts of permeant acids or substrate acids alone in a Mg^{2+}-free medium.[16] The fact that substrate oxidation did not promote swelling with $Tris^+$ in such a medium, even although respiration was uncoupled, was interpreted to indicate that exchange with the large $Tris^+$ molecule was the rate-limiting factor. The findings suggested that neutralization of the charged state of the membrane may occur in the absence of or prior to osmotic swelling in a Mg^{2+}-free system and this may be responsible for the establishment of a pH gradient and the resultant swelling. But such a respiratory uncoupling does not occur in the pre-

TABLE II. ATP-dependent pH changes under swelling and non-swelling circumstances

	Incubation conditions	Initial pH	Final pH*	pH change (pH units)	pH change (%)
1	ATP no Mg^{2+} (20 mM K^+ acetate, 106 mM KCl)	7·375	7·319	0·056	0·76
2	ATP no Mg^{2+} (20 mM Na^+ acetate, 106 mM NaCl) + oligomycin	7·360	7·306	0·054	0·73
3	ATP no Mg^{2+} (20 mM Na^+ acetate, 106 mM NaCl)	7·365	7·100	0·265	3·60
4	ATP no Mg^{2+} no Mito (20 mM Na^+ acetate, 106 mM NaCl) + Mg^{2+}, no Mito (change complete at 1·5 min)	7·366	7·294	0·072	0·97
	+ mito (added at 3·0 min)	7·305	7·290	0·015	0·20
5	ATP + Mg^{2+} (10 mM Tris PO_4, 110 mM KCl)	7·227	7·164	0·063	0·87
	+ thyroxine (added at 3·0 min)	7·180	7·123	0·057	0·80
6	ATP + Mg^{2+} (10 mM Tris PO_4, 110 mM NaCl)	7·220	7·138	0·082	1·13
	+ thyroxine (added at 3·0 min)	7·164	7·110	0·054	0·75
7	ATP no Mg^{2+} (10 mM Tris PO_4, 110 mM KCl)	7·324	7·314	0·010	0·14
	+ thyroxine (added at 3·0 min)	7·330	7·270	0·060	0·82
8	ATP no Mg^{2+} (10 mM Tris HCl, 110 mM KCl)	7·267	7·245	0·022	0·30
	+ thyroxine (added at 3·0 min)	7·270	7·230	0·040	0·55
9	ATP no Mg^{2+} (20 mM Tris HCl, 110 mM KCl)	7·240	7·196	0·044	0·61
	+ DNP (added at 3·0 min)	7·196	6·915	0·281	3·90
10	ATP no Mg^{2+} (20 mM Tris PO_4, no alkali cation + valino	7·402	7·390	0·012	0·16
	+ KCl (added at 3·0 min)	7·390	7·210	0·180	2·43
11	ATP + Mg^{2+}† (20 mM Tris PO_4, no alkali cation)	7·350	7·085	0·265	3·60
	+ valino + KCl (added at 3·0 min)	7·085	7·010	0·075	1·05
12	ATP no Mg^{2+} (20 mM Tris acetate, no alkali cation + valino	7·370	7·340	0·030	0·40
	+ KCl (added at 3·0 min)	7·340	7·022	0·318	4·33
13	ATP no Mg^{2+} (no anion, no alkali cation + valino)	7·290	7·260	0·030	0·41
	+ KCl → 5 mM (added at 3·0 min)	7·260	6·800	0·460	6·33
	+ Tris HCl, pH 7·4 → 20 mM	6·800	6·965	+ 0·295	4·34

* pH after 20 min. incubation.
† Mg^{2+} added in increments.
The reaction was started in all cases by the addition of mitochondria at zero time. In addition to the components outlined, all media also contained the following: bovine serum albumin, 0·20%; Tris HCl, pH 7·4, 2·0 mM; Tris ATP, 5·0 mM; mannitol, to bring to isotonicity; mitochondrial protein, 1 mg/ml. Other reagents were added, where indicated, to give the following concentrations: $MgCl_2$, 5·0 mM; oligomycin, 6 μg/ml; thyroxine-albumin complex, 5×10^{-4} M; DNP, 5×10^{-4} M; KCl, 5·0 mM; valinomycin, 83 ng/ml)

sence of Mg^{2+}, even when valinomycin is added. Indeed, respiration is controlled by phosphorylation in the presence of Mg^{2+}. It would seem unlikely then that equilibration of H^+ across the membrane with its attendant anionic electrogenic buildup can occur in these circumstances. Consequently, the activation of swelling which results when thyroxine is superimposed in such a Mg^{2+}-containing medium must be the result of the selective change in accessibility of cations which is promoted by the hormone. A similar selective change in cation accessibility is promoted by valinomycin, even in the presence of Mg^{2+}. Such a change in cation permeability could alter the status of electrical gradients in the membrane since the valinomycin-catalysed translocation of K^+ in phospholipid micelles appears to be electrogenic.[48] Further, a relationship has been demonstrated

between the increase in internal alkalinity which accompanies uptake of cations and the accumulation of anions.[49] Indeed, Mitchell's hypothesis[37] includes a cation influx leading to charge neutrality and an anion exchange down a pH gradient leading to pH neutrality. Thus it is apparent that a change in cation permeability, by altering the status of electrical gradients in the membrane, could cause large volume changes. The findings suggest that thyroxine promotes swelling by promoting such an energy-dependent change in accessibility to cations, possibly by effecting a change in membrane tertiary structure.

Summary

Divalent cation-depleted skeletal muscle mitochondria undergo energy-dependent swelling in the presence of thyroxine analogues + Mg^{2+}, as well as in the presence of valinomycin or the absence of Mg^{2+}. ATP-supported swelling shows a K^+-specificity in the presence of thyroxine analogues or valinomycin, in contrast to a Na^+-specificity in the absence of Mg^{2+}. Substrate-supported swelling shows a K^+-specificity in the presence of valinomycin but fails to show an alkali metal cation specificity under the other two swelling conditions. All three kinds of swelling show a permeant anion dependency. Although Mg^{2+} inhibits the swelling which occurs in its absence and also inhibits uncoupling of respiration, even in the presence of valinomycin, nevertheless Mg^{2+} does not inhibit the energy-dependent swelling which occurs in the presence of valinomycin or thyroxine analogues. The findings show that thyroxine does not promote swelling simply because it chelates Mg^{2+}. Rather, they show that thyroxine promotes a selective change in accessibility of monovalent cations. They suggest that thyroxine in the presence of Mg^{2+} acts at the first coupling site as an electron acceptor. An observed inhibition of oxygen uptake would appear to be explained on the basis of thyroxine in higher concentration acting as an electron sink. The findings suggest that, as with the lipid-soluble K^+ carrier, valinomycin, in the presence of Mg^{2+}, a change in the status of electrical gradients in the membrane can account for the osmotic swelling observed in the presence of thyroxine analogues.

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