Mitochondrial Energy Flux and Ion-Induced Adenosine Triphosphatase Activity and Light-Scattering Changes Mediated by Gramicidin*

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ABSTRACT: The importance of energy flux to the uptake of monovalent cations, such as Na^+ and K^+, was examined with the use of light scattering and ion specific electrodes, and correlated with adenosine triphosphatase activity measured by H^+ release. K^+, but not Na^+, induces an appreciable increase in light transmission in the presence of Gramicidin when supported by adenosine triphosphate or succinate in a P_i-free medium. Furthermore, Na^+ prevents the K^+-induced change supported by adenosine triphosphate. However, Na^+ induces a light-scattering change when both adenosine triphosphate and 4 mM succinate are present, but with 1 mM malonate present or with lower concentrations of succinate the observed changes in light scattering are not maintained. This requirement for adenosine triphosphate in addition to succinate for an optimal Na^+-induced Gramicidin-mediated light-scattering change is also indicated by the finding that oligomycin caused the Na^+-induced light-scattering change supported by adenosine triphosphate and succinate in phosphate medium to return to the level observed prior to adenosine triphosphate addition. Further, the K^+-induced Gramicidin-mediated light-scattering change is greater than that induced by Na^+, and correspondingly the uptake of K^+ is larger than Na^+ uptake as measured with the ion-sensitive electrode. Analogous to the light-scattering changes, K^+, but not Na^+, stimulates adenosine triphosphate mediated H^+ release of Gramicidin-treated mitochondria when energy flux is low (*i.e.*, with ATP alone), and Na^+ prevents this stimulation by K^+. Furthermore, progressive changes in light scattering induced by successive additions of Na^+ or K^+ in the presence of succinate are comparable to progressive changes in adenosine triphosphate mediated H^+ release. These findings are compatible with the interpretation that in the presence of Gramicidin (and possibly in the absence of antibiotic) the energy flux which is required for K^+ transport is less than that for Na^+ transport, and that ion transport with its associated volume and/or internal structural changes as well as adenosine triphosphatase activity is not only dependent upon the energy supply but also on the total ionic environment.

In an attempt to correlate mitochondrial ATPase activity with ion movements, we have examined under varied conditions hydrogen ion release accompanying ATP breakdown and light-scatter changes associated with energy-dependent cation movements. A decrease in light scattering at 90° or an increase in transmission (0°) is observed due to the uptake of water, which occurs simultaneously with cation uptake (Pressman, 1967; Packer, 1967). The availability of transport-mediating antibiotics is extremely useful to study the relationship of ion movements to ATPase activity since they promote accelerated movements of selective monovalent cations which are normally slow and difficult to measure. Gramicidin is of especial interest since it promotes the movement of several monovalent cations, and for this study permits comparison of Na^+ and K^+ in inducing light-scattering changes and ATPase activity.

In our studies, we observed, that while K^+ ions induce a Gramicidin-mediated ATP-supported light-scattering change and stimulation of H^+ release in P_i-free media, Na^+ did not induce a light-scattering change supported by ATP. In contrast, when the respiratory substrate, succinate, was also included in the reaction medium, a marked light-scattering change and uptake of Na^+ as measured with the ion-sensitive electrode was noted. These findings suggest that the energy flux is crucial to the uptake of specific ions, and that the influence of the energy flux is also dependent upon the ionic environment.

In order to test further the energy requirements for ion movements, the effect of varying the energy flux on Na^+- or K^+-induced light-scattering changes was first examined with the use of alternate energy sources, and selective inhibitors of energy utilization. Finally the influence of K^+ and Na^+ on ATP-mediated H^+ release by Gramicidin-treated mitochondria was compared and attempts were made to establish the relationship between ion movements as measured by light-scattering changes and "ATPase" activity.

Materials and Methods

Mouse liver mitochondria were prepared as described earlier with the exception that 0.1 mM EDTA and 1.0 mM Tris were included in the mannitol–sucrose

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medium used for the isolation of mitochondria (Parsons *et al.*, 1966; Wenner, 1966). The twice washed mitochondria were suspended in a 225 mM mannitol plus 75 mM sucrose medium prior to use.

Several parameters were measured simultaneously in the study of antibiotic-induced ion movements by insertion of ion-sensitive electrodes in an apparatus which permitted measurement of both fluorescence and light scattering (Pressman, 1967), and which was built by the electronics instrument shop of the Johnson Research Foundation, University of Pennsylvania. The mixture was stirred with the use of a vibrator.

The method of measurement of light scattering was dependent upon the illumination of the sample with a collimated beam of light, wavelength 546 mμ, obtained with the use of Wratten filters 57 plus 21 and transmission which provides an indirect measurement of scattering was measured at 180° with the use of filters of the same wavelengths. An increase in transmission (decrease in optical density) reflects mitochondrial swelling (Gotterer *et al.*, 1961; Bartley and Enser, 1964), and under the conditions of our experiments, changes in transmission were observed to be inversely related to light-scatter changes at 90° as determined by independent measurements (*cf.* Packer, 1967). The transmission changes were calibrated by the insertion of appropriate neutral density filters of known optical density in the incident light path.

Sodium ions were monitored with the use of a sodium ion electrode (catalog no. 39046) obtained from Beckman Instruments, Inc., and the Beckman Research pH meter. Hydrogen ion changes were followed with a 5-mm diameter combination pH electrode probe (Arthur H. Thomas) and the use of the Beckman Research pH meter and a suitable recorder as described previously.

Valinomycin was kindly donated by Drs. J. C. MacDonald and B. C. Pressman, and Gramicidin (a mixture of A, B, and C) was obtained from Nutritional Biochemicals Corp. Rotenone was obtained from S. B. Penick. The di-Tris salt of ATP from equine muscle and the disodium salt of ATP (99–100%) purified to remove trace metals were purchased from Sigma Chemical Co. When Tris was required for neutralization purposes, the product used was the three-times-recrystallized base (initially with EDTA (Na) and two subsequent crystallizations with distilled water to remove traces of EDTA) obtained from General Biochemicals Corp. With the exception of mannitol obtained from Matheson Coleman and Bell, other reagents were obtained from Sigma Chemical Co.

Rotenone (3×10^{-5} M) was included in the reaction medium to inhibit DPNH oxidation and to minimize secondary contributions by the further metabolism of the products of succinate oxidation which might result in an auxiliary release of H^+.

Results

Influence of Energy Flux on Ion Movements and Light-Scattering Changes. Since both respiration and ATP can serve as an energy source, the influence of succinate and/or ATP on Na^+ and K^+ movements and the associated light-scattering changes were first compared. In Figure 1, the requirement of both ATP and succinate to support the Na^+-induced Gramicidin-mediated light-scattering change in P_i-free medium is demonstrated. In contrast to our finding as well as to a previous finding (Pressman, 1965) that respiratory substrates or ATP supported a K^+-induced light-scattering change with Gramicidin, neither succinate nor ATP alone supported a light-scattering change when Na^+ served as the added cation. However, the addition of Na^+ to ATP-treated mitochondria when succinate was present produced a pronounced decrease in light scattering, and the subsequent addition of an energy-depleting agent as *m*-chlorocarbonyl cyanide phenylhydrazone caused a reversal of this process. Succinate itself produced a small change in the light-scattering pattern in the absence of added cations (which could be reversed by the addition of an uncoupling agent), but this can probably be attributed to the monovalent cations present in trace amounts in the Tris salts.

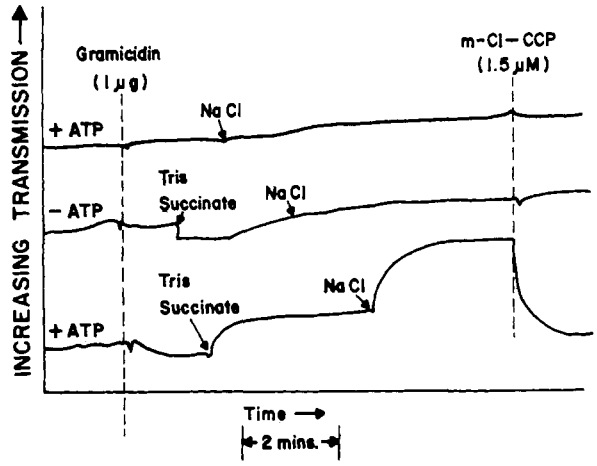

FIGURE 1: Requirement of both ATP and succinate to support Na^+-induced light-scattering changes in the presence of Gramicidin. The basic medium consisted of 225 mM mannitol, 75 mM sucrose, and 3.0×10^{-5} M rotenone. "Tris"-neutralized ATP at pH 7.4 was added to a final concentration of 3.0 mM. Mitochondria equivalent to 0.07 mg of nitrogen/ml were suspended to a total volume of 8.0 ml and the pH was adjusted to 7.4 with saturated "Tris." Sodium chloride when added was in a final concentration of 1.25 mM; succinate ("Tris"), 4.0 mM. The volume of the Gramicidin solution (in alcohol) added did not exceed 10 μl.

The contrast in the results obtained when K^+ serves as the added cation is seen in Figure 2. The addition of K^+ but not Na^+ to Gramicidin-treated mitochondria induced an appreciable decrease in light scattering which was reversed by *m*-chlorocarbonyl cyanide phenylhydrazone. Furthermore, Na^+ prevented the K^+-induced change by the Gramicidin-treated mitochondria when ATP served as the energy source. Moreover, the subsequent addition of valinomycin did not initiate a light-scattering response whereas the addition of valinomycin to mitochondria in the absence of Gramicidin with K^+ present produced a significant decrease in light scattering.

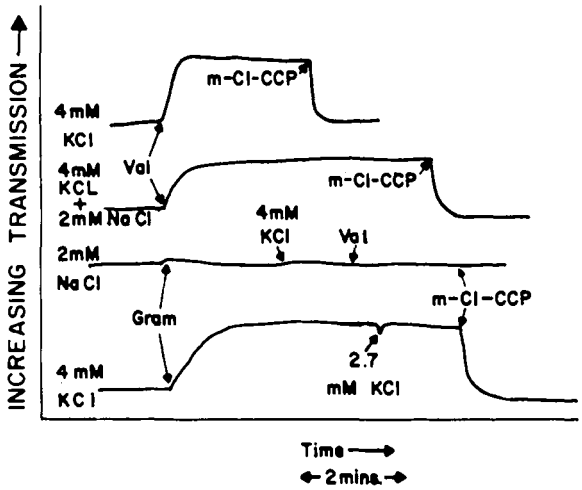

FIGURE 2: Prevention of valinomycin-induced light-scattering change by the introduction of Na^+ and Gramicidin. In each experiment, 3.0 mM ATP ("Tris") was added to the basic medium described in Figure 1. Mitochondria equivalent to 0.10 mg of nitrogen/ml were added to a total volume of 8.0 ml. Valinomycin was added in a final concentration of 10^{-8} M, Gramicidin and *m*-chlorocarbonyl cyanide phenylhydrazone as in Figure 1.

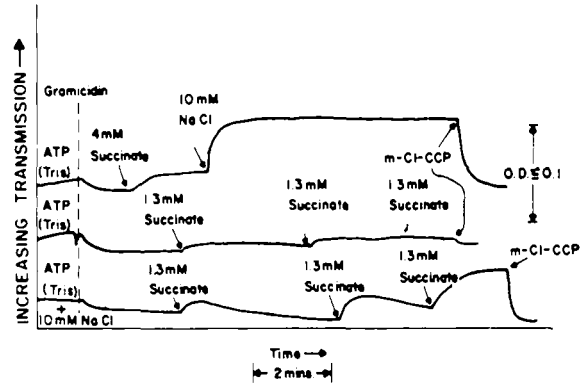

FIGURE 4: Failure of low succinate concentrations to maintain light-scattering changes induced by Na^+. ATP ("Tris"-neutralized) when added was in a final concentration of 3.0 mM. Succinate additions were also "Tris"-neutralized at pH 7.4. The final concentration of Gramicidin or *m*-chlorocarbonyl cyanide phenylhydrazone when added was as in Figure 1. Mitochondria were equivalent to 0.13 mg of nitrogen/ml.

The greater energy requirement for a Na^+-induced light-scattering response is further indicated by the release of the Na^+ inhibition of light-scattering responses by subsequent additions of succinate. As described in Figure 3, the middle trace, which represents a continuation of the trace reported in Figure 2, indicates that the effect of succinate in evoking a significant light-scattering response is concentration dependent. Further, it can be seen from the lower trace that the light-scattering change is not observed in the absence of Gramicidin. Nor in this control run did the uncoupling agent produce an increase in light scattering.

The requirement of a respiratory substrate in addition to ATP to initiate and maintain a Na^+-induced light-scattering change is further indicated by an experiment with different concentrations of respiratory substrate. When the succinate concentration is varied as in Figure 4, it is seen that concentrations of succinate lower than 4 mM fail to support an appreciable Na^+-induced light-scattering change in the presence of Gramicidin plus ATP.

The possibility that this failure is primarily due to energy flux rather than a critical anion concentration could be examined with the use of a competitive inhibitor of succinate oxidation as malonate to vary the energy supply. As seen in Figure 5, the Na^+-induced Gramicidin-mediated light-scattering change observed when supported by 3 mM ATP and 4 mM succinate is compared with that obtained when malonate is also present. With either concentration of malonate examined, the scattering changes were incomplete and

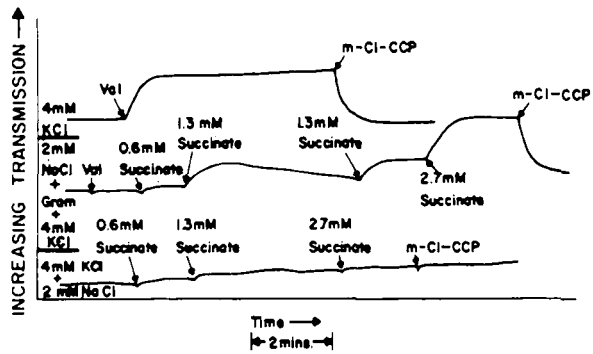

FIGURE 3: Restoration of valinomycin-induced light-scattering change in the presence of Na^+ and Gramicidin by substrate additions. In each experiment 3.0 mM ATP ("Tris") was added to the basic medium described in Figure 1. Mitochondria equivalent to 0.10 mg of nitrogen/ml were added to a total volume of 8.0 ml, and valinomycin, Gramicidin, or *m*-chlorocarbonyl cyanide phenylhydrazone when added were in a final concentration as reported in Figure 1.

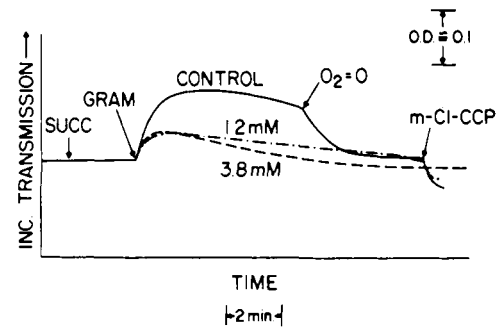

FIGURE 5: Effect of malonate on Gramicidin-induced light-scattering change in the presence of Na^+. "Tris" ATP (3.0 mM) was added to the basic medium described in Figure 1, in which mitochondria equivalent to 0.10 mg of nitrogen/ml were suspended. NaCl (6.25 mM) was also added to the suspension medium in a final volume of 8.0 ml. The dashed lines represent malonate additions at the designated final concentrations. Gramicidin was added as in Figure 1; *m*-chlorocarbonyl cyanide phenylhydrazone was in a final concentration of 0.7 μM.

were poorly maintained, particularly at the higher concentration. Thus, the energy supply is critical to Na^+-induced light-scattering changes.

The requirement of succinate in addition to ATP for Na^+-induced light-scattering changes is not specific as a number of respiratory substrates will suffice. The use of a substrate which enters at the terminal components of the respiratory chain, namely, N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine, to support a Na^+-induced light scattering is demonstrated in Figure 6. It is apparent from this figure that with N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine alone, a greater light-scattering change is observed than with ATP alone. Further, the combination of both energy sources supports the ion-induced changes better than with either source by itself. However, the terminal electron transport system appears to be less effective in promoting the light-scattering change than the succinate respiratory chain.

A comparison of the K^+- *vs.* Na^+-induced light-scattering change when supported by the N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine–ATP system is also included in this figure. In accord with earlier studies which suggest that energy requirements for Na^+ movements are greater than those of K^+, the Na^+-induced change is less pronounced than that of K^+ when N, N, N^1, N^1 - tetramethyl - *p* - phenylenediamine – ATP serves as the energy source.

The requirement of ATP in addition to a respiratory substrate can be further examined with the use of an inhibitor of ATP utilization such as oligomycin. The necessity for ATP as well as for succinate is demonstrated in Figure 7 where the effect of succinate and ATP is compared, and where the combined energy sources are then treated with oligomycin. While succinate alone did not support an appreciable Na^+-induced light-scattering change in the previous studies with P_i-free media, succinate did initiate a greater but suboptimal light-scattering change in phosphate medium. In this experiment, succinate was already present in a 5 mM P_i media, and it can be seen that Gramicidin induced an appreciable change in light scattering, which was further stimulated by the addition of ATP. Furthermore, when oligomycin was added the light-scattering change returned to the level observed prior to ATP addition.

The likelihood that the observed changes in light scattering were related to the uptake of monovalent cations was examined by simultaneous measurement of light scattering and Na^+ uptake monitored with the Na^+ ion sensitive electrode. In Figure 8, an experiment is described in which these parameters were compared in the presence of several concentrations of Na^+. It is to be noted that successive additions of Na^+ produced increasingly larger changes in light scattering and successive increases in Na^+ uptake as measured with the ion-sensitive electrode with concentrations of NaCl up to 1.25 mM. It is concluded from experiments of this type that the measurement of light-scattering changes can provide a qualitative measure of cation uptake under conditions where the electrode measurements are barely detectable.

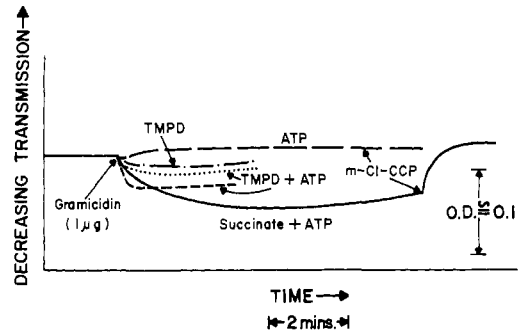

FIGURE 6: Comparison of N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine with other energy sources in the support of K^+- and Na^+-induced Gramicidin-mediated light-scattering changes. Mitochondria equivalent to 0.22 mg of N/ml were suspended in the basic medium described in Figure 1. "Tris"-neutralized ATP when added was in a final concentration of 3.0 mM. NaCl (2.5 mM) was included with the basic medium except for one run where 2.5 mM KCl was substituted for NaCl as indicated by the short dashed line. In those cases where N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine was used, 3.0 mM ascorbate (Tris-neutralized) was added 1 min prior to 0.3 mM N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine. Succinate (Tris) when added was in a final concentration of 3.0 mM.

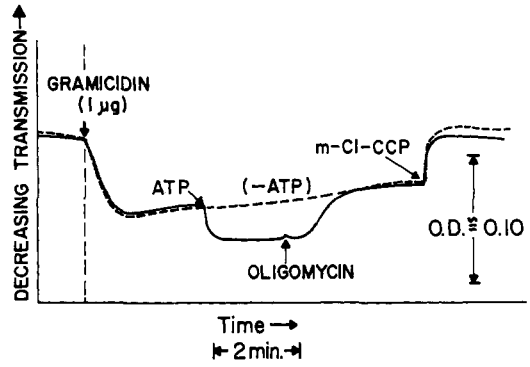

FIGURE 7: Stimulation of Gramicidin-induced succinate-supported light-scattering change by ATP in phosphate medium and reversal by oligomycin. In addition to the basic medium described in Figure 1, 5 mM phosphate (Tris-neutralized) buffer (pH 7.4) and 8 mM NaCl were added, followed by 4 mM succinate (Tris). ATP (Tris) when added was in a final concentration of 3 mM. Oligomycin (10 μg) in an ethanol solution was added when indicated. The total volume in this experiment was 10.0 ml, and mitochondria were equivalent to 0.24 mg of N/ml.

Relationship of Light Scattering to ATPase Activity. A. COMPARISON OF Na^+ AND K^+ IN INITIATING LIGHT-SCATTERING CHANGES AND ATPase ACTIVITY. In the course of study, it had been noted that the Gramicidin-stimulated ATP(Na)-mediated H^+ release was further enhanced by subsequent additions of K^+ but comparable additions of Na^+ caused H^+ release to decline. These findings suggested that comparison of light-scattering changes with "ATPase" activity as measured by the technique of H^+ release would require rate studies with several concentrations of K^+ or Na^+ for extended times in order to obtain a profile which would allow the derivation of meaningful relationships be-

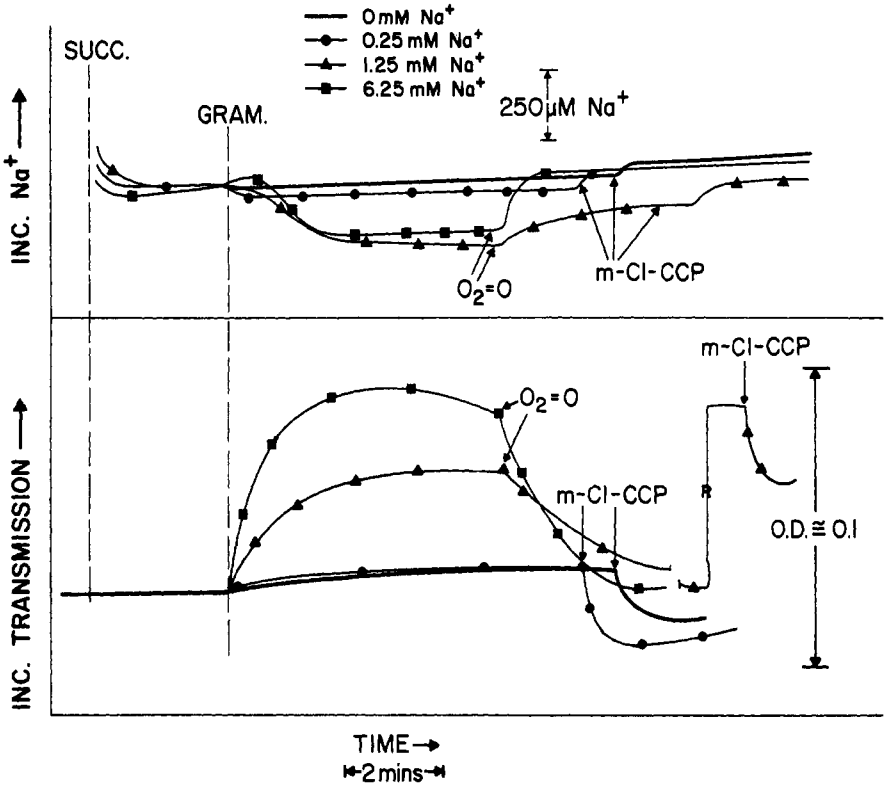

FIGURE 8: Comparison of Gramicidin-induced light-scattering changes with sodium ion sensitive electrode changes at different Na^+ concentrations. Mitochondria in a final concentration equivalent to 0.11 mg of nitrogen/ml were suspended in the basic medium described in Figure 1 with 3.0 mM ATP ("Tris") to a final total volume of 8.0 ml. When added, NaCl at the specified concentration was included with the reaction medium. The upper curves represent traces obtained with the Beckman cation-sensitive electrode (39046) which were made equivalent by redrawing each curve so that identical calibrations could be compared. Each calibration of the original ion-sensitive electrode trace was made with several known additions of NaCl. The lower curves represent uncorrected light-scattering traces obtained simultaneously with the ion-sensitive electrode traces at each concentration of NaCl.

tween these two parameters. Prior to studies of that type, which will be the subject of another report, an examination of the effect of a fixed concentration of Na^+ or K^+ on ATP-mediated H^+ release and light-scattering change is desirable to establish the relative effects of Na^+ and K^+ in eliciting changes in light scattering and H^+ release under these specified conditions. The light-scattering change and stimulation of ATP-mediated H^+ release induced by sodium or potassium ions were therefore compared in the presence of respiratory substrate where the responses were less likely to be limited by energy flux. As seen in Figure 9, either cation induced an appreciable change in light scattering of Gramicidin-treated mitochondria when both energy sources were present, in agreement with our earlier findings. However, it was possible to directly compare in this experiment the relative differences in magnitude of the light-scattering change, and it was noted that K^+ produced more profound changes in light scatter than does Na^+.

The finding that K^+ produces a greater change in light scattering raises the question as to whether more K^+ than Na^+ is accumulated or whether a given concentration of K^+ evokes a larger response than a given concentration of Na^+. It was possible to measure cation uptake in experiments where Na^+ or K^+ were added prior to Gramicidin, and under these conditions, the cation-sensitive electrode traces reflected ionic changes in the medium rather than electrode equilibration changes. When the concentration of Na^+ or K^+ was 1.2 mM as in Figure 9, significantly greater uptake of K^+ than Na^+ was observed in accord with the idea that Na^+ transport requires a larger energy flux.

The contribution of substrate to K^+- *vs.* Na^+-induced ATP-mediated H^+ release is in part indicated by the experiment described in Figure 9. It is seen that the addition of succinate to ATP-treated mitochondria in the presence of Gramicidin but absence of added cations completely prevents the appearance of H^+ in the medium thus providing a convenient base line for studying and comparing progressive changes brought about by successive additions of cations. Following the addition of either 1.2 mM K^+ or Na^+ to these Gramicidin-treated mitochondria, a reversal of the substrate-inhibited ATP-mediated H^+ release was observed, and the added cations actually promoted a fivefold stimulation of the initial rate of H^+ production observed prior to the addition of Gramicidin. At this concentration of cations, the rates of H^+ release observed with Na^+ and with K^+ were similar.

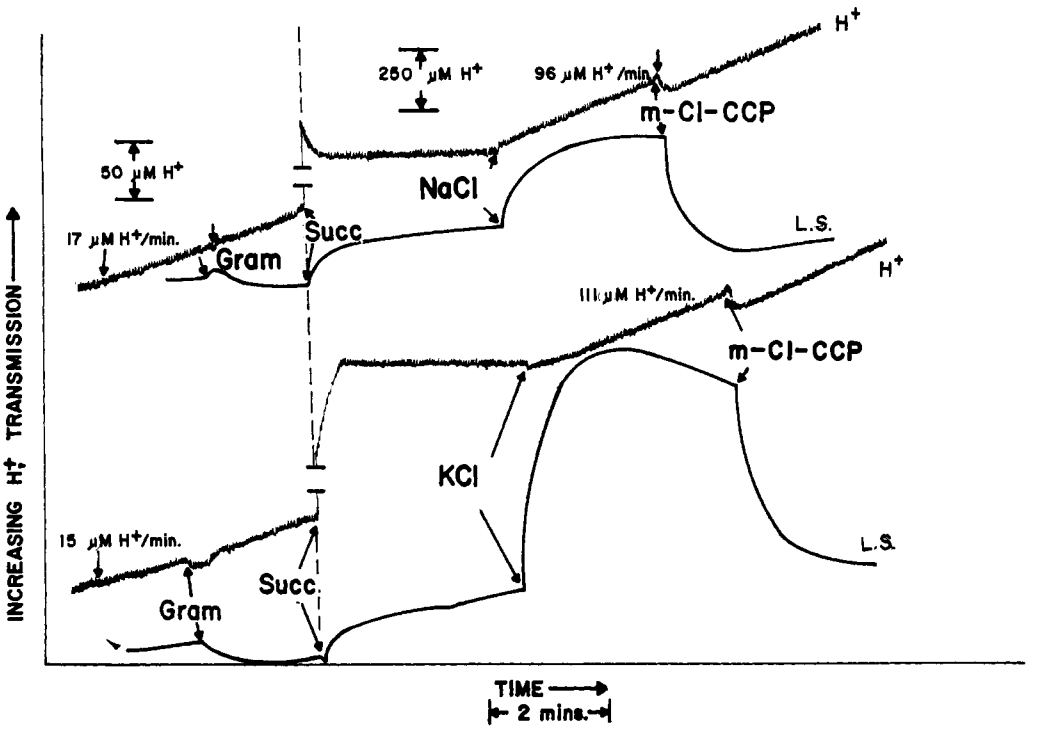

FIGURE 9: Comparison of K^+- and Na^+-induced light-scattering changes and H^+ release in the presence of ATP and respiratory substrate. To the basic medium described in Figure 1, ATP ("Tris") in a final concentration of 3.0 mM was added. Succinate ("Tris") when added in a final concentration of 4.0 mM. Mitochondria were in a final concentration equivalent to 0.10 mg of nitrogen/ml.

Finally, it is considered relevant to summarize the results of our experiments with successive additions of cations on light-scattering changes and on ATP-mediated H^+ release since both parameters were observed to be similarly dependent upon energy fluxes. As seen in Table I, K^+ but not Na^+ (1.0–10 mM) stimulates ATP-mediated H^+ release of Gramicidin-treated mitochondria when energy flux is low (*i.e.*, with ATP in the absence of respiratory substrate). This analogy to the presently reported light-scatter changes was further extended by the finding that Na^+ prevents this K^+-induced stimulation of H^+ release. Additionally, the increasing stimulation of ATP-mediated H^+ release observed with successive additions of Na^+ in the presence of succinate was comparable with the increasing changes in light scattering. When no further increases in light scattering were detected upon subsequent additions of Na^+, no further stimulations of ATP-mediated H^+ release could be observed. Thus our findings indicate that changes in ion-induced ATP-mediated H^+ release are correlated with ion movements as reflected by light-scatter changes. The similarities noted also support the earlier conclusions that the critical energy flux required for Gramicidin-mediated Na^+ movements is greater than that for K^+ movements.

Discussion

Requirement of a Greater Energy Flux for Na^+-Induced Light-Scattering Change than for K^+-Induced Change. The use of light scattering to follow ion movements of Na^+ and K^+ in the present experiments is considered valid since it has been demonstrated that

TABLE I: Failure of Na^+ to Stimulate Gramicidin-Induced ATP-Mediated H^+ Release.[a]

	μM H^+ Released/min				
Additions	0	1 mM	2 mM	5 mM	10 mM
KCl	25	56	101	119	164
NaCl	25	27	45	28	48

[a] Mitochondria equivalent to 0.20 mg of N/ml were added to the basic medium and ATP (Tris) as described in Figure 1. The cations were added 3 min after Gramicidin addition.

the changes in light scattering and Na^+ uptake are parallel when simultaneous measurements of light scattering and of electrode changes with the less-sensitive Na^+ probe are made. The observation that both succinate and ATP are required to support Na^+-induced Gramicidin-mediated light-scattering changes in P_i-free media indicates that energy derived from each process alone is insufficient to support optimal Na^+ uptake in P_i-free media. This proposal is derived from observations that the light-scattering change is small and not maintained when supported by low concentrations of the respiratory substrate and that succinate supports poorly or not at all the light-scattering changes when malonate is present. These findings are in contrast to the results obtained with K^+ where light-scattering changes are supported either by succinate or ATP. It is presumed therefore that Na^+ uptake imposes a larger energy requirement than that for K^+ uptake.

This proposal is also in accord with the observations that the K^+-induced light-scattering changes supported by ATP are prevented by the presence of ions such as Na^+ but it is not certain whether Na ions tax the transport system by virtue of their larger energy requirements for transport or whether sodium ions alter the selectivity of Gramicidin for K^+.

Although not demonstrated here, this inhibition by Na^+ of a K^+-induced light-scattering change has been observed to be reversed by the introduction of increased levels of K^+. These findings indicate that an increased K^+ concentration restores conditions to where the energy requirements for net K^+ uptake do not exceed the availability of energy from ATP. They further indicate that a high Na^+/K^+ ratio rather than an absolute Na^+ concentration appears to be responsible for the inhibitory effects.

The validity of the conclusion that succinate acts primarily as an auxiliary energy source for cation uptake and that alternate energy sources such as ATP are not equivalent in contributing to the energy pool is reinforced by experiments where the energy flux was altered by the use of respiratory substrates which enter at the terminal energy conservation step. The Na^+-induced change was better supported by both *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylenediamine and ATP than with either alone, but less than with succinate plus ATP. The finding that K^+ induces a more pronounced light-scattering change than does Na^+ when supported by the *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylenediamine–ATP system (Figure 6) or with succinate plus ATP (Figure 9) is also in accord with the proposal of a larger energy requirement for Na^+ transport than that of K^+.

It could be argued that ATP does not act as an auxiliary energy source and that ATP solely provides permeant anions such as phosphate necessary for optimal light-scattering change but this possibility appears remote in view of the findings obtained in the experiment described in Figure 7 where both ATP and succinate were required to produce an optimal Na^+-induced light-scattering change in *phosphate medium*. The idea that ATP acts to provide a supplemental energy supply is further supported by the finding that in this experiment oligomycin, an inhibitor of ATP utilization, caused the Na^+-induced light-scattering change supported by succinate and ATP to return to the level observed prior to ATP addition.

It is to be noted, however, that the Na^+-induced light-scattering change supported by 4 mM succinate is greater in phosphate medium than in P_i-free medium, and a possible explanation is that a higher endogenous ATP level is favored by the phosphate medium. This observation that Gramicidin will induce an appreciable Na^+ accumulation when supported by the oxidation of 4 mM succinate alone in the presence of P_i is in accord with the finding of Pressman (1965) from experiments carried out in P_i media where 3 mM glutamate and malate served to support a Gramicidin-induced Na^+ accumulation without the necessity for ATP addition. We have confirmed these results and conclude that it is not essential that ATP be present for optimal Na^+-induced changes under conditions where the energy flux is presumed to be maximal as with the use of substrates in combination or with higher concentrations of succinate.

"*ATPase*" *Activity and Its Relationship to Ion Movements.* It is recognized that ATP-mediated H^+ release is not a strict measure of ATPase activity but the advantages of measurement of dynamic changes with the use of this simple technique outweighs disadvantages as the masking of ATPase activity by secondary contributions to H^+ release. As an example of the interference by the latter, it has been demonstrated that the initial addition of antibiotics as valinomycin in short term experiments (*cf.* Figure 7 of Pressman, 1963) produced some initial H^+ release conceivably due to cation–H^+ exchange, but the change in pH during the last segment of the curve was fully accountable on the basis of the known acid liberation accompanying the splitting of ATP. Further, in our experiments, H^+ release has been observed to compare favorably with ATP breakdown measured independently by P_i formation indicating that secondary contributions of H^+ release are minor. Also, in the reverse reaction of ATP dephosphorylation, the measured H^+ changes of ADP phosphorylation have been found to be in reasonable accord with the predicted theoretical values (Nishimura *et al.*, 1962; Wenner, 1966). The ATP-mediated H^+ release is therefore referred to as "ATPase" activity.

The present studies emphasize the importance of stating and controlling the ionic composition of buffer used for measurement of "ATPase" as well as for transport experiments, and particularly the concentration of Na^+ and K^+. In this respect, the present experiments are in accord with the differential activation of ATPase by Na^+ and K^+ reported by Lardy and Wellman (1953) who stated that "the phosphate-liberating activity of the soluble enzyme "(ATPase)" was usually slightly greater in the presence of KCl than of NaCl."

It might be anticipated that the energy flux is critical to "ATPase" as well as to changes in light scattering. Comparisons of both ATP-mediated H^+ release and

light-scattering changes by Gramicidin-treated mitochondria under conditions where the ionic environment is varied indicate that when the energy supply fails to accommodate ion movements as measured by light-scattering changes, the mitochondria are unable to maintain the stimulated "ATPase" activity.

Relevance of Energy-Dependent Gramicidin-Mediated Transport to Cation Movements in the Absence of Antibiotics. In one current mechanism which has been offered to explain antibiotic-mediated cation transport, the cation is presumed to form a desolvated metal ion complex with the ionophore, allowing the ion to pass through a relatively impermeable barrier to a mitochondrial ion pump which exists in series with a portion of the membrane (Pressman, 1968). The ionophore is presumed to react with a component preexisting in the membrane, conceivably a valinomycin-like ionophore which is an element of a mitochondrial ion pump. This mechanism is supported by studies with mock lipids where evidence has been presented that ionophores such as valinomycin, nigericin, and actins form complexes with selective cations in mock lipid systems. The basis for ionic selectivity of these antibiotics is presumed to reside in the association of the cation with the peptide ring in a manner analogous to that found from X-ray diffraction studies for the K^+ complex of nonactin (Kilbourn *et al.*, 1967).

There is evidence that Gramicidin lowers the membrane resistance of phospholipid bilayers (Mueller and Rudin, 1967) but demonstration of Gramicidin complexes with monovalent cations in the mock lipid systems has not been reported, and our attempts to show this have been unsuccessful.[1] Thus, the mechanism by which Gramicidin mediates ion transport is not yet established but the finding that the substrate flux influences ion uptake and ATPase in the presence of Gramicidin when considered with some unpublished work appears relevant to an understanding of the significance of the energy flux to ion uptake of untreated mitochondria. In unreported experiments, we have observed that in the presence of K^+ but not Na^+, particularly at pH 6.0–7.0, the addition of succinate to ATP-treated mitochondria in the absence of Gramicidin will produce a decrease in light scattering. It is suggested from these observations that the energy required for monovalent cations to transverse the hydrophobic barrier is less at lower pH, and that under conditions where the charge of the phospholipid barrier is minimal, the energy required for K^+ to cross the lipid barrier in the absence of added Gramicidin is less than that for Na^+. Thus, these findings introduce the possibility that specificity of ion transport in the absence of added ion-inducing antibiotic may be due in part to the energy requirement of individual ions for transversing the physical barrier set up by the membrane.

The question is also raised whether the energy flux is in part responsible for specificity in the presence of antibiotics. While the basis for ionic selectivity of the antibiotic is probably dependent on its electronegative center, one need not postulate a shuttle carrier model to explain antibiotic-accelerated ion transport. The possibility is not excluded that Gramicidin in association with the cation modifies the lipid environment possibly at the interphase and/or the electrochemical state to favor an accelerated transport of ions. A lowered energy barrier could then allow transport of those ions whose energy requirements are not met in the absence of Gramicidin.

Relevance of the Present Study to the Reversal by Increased Substrate Concentration of Respiratory Inhibition by Uncoupling Agents. There is considerable evidence for the observation that increased concentration of substrate reverses inhibition of respiration induced by uncoupling agents. Preliminary studies (Wenner and Hackney, 1968) have also indicated that inhibition of respiration which develops with uncoupling concentrations of Ca^{2+} or Sr^{2+} is also reversed by the introduction of an increased concentration of substrate. While several explanations have been advanced for the former observation (*cf.* van Dam and Slater, 1967), unequivocal experimental evidence supporting a specific mechanism is lacking. Previous studies (Harris *et al.*, 1967; van Dam and Slater, 1967) propose that respiratory inhibition by uncoupling agents is due to the lack of availability of substrate anion but this explanation fails to provide an explanation for respiratory control of endogenous substrates (*cf.* Cereijo-Santalo, 1968). There is also increasing evidence that uncoupling agents might alter respiration by their influence on proton conductance across phospholipid membranes (Hopfer *et al.*, 1968; Mitchell, 1961; Skulachev, 1967; Wenner, 1966).

The present studies point to the importance of the *cation*, as well as the anion (substrate or other anion), in the explanation for the reversal by increased substrate concentration of respiratory inhibition produced by uncoupling agents. When the energy-generating system is incapable of promoting cation uptake as in the presence of uncoupling agents, *e.g.*, dinitrophenol, or when ion concentrations are in excess of energy supply, ATPase which can regulate respiratory activity becomes inhibited. However, the introduction of a substrate which provides an additional energy source can now promote cation uptake and conceivably reverse the inhibition of ATPase and restore respiration to its original level. The mechanism by which ion movements influence ATPase activity is not clear but possibly an electrochemical gradient is responsible for ion movements (*cf.* Robertson, 1960; Mitchell, 1961) and when the critical energy state necessary to move the ions present is not maintained, as with ATP alone, the ATPase becomes inactivated. Whether the ions

[1] The influence of Gramicidin on the distribution of ^{42}KCl or $^{22}NaCl$ in a two-phase system (water *vs.* butanol–toluene) was examined in an experiment analogous to that reported in Figure 5 of Pressman (1968) with the exception that potassium or sodium thiocyanate, respectively, served as the source of the compatible organic anion. However, no increase in radioactivity was observed in the mock lipid phase when Gramicidin (0.1–1.0 mg/ml) was introduced. It is recognized, however, that the failure to observe stable complex formation in mock lipid systems does not exclude the possibility that transient complexes are formed but the equilibrium conditions favor dissociation of the complex.

themselves activate ATPase which is normally protected from the ionic environment and/or whether the energy process itself may induce a conformational change of the ATPase necessary for the expression of its activity remains unanswered.

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The Attachment Site of Carbohydrate in a Mouse Immunoglobulin Light Chain*

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ABSTRACT: An immunoglobulin light chain produced and secreted by a plasma cell tumor in mice (MOPC 46) is unusual in containing about 12% by weight of carbohydrate covalently attached to an asparagine or aspartic acid residue in the polypeptide chain. Glycopeptides from tryptic and chymotryptic digests of this light chain have been purified. Three forms each of the tryptic and the chymotryptic peptide, to which carbohydrate is attached, can be separated. Analysis of these glycopeptides give the unique sequence for all three forms:

carbohydrate

(Ser, Cys)-Arg-Ala-Ser-Gln-Asx-Ile-Ser-Asn-Asn-Leu-His-Trp-Tyr-Gln-Gln-Lys. Comparison of this to other sequences of mouse light chains (Gray, W., Dreyer, W., and Hood, L. (1967), *Science 155*, 465) suggests the attachment site of carbohydrate to be residue 28 within the variable part of the light chain using the numbering of mouse 41.

Immunoglobulins are glycoproteins formed of heavy chains of mol wt 55,000, and of light chains of mol wt 23,000. Carbohydrate is usually attached to a site in the carboxy-terminal half of the heavy chain, while the light chains seem not to contain carbohydrate (Fleischmann *et al.*, 1962; Edelman and Gally, 1964).

Recently we reported that the light chain produced and secreted by the mouse plasma cell tumor MOPC 46 does contain carbohydrate. The monomeric form of the light chain appears in three forms containing either two, one, or zero molecules of sialic acid (Melchers *et al.*, 1966). We show in this paper that, from tryptic or chymotryptic digests of the three forms of the light chain, three tryptic or three chymotryptic glycopeptides can be obtained. We have determined the amino acid

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