Ehrenberg, A., and Kamen, M. D. (1965), *Biochim. Biophys. Acta 102*, 333.
Fluck, E., Kerler, W., and Neuwirth, W. (1963), *Angew. Chem. 2*, 277.
Fry, K. T., Lazzarini, R. A., and San Pietro, A. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 652.
Fry, K. T., and San Pietro, A. (1963) *in* Photosynthetic Mechanisms in Green Plants, Kok, B., and Jagendorf, A., Ed., National Academy of Science, National Research Council, Publication 1145.
Goldanski, V. (1963), *Atomic Energy Rev. 1*, 3.
Gonzer, U., and Grant, R. W. (1965), *Biophys. J. 5*, 823.
Kagan, Yu (1961), *J. Exptl. Theor. Phys. USSR 41*, 659.
Lang, G., and Marshall, W. (1966), *Proc. Phys. Soc. (London) 87*, 3.
Lovenberg, W., Buchanan, B. B., and Rabinowitz, J. C. (1963), *J. Biol. Chem. 238*, 3899.
Matsubara, H., Sasaki, R. M., and Chain, R. K. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 439.
Muir, A. H., Jr., Ande, K. J., and Coogan, H. M. (1966), Mössbauer Effect Data Index 1958–1965, New York, N. Y., Interscience.
Palmer, G., and Sands, R. H. (1966), *J. Biol. Chem. 241*, 253.
Sasaki, R. M., and Matsubara, H. (1968), *Biochem. Biophys. Res. Commun.* (in press).
Van Gelder, B. F., and Slater, E. C. (1962), *Biochim. Biophys. Acta 58*, 593.
Wertheim, G. K. (1964), *in* Mössbauer Effect Principles and Application, New York, N. Y., Academic.

Studies on the Mitochondrial Adenosine Triphosphatase System. III. Isolation from the Oligomycin-Sensitive Adenosine Triphosphatase Complex of the Factors Which Bind F_1 and Determine Oligomycin Sensitivity of Bound F_1*

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ABSTRACT: The oligomycin-sensitive adenosine triphosphatase (ATPase) complex after extraction with salt solutions in relatively high concentrations (>2 M) is resolvable into a soluble and particulate fraction. The soluble fraction contains protein which can be identified with F_1 subunits. The particulate fraction contains 20% by weight of lipid and is devoid of ATPase activity. When F_1 is added back to the particulate fraction, there is rebinding of F_1 to the particles and this rebinding can be correlated with the restoration of oligomycin-sensitive ATPase activity. The salt-extracted, ATPase-free residue after exposure to dilute alkali (pH 11.5) can still bind F_1 but the particle-bound ATPase thus reconstituted is not oligomycin sensitive. The thesis that there is a specific protein required for binding of F_1 as well as a specific protein for conferring oligomycin sensitivity is compatible with the experiments described in the present communication.

We have previously reported the isolation from bovine heart mitochondria of an oligomycin-sensitive ATPase complex (O-S ATPase)[1] which was nearly free of cytochromes and which was particulate in the absence of bile salts (T agoloff *et al.*, 1968). The enzyme complex, purified fivefold, retained the same sensitivity to inhibitors, the same degree of activation by various divalent metals, and the same specificity toward various triphosphonucleotides as the ATPase of submitochondrial particles. This preparation has proved to be an excellent starting material for studies of the components of the oligomycin-sensitive ATPase.

The separation, from the O-S ATPase, of a soluble, oligomycin-insensitive, cold-labile ATPase identified

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[1] Abbreviations not listed in *Biochemistry 5*, 1445 (1966), are: O-S ATPase, oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff *et al.* (1968); ATPase (NaCl), O-S ATPase extracted with a solution 2 M in NaCl; ATPase (NaCl–urea), O-S ATPase extracted with a solution 2 M in NaCl and 2 M in urea; ATPase (NaBr), O-S ATPase extracted twice with solutions 3.5 M in NaBr; ATPase (NaBr, NH_4OH), ATPase (NaBr) extracted twice with solutions 0.4 M in NH_4OH; F_1, coupling factor I (Pullman *et al.*, 1960); sucrose-Tris, a solution 0.25 M in sucrose and 0.01 M in Tris-acetate (pH 7.5).

with F_1 (Pullman *et al.*, 1960) has already been reported (Tzagoloff *et al.*, 1968). In this communication we report further studies on the dissection of the complex. When soluble F_1 protein is separated from the O-S ATPase by extraction with media of high salt concentration, the lipoprotein residue retains the capacity both to rebind F_1 and to confer oligomycin sensitivity upon bound F_1. If the salt-extracted lipoprotein residue is further extracted with media of alkaline pH, the capacity to bind F_1 is retained but the capacity to confer oligomycin sensitivity upon the ATPase activity of bound F_1 is lost.

Materials and Methods

Preparation of the O-S ATPase Complex. The oligomycin-sensitive ATPase complex was prepared from bovine heart mitochondria by a modification of the method described previously (Tzagoloff *et al.*, 1968). The concentration of ammonium sulfate in step 4 was taken directly to 40% saturation at 4°. The lipoprotein precipitate, so formed, was washed by resuspension and centrifugation in a medium 0.25 M in sucrose and 0.01 M in Tris-acetate (pH 7.5) (sucrose-Tris). The precipitate contains oligomycin-sensitive ATPase activity. This method of preparation gave a higher total yield of the enzyme complex than did the original method although the specific ATPase activity was not as high as previously reported (Tzagoloff *et al.*, 1968). Protein analysis by electrophoresis on polyacrylamide gel (Takayama *et al.*, 1966) indicated that the two preparations contained the same protein components.

Extraction of the O-S Complex with NaCl. The precipitate containing the O-S ATPase complex was suspended in a solution which was 0.25 M in sucrose, 0.01 M in Tris-acetate (pH 7.5), and 1 mM in dithiothreitol at a protein concentration of 5–10 mg/ml. To this suspension was added solid NaCl to a final concentration of 2 M. The suspension was incubated at room temperature for 10 min and centrifuged at 156,000*g* for 10 min. The pellet was washed with the original volume of sucrose-Tris by resuspension and centrifugation. The washed pellet was then suspended in sucrose-Tris at a protein concentration of 10–20 mg/ml. The particles in this fraction will be referred to as ATPase (NaCl).

Extraction of the O-S ATPase Complex with NaCl and Urea. The extraction of the O-S ATPase complex with NaCl and urea was carried out under the same conditions as for the NaCl extraction except that urea was also added (to a concentration of 2 M). The particles obtained by this procedure are referred to as ATPase (NaCl–urea).

Extraction of the O-S Complex with NaBr. The O-S ATPase complex at a protein concentration of 10 mg/ml was suspended in a solution which was 0.25 M in sucrose, 0.01 M in Tris-acetate (pH 7.5), 1 mM in DTT, and 3.5 M in NaBr. The suspension was incubated at 0° for 20 min and was then centrifuged at 156,000*g* for 20 min. The extracted residue separated as a floating layer. The infranatant solution was decanted and the residue was collected and extracted a second time with 3.5 M NaBr. The twice-extracted material was then washed twice by resuspension in sucrose-Tris. The twice-washed pellet was finally suspended in sucrose-Tris at a protein concentration of 10 mg/ml. The particles obtained by this procedure are referred to as ATPase (NaBr).

Extraction of ATPase (NaBr) with NH_4OH. ATPase (NaBr) was suspended in a solution which was 0.3 M in KCl and 1.5 mM in EDTA (KCl-EDTA) at a protein concentration of 15 mg/ml. To this suspension was added one-half volume of 1.2 N NH_4OH. After incubation at 0° for 30 min, the suspension was centrifuged and the pellet was resuspended in KCl-EDTA and was extracted a second time with NH_4OH. The twice-extracted material was washed twice with sucrose-Tris and the resulting washed pellet was finally suspended in sucrose-Tris at a protein concentration of 10 mg/ml. The particles obtained by this procedure are referred to as ATPase (NaBr, NH_4OH).

Preparation of F_1 and of ETP_H. F_1 (preparation B) was obtained from submitochondrial particles by the method of Pullman *et al.* (1960) as modified by MacLennan *et al.* (1968). ETP_H was prepared by the method of Hansen and Smith (1964).

Other Methods. ATPase activity was measured under the conditions previously described (Tzagoloff *et al.*, 1968). Electrophoresis on polyacrylamide gel was performed by the procedure of Takayama *et al.* (1966). Protein concentration was estimated by the biuret method of Gornall *et al.* (1949).

Results

In another study (MacLennan *et al.*, 1968) we reported that subunits of F_1 could be extracted by exposing submitochondrial particles to salt solutions. The extraction of subunits was effected through depolymerization of particle-bound F_1. In this communication, we describe extraction procedures, involving salt, which selectively remove F_1 (in the form of subunits) from the O-S ATPase complex. When the O-S ATPase complex was extracted with NaCl, NaBr, or NaCl plus urea, it could be resolved into a soluble, lipid-free solution and an insoluble lipoprotein fraction. This particulate fraction had low, or no, ATPase activity but oligomycin-sensitive ATPase activity could be reconstituted by interaction of the particle with either F_1 or subunits of F_1.

Properties of ATPase (NaCl). The ATPase activity of the O-S ATPase complex was reduced from values of 5–8 μmoles of ATP hydrolyzed/min per mg of protein to values of about 0.1 to 0.5 following extraction with 2 M NaCl. Figure 1 shows that part of this activity could be restored by the addition to the particle of either F_1 or of subunits of F_1. The titration curves show that the extent of reactivation of ATPase (NaCl) by F_1 or subunits of F_1 was essentially the same. Restoration of ATPase activity by subunits is believed to be due to the reincorporation of the subunits into the residue which is deficient in these very subunits (see MacLennan *et al.*, 1968).

Properties of ATPase (NaCl–Urea). The ATPase activity of the O-S ATPase complex was also lowered in consequence of extraction with solutions 2 M in NaCl

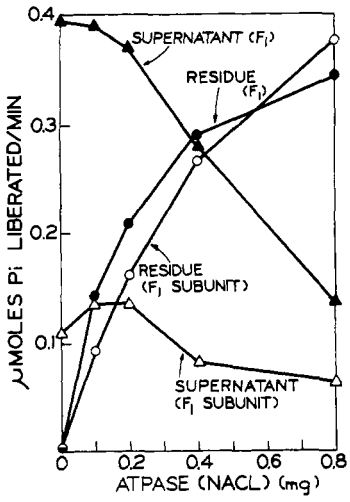

FIGURE 1: Reactivation of the ATPase activity of O-S ATPase (NaCl) after interaction with F_1 and depolymerized F_1, respectively. O-S ATPase (NaCl) was prepared by the procedure described in the text. F_1 was dissolved at a protein concentration of 2 mg/ml in a solution that was 0.02 M in potassium phosphate (pH 7.5), 2 mM in ATP, 1 mM in EDTA, and 0.2 M in KBr. F_1 (100 μg) was then mixed with the indicated amounts of O-S ATPase (NaCl) in a final volume of 1.0 ml of sucrose-Tris. The mixture was incubated at room temperature for 10 min and then centrifuged. The pellets were resuspended in 0.3 ml of sucrose Tris; the ATPase activity in a 0.1-ml sample of this suspension was assayed at 30°. The ATPase activity of the supernatant fluids (0.1 ml) were also assayed. The ATPase activity of the residue particles is indicated by closed circles, and of the supernatant solutions by closed triangles. F_1 was depolymerized by exposure to 0° for 30 min. ATPase (NaCl) was then mixed with the depolymerized F_1 just as described above for F_1. The ATPase activity of the residue particles is indicated by open circles and of the supernatant solutions by open triangles. The values plotted in this graph have been corrected for the residual ATPase rate in the particle in absence of added F_1 (0.12 μmole of ATP hydrolyzed/min per mg of protein).

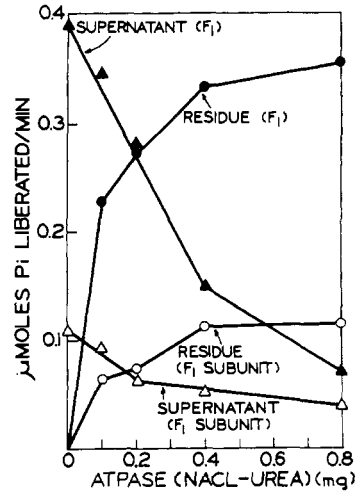

FIGURE 2: Reconstitution of ATPase activity by interaction of O-S ATPase (NaCl–urea) with F_1 and depolymerized F_1. O-S ATPase (NaCl–urea) was prepared by the procedure described in the text. All of the conditions were identical with those described in the legend to Figure 1 except that O-S ATPase (NaCl–urea) was used. The specific activity of the ATPase function in O-S ATPase (NaCl–urea) (0.05 μmole of P_i liberated/min per mg) has been subtracted as a correction to the values reported in this figure. Reconstitution with F_1: (▲—▲) ATPase in supernatant and (●—●) ATPase in residue. (△—△) ATPase in supernatant and (○—○) ATPase in residue.

and 2 M in urea. ATPase activity could be restored by the addition of F_1 but only slightly by the addition of subunits of F_1. This observation is documented in Figure 2. Apparently ATPase (NaCl–urea) is deficient in F_1 units rather than in subunits of F_1.

Table I shows that F_1 protein is removed from solution following interaction of F_1 with the variously extracted O-S ATPase complex. These data, together with the data presented in Figures 1 and 2, show that a transfer of both protein and of ATPase activity from the soluble phase to the particulate phase is demonstrable. Table II shows that the ATPase activity resulting from the interaction of F_1 with ATPase (NaCl) or with ATPase (NaCl–urea) was about 83% sensitive to oligomycin.

Properties of ATPase (NaBr). ATPase (NaBr) is able to bind a high level of F_1 (see Table III). In general, we have found that the addition of 400 μg of F_1 was required to saturate 1 mg of ATPase (NaBr). The activity of the reconstituted ATPase was sensitive to rutamycin to about 85–87%. Freezing of ATPase (NaBr) often reduced the oligomycin sensitivity of ATPase activity reconstituted upon rebinding of F_1.

Gel Electrophoresis of the Proteins in the ATPase Complex After Extraction with NaCl–Urea or NaBr. In another study (Tzagoloff *et al.*, 1968) we showed that one component of the oligomycin-sensitive ATPase complex was a protein with properties similar to those of the soluble ATPase enzyme, F_1, which had been isolated and characterized by Pullman *et al.* (1960). The F_1 component could be identified among the bands into which the proteins of the O-S ATPase complex were separated by electrophoresis. In addition to the four protein components of F_1, two other proteins (bands 5 and 6) were

TABLE I: Binding of F_1 Protein by Extracted O-S ATPase Complex.[a]

Particle	F_1 in Supernatant (μg)	F_1 Subunits in Supernatant (μg)
None	140	120
ATPase (NaCl)	80	80
ATPase (NaCl–urea)	80	90
ATPase (NaBr)	100	100
ATPase (NaBr, NH_4OH)	100	90

[a] The experiment was identical with those described in the legends to Figures 1 and 2. Particle protein (400 μg) was incubated with 140 μg of F_1 or 120 μg of F_1 subunits for 10 min at 23° in a volume of 1 ml. The particulate protein was removed by centrifugation and soluble protein remaining in the supernatant was estimated by a biuret reaction (Gornall *et al.*, 1949).

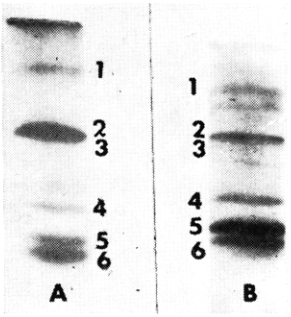

FIGURE 3: Comparison of the electrophoretic profile of the O-S ATPase complex (A) and that of the complex after extraction with a solution 2 M in NaCl and 2 M in urea (B).

identified. In Figure 3 the electrophoretic profile for the O-S ATPase complex is compared with that for ATPase (NaCl–urea). The relative intensities of protein bands 1–4 (components of F_1) were greatly diminished after extraction of the particle while the relative intensities of protein bands 5 and 6 were greatly increased.

A similar effect was noted when the O-S ATPase complex was extracted with NaBr (Figure 4). The proteins corresponding to bands 1–4 were largely lost whereas the proteins bands 5 and 6 were unaffected. Protein bands 1–4 plus small amounts of additional components were recovered in the NaBr extract. Electrophoretic profiles of ATPase (NaCl) not shown here still contained a considerable amount of protein bands 1–4 as well as protein bands 5 and 6.

Extraction of ATPase (NaCl–Urea) or ATPase (NaBr) at Alkaline pH. The ability of the lipoprotein residue to bind F_1 and also to impose oligomycin sensitivity upon

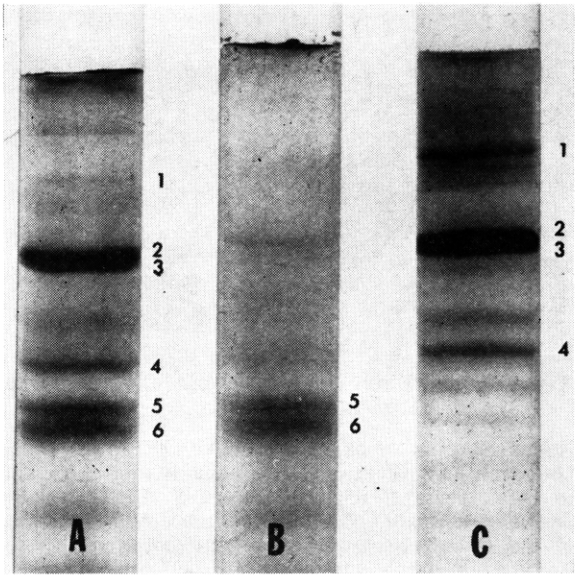

FIGURE 4: Electrophoretic profiles of: (A) O-S ATPase complex; (B) O-S ATPase complex after extraction with 3.5 M NaBr; (C) NaBr extract.

the ATPase activity reconstituted after rebinding of F_1 made it of interest to determine whether conferral of oligomycin sensitivity and binding were dependent or independent properties. Table IV shows that the independence of the two properties could, indeed, be demonstrated in the lipoprotein residue obtained by extraction of the O-S ATPase serially with NaCl–urea and then with dilute alkali. The particle extracted at pH 11.5 retained the capacity to bind F_1 but the ATPase activity after such rebinding was oligomycin insensitive.

TABLE II: Oligomycin Sensitivity of Reconstituted ATPase Complexes.[a]

Particle	Addn	ATPase Act. (μmoles of P_i liberated/min per mg)	
		−Rutamycin	+Rutamycin
O-S ATPase (NaCl)	None	0.75	0.04
O-S ATPase (NaCl)	F_1	3.70	0.63
O-S ATPase (NaCl–urea)	None	0.01	0.00
O-S ATPase (NaCl–urea)	F_1	2.60	0.43

[a] O-S ATPase (NaCl) and O-S ATPase (NaCl–urea) were prepared as described in the text. The particles were mixed with F_1 at a ratio of 0.1 mg of F_1 protein/mg of particle protein. The mixture was incubated at room temperature for 10 min and was centrifuged. The pellets were resuspended in sucrose-Tris and assayed for ATPase activity. Rutamycin was added to a final concentration of 1 μg/ml. The ATPase activity of the added F_1 was oligomycin insensitive.

TABLE III: ATPase Activity Derived by Interaction of the Extracted ATPase Complex (ATPase (NaBr)) with F_1.[a]

μg of F_1 Protein Added/mg of O-S ATPase (NaBr) Protein	ATPase Act. (μmoles of P_i liberated/min per mg)	
	−Rutamycin	+Rutamycin
0	0	0
200	2.0	0.16
400	2.95	0.42
600	3.35	0.42
800	3.35	0.52

[a] O-S ATPase (NaBr) protein (1 mg) was incubated with the indicated amounts of F_1 protein for 10 min at 23° in 0.2 ml of a solution which was 0.25 M in sucrose, 0.01 M in Tris-acetate (pH 7.5), 2 mM in ATP, and 1 mM in EDTA. Sucrose-Tris (1 ml) was then added to the solution and the particles were collected by centrifugation in a microcentrifuge at room temperature. The pellet was suspended in 0.5 ml of sucrose-Tris and a 0.02-ml sample was assayed for ATPase activity.

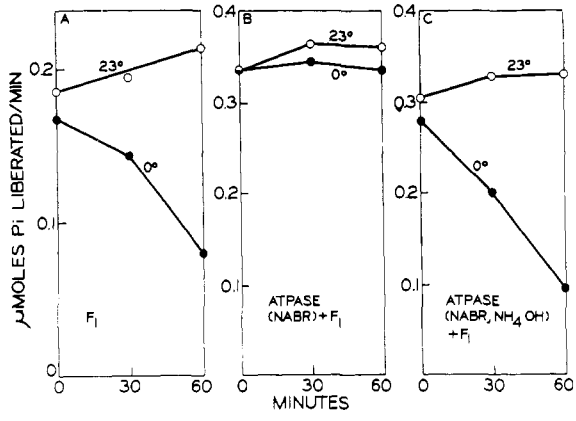

FIGURE 5: Cold lability studies. (A) Of F_1; (B) of F_1 bound to O-S ATPase (NaBr); and (C) of F_1 bound to O-S ATPase (NaBr, NH_4OH). O-S ATPase (NaBr) and ATPase (NaBr, NH_4OH) were prepared as described in the text. The particles were suspended in sucrose-Tris at a protein concentration of 10 mg/ml. F_1 was dissolved at a protein concentration of 5 mg/ml in a solution of sucrose-Tris which was 4 mM in ATP and 1 mM in EDTA. The particles were mixed with F_1 at a protein ratio of 0.21 mg of F_1/mg of particle and the respective mixtures were incubated at room temperature for 10 min. The suspensions were then centrifuged; the supernatant solution was removed; and the particles were resuspended in two volumes of sucrose-Tris which was 1 mM in EDTA and 4 mM in ATP. The samples were divided; one half was incubated at room temperature and the other half at 0° for the periods of time indicated prior to assay. The ATPase activities of the samples (0.04 ml) were assayed at 30° in a final volume of 1 ml. F_1 was incubated either at 0 or 23° at a protein concentration of 1 mg/ml; ATPase activity was then assayed in a sample (0.01 ml) thus treated.

TABLE IV: Effect of pH on the Ability of ATPase (NaCl-urea) to Determine Oligomycin Sensitivity of Bound F_1.

pH of Extraction of O-S ATPase (NaCl-urea)	Addn	ATPase Act. (μmoles of P_i liberated/min per mg) −Rutamycin	+Rutamycin
7.5	None	0.16	0.08
7.5	F_1	1.80	0.19
10.4	F_1	2.90	0.30
11.0	F_1	2.25	1.21
11.5	F_1	2.68	2.50

[a] ATPase (NaCl-urea) was prepared as described in the text. Suspensions of ATPase (NaCl-urea) at a protein concentration of 10 mg/ml in sucrose-Tris were adjusted with KOH to pH 7.5, 10.4, 11.0, and 11.5, respectively, and then centrifuged at 105,000*g* for 10 min. The pellets were resuspended in the original volume of sucrose-Tris and mixed with F_1 (0.1 mg of F_1/mg of particle). The mixture was incubated for 10 min at 23° and then centrifuged. The pellet was resuspended in the original volume of sucrose-Tris and assayed for ATPase activity. Rutamycin was added at a concentration of 1 μg/ml.

A similar phenomenon was observed after alkaline extraction of ATPase (NaBr). ATPase (NaBr) after extraction with ammonia could still bind F_1 but the ATPase function after rebinding was oligomycin insensitive (see Table V).

Cold Stability of the ATPase of F_1 and of the Reconstituted Oligomycin-Sensitive ATPase. As first reported by Pullman *et al.* (1960) the ATPase activity of F_1 is cold labile. This lability was also shown to apply to the oligomycin-insensitive ATPase activity of the soluble enzyme obtained by fragmentation of the O-S ATPase complex (Tzagoloff *et al.*, 1968). Since the oligomycin-sensitive ATPase activity was not cold labile it was of interest to examine whether the ATPase activity, reconstituted by rebinding of F_1 to ATPase (NaBr) or to ATPase (NaBr, NH_4OH), was cold labile. F_1 was incubated with ATPase (NaBr) and ATPase (NaBr, NH_4OH), respectively. The particles were reisolated by centrifugation and the cold lability of the respective reconstituted enzymes was tested over a period of 1 hr. Figure 5 shows that the oligomycin-sensitive ATPase activity was stable to cold whereas the oligomycin-insensitive ATPase activity reconstituted by the rebinding of F_1 to ATPase (NaBr, NH_4OH) was cold labile.

Phospholipid Composition of the Various Particles. The phospholipid content of the ATPase complex after extraction with the various salts was calculated from the values for total phosphorus (Fleischer *et al.*, 1961). Table VI shows that the phospholipid concentration was not diminished by any of the salt extraction procedures nor by exposure of ATPase (NaBr) to alkaline pH.

Effect on Submitochondrial Particles of Extraction with NaCl, NaCl-Urea, and NaBr. The extraction procedures described above were applied to submitochondrial particles (ETP_H; Hansen and Smith, 1964) to determine whether the phenomena observed with the isolated O-S ATPase complex could be reproduced with submitochondrial particles.

Although NaCl and NaCl-urea selectively removed subunits of F_1 from ETP_H, NaBr extraction removed not only F_1 but also certain electron transfer components. In particular, succinic dehydrogenase was quantitatively extracted from ETP_H. Data showing that the NaCl-extracted ETP_H retained masked ATPase have been presented (MacLennan *et al.*, 1968). This was not true, however, of the NaBr-extracted particle which was free not only of masked ATPase activity but also of the headpieces of the inner membrane (D. H. MacLennan, J. Asai, A. Tzagoloff, and T. Oda, in preparation).

The curves in Figure 6 show that F_1 and subunits of F_1 reconstituted the ATPase activity of each of the following three treated particles. The ATPase activity of ETP_H (NaCl) was maximally reconstituted by addition of 30 μg of F_1, that of ETP_H (NaCl-urea) with 120 μg of F_1, and that of ETP_H (NaBr) with 160 μg of F_1. The specific ATPase activity of the three particles thus reconstituted were, respectively, 1.3, 2.5, and 3.2. The values for the amount of F_1 required for maximal reac-

TABLE V: Binding of F_1 by ATPase (NaBr) and ATPase (NaBr, NH_4OH).[a]

Particle	Addn	ATPase Act. (μmoles of P_i liberated/min per mg) −Rutamycin	ATPase Act. (μmoles of P_i liberated/min per mg) +Rutamycin
O-S ATPase (NaBr)	None	0.04	0.00
O-S ATPase (NaBr)	F_1	2.15	0.27
O-S ATPase (NaBr, NH_4OH)	None	0.00	0.00
O-S ATPase (NaBr, NH_4OH)	F_1	1.48	1.43

[a] The preparations, respectively, of O-S ATPase (NaBr) and of O-S ATPase (NaBr, NH_4OH) are described in the text. The particles were incubated in the presence of 0.25 mg of F_1 protein/mg of particle protein for 10 min at room temperature. The pellets collected by centrifugation were resuspended in sucrose-Tris, and 225 μg of each sample was assayed at 30° for ATPase activity. The inhibitor rutamycin was added, where indicated, at a concentration of 1 μg/ml.

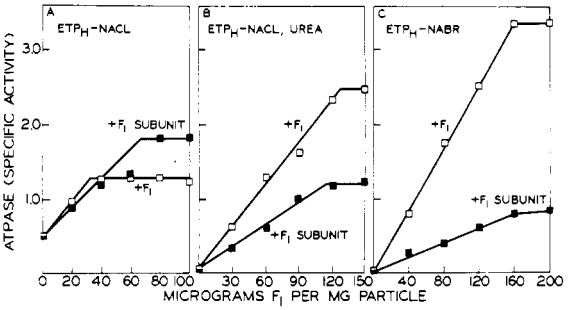

FIGURE 6: Effect of increasing levels of F_1 or depolymerized F_1 on ATPase activity of ETP_H (NaCl), ETP_H (NaCl–urea), and ETP_H (NaBr). A corresponds to ETP_H (NaCl), B to ETP_H (NaCl–urea), and C to ETP_H (NaBr); each of the respective particles was prepared as described in the text. ETP_H (NaCl) or ETP_H (NaCl–urea) was incubated with the indicated levels of F_1 (per 2 mg of particle) at 23° for 10 min in 0.2 ml of a solution which was 0.12 M in sucrose, 0.005 M in Tris-acetate (pH 7.5), 0.010 M in potassium phosphate (pH 7.5), 2 mM in ATP, 1 mM in EDTA, 2.5 mM in $MgCl_2$, and 0.1 M in KBr. Sucrose-Tris (1 ml) was then added to the suspension and the particle was collected by centrifugation at 156,000g. The pellet was suspended in 0.5 ml of sucrose-Tris, 0.05 ml being taken for the ATPase assay. The assay of ETP_H (NaBr) was carried out in the same manner except that 1 mg of protein was used. F_1 was prepared at a concentration of 2 mg/ml in a solution which was 20 mM in potassium phosphate (pH 7.5), 4 mM in ATP, 2 mM in EDTA, and 200 mM in KBr. The complex was depolymerized by incubation at 0° for 30 min; 80% inactivation was attained during this treatment.

tivation of the ATPase activity of the three respective particles may correspond to the amounts of F_1 which were extracted from these respective particles by the various salt treatments. When these three extracted particles were reacted with subunits of F_1, the ATPase function of ETP_H (NaCl) was restored (specific activity 1.9), but the ATPase activity of ETP_H (NaCl–urea) was smaller and that of ETP_H (NaBr) was negligible.

TABLE VI: Concentration of Phospholipid in the Particulate ATPase Complex before and after Various Extraction Procedures.[a]

Particle	Phosphorus (μg/mg of protein)	Phospholipid (% by wt)
O-S ATPase	6.0	16
O-S ATPase (NaCl)	6.7	17
O-S ATPase (NaCl–urea)	7.1	18
O-S ATPase (NaBr)	7.7	19
O-S ATPase (NaBr, NH_4OH)	7.8	19

[a] Phospholipid was extracted by the method of Fleischer *et al.* (1961). Phospholipid phosphorus was determined by the method of Chen *et al.* (1956). The phospholipid content was calculated on the assumption that 33 μg of phosphorus is equivalent to 1 mg of phospholipid (Fleischer *et al.*, 1961).

Discussion

In the studies described above on the resolution of the isolated oligomycin-sensitive ATPase complex, we have succeeded in obtaining lipoprotein particles which have lost the ATPase function but which, after interaction with F_1, can reacquire this activity. The reconstituted ATPase function can be oligomycin sensitive or insensitive depending upon the conditions of extraction. In these reconstitution experiments the extracted residue was similar to the F_0 reported by Kagawa and Racker (1966). The fraction is lipoprotein in nature and remains insoluble after most of the F_1 subunits have been removed by extraction either with NaCl plus urea or with NaBr. Electrophoretic analysis of the extracted residues indicated two major protein components which correspond electrophoretically to bands 5 and 6 of the O-S ATPase complex. These proteins are absent in preparations of F_1 and are, therefore, concerned only with binding of F_1 and the determination of oligomycin sensitivity. The protein corresponding to band 6 has, in fact, been purified; and in another communication (MacLennan and Tzagoloff, 1968) it was shown to be the determinant of the oligomycin sensitivity of the reconstituted ATPase activity.

It was also possible to extract F_1 protein from the O-S ATPase complex with 2 M NaCl. The residue, ATPase (NaCl), still contained protein components of F_1. Unlike ATPase (NaCl–urea) or ATPase (NaBr), the ATPase function of which was reconstituted only with F_1, the ATPase activity of ATPase (NaCl) could

be reconstituted either with F_1 or with depolymerized F_1. A similar phenomenon was observed with submitochondrial particles extracted with NaCl (MacLennan *et al.*, 1968). Since F_1 is a polymeric unit (Penefsky and Warner, 1965), a possible explanation for this phenomenon is that NaCl extracts some of the subunits from the particle-bound F_1. The subunits remaining in the particle could then act as a point of entry for the reincorporation of the missing protein(s). However, when the particle was extracted with NaCl–urea or NaBr most of the F_1 protein was removed; then only F_1 could reconstitute the ATPase activity of these particles. This hypothesis has been discussed previously (MacLennan *et al.*, 1968).

The fact that the ATPase function of ATPase (NaBr, NH_4OH) and ATPase (NaCl–urea, NH_4OH), reconstituted by the interaction with F_1, is oligomycin insensitive, indicates that the two properties (namely, binding of F_1 and determination of oligomycin sensitivity) are separable. This conclusion is also supported by a previous observation that when the O-S ATPase complex was exposed to high concentrations of tributyltin chloride or to heat (Tzagoloff *et al.*, 1968), the loss of sensitivity to oligomycin was not accompanied by release of F_1 from the particle. Similar findings have previously been reported by Kagawa and Racker (1966).

The ATPase activity reconstituted by the rebinding of F_1 to alkali-extracted ATPase (NaBr) not only is insensitive to oligomycin but also is cold labile. This is not the case for the ATPase function reconstituted by the rebinding of F_1 to ATPase (NaBr), a rebinding which reconstitutes an oligomycin-sensitive ATPase function. Cold lability and oligomycin sensitivity of the ATPase function appear to be determined not by rebinding of F_1 to the particle but by yet another factor.

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References

Chen, P. S., Toribara, T. Y., and Warner, H. J. (1956), *Anal. Chem. 28*, 1756.

Fleischer, S., Klouwen, H., and Brierley, G. P. (1961), *J. Biol. Chem. 236*, 2936.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hansen, M., and Smith, A. L. (1964), *Biochim. Biophys. Acta 81*, 214.

Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241*, 2467.

MacLennan, D. H., Smoly, J. M., and Tzagoloff, A. (1968), *J. Biol. Chem.* (in press).

MacLennan, D. H., and Tzagoloff, A. (1968), *Biochemistry 7*, 1603 (this issue; following paper).

Penefsky, H. S., and Warner, R. C. (1965), *J. Biol. Chem. 240*, 4694.

Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.

Takayama, K., MacLennan, D. H., Tzagoloff, A., and Stoner, C. D. (1966), *Arch. Biochem. Biophys. 114*, 223.

Tzagoloff, A., Byington, K. H., and MacLennan, D. H. (1968), *J. Biol. Chem.* (in press).