IMMUNOGENIC PROPERTIES OF MEMBRANE-BOUND ATPase FROM STABLE PROTEUS P 18 L-FORMS : A KINETIC STUDY OF INHIBITION BY SPECIFIC ANTIBODIES

Henri MONTEIL and Guy ROUSSEL
Institut de Bactériologie de la Faculté de Médecine
Université Louis Pasteur - 3, rue Koeberlé - 67000 STRASBOURG - FRANCE

Received January 13,1975

SUMMARY

Antisera to Mg^{2+} dependent ATPase isolated and purified from membranes of Proteus L-Forms were prepared. These antibodies caused growth inhibition of L-Forms on solid media and complete inhibition of the soluble ATPase activity. Kinetic studies showed an uncommon type of inhibition : Anti-ATPase antibodies acted as uncompetitive inhibitor with respect to the substrate.

In a recent publication (6), we have reported that membrane-fractions from Proteus P 18 L-Forms induce rabbit antibodies which inhibit the growth of these bacteria ; furthermore, these antibodes decrease oxygen uptake, flavoprotein respiration, and ATP hydrolysis by growing cells. We proposed that ATPase was the common base of all observed metabolic inhibitions, and we tried to isolate it. ATPase (E.C. 3.6.1.3.) can be released from the cell membrane in a soluble and purified form. We have undertaken the study of immunogenicity of purified enzyme and the modalities of its inhibition by specific antibodies.

MATERIALS AND METHODS

Stable Proteus P 18 L-Forms were grown and harvested as previously described (6). ATPase was solubilized according to Abram's method (1) modified (7). Solubilized ATPase was centrifuged for 90 minutes at 105,000 x g. The supernatant holding the soluble enzyme was then concentrated through a Pellicon membrane (Millipore PSJM 02510) ; the ultrafiltration retains components with a molecular weight over 100,000. The enzyme was purified by gel filtration through an indubiose column (AcA 3-4, 3 % acrylamide, 4 % agarose), which separated proteins between 40,000 and 400,000 daltons. Enzyme or fractions were mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously into young rabbits (one injection each week for 5 or 6 weeks). The animal's blood was colleted 8 days after the last injection.

Gamma-globulins were separated from sera and from control serum by precipitation with ammonium sulphate. Gamma-globulins solutions were dia-

TABLE I : ANTIGEN FRACTIONS AND PROPERTIES OF IMMUNESERA.

SERA No.	IMMUNIZING ANTIGEN		PROPERTIES OF ANTIBODIES		
	Bacteria or Fractions	Total amount injected	ATPase activity µmol Pi x min^{-1}	Immunodiffusion	Growth inhibition
31	Proteus P 18	6 ml of a 24 hours culture	-	-	+ +
30	Proteus P 18 L-Forms	6 ml of a 24 hours culture	-	-	+ + +
60	Washed L-Forms membranes	10 mg proteins	1,6	+	+ + +
32	Crude ATPase	1,20 mg proteins	6	+	+ +
28	Purified ATPase (peak 1)	0,50 mg proteins	4	+	+
29	Associated Proteins (peak 2)	0,44 mg proteins	0	-	-

lyzed against several changes of 33 mM Tris-HCl buffer (pH = 7.5) to eliminate any ions which non-specifically activate or inhibit ATPase.

An estimate of inorganic phosphate released throughout the enzyme reaction was carried out with Baginsky's method (2) applied (7) to bacterial ATPase assays. The serum or corresponding gamma-globulins was preincubated for 10 minutes in the presence of ATPase prior to titration. The reaction medium was then brought to 1.6 ml by addition of 33 mM Tris-HCl buffer, pH = 7.5, 0.75 mM $MgCl_2$, ATP (100 µl of a 25.5 mM solution) was added at the zero point and left at 37° for 12 minutes. The reaction was stopped by adding 1.7 ml of a 4 % ascorbic acid solution in 20 % trichloracetic acid. Centrifugation eliminated the precipitate. 1 ml of supernatant was tested for inorganic phosphate under the conditions described (7). All experiences yielded an Mg/ATP ratio of 0.5.

Double diffusion in agar following Ouchterlony's classical method was used. We have previously described the growth inhibition of L-Forms on an agar medium enriched with horse serum (6).

RESULTS

A soluble form of Mg^{2+} dependent ATPase was obtained from stable Proteus P 18 L-Forms, and centrifuged at 105,000 x g. Solubilization was caused by shock-wash in a 33 mM Tris-HCl buffer, pH = 7.5, free of divalent cations. After purification in an acrylamide-agarose gel (disc electrophoresis displayed only one band), the enzyme was shown to have a molecular weight of 360,000 and two subunits α and β (64,000 and 58,000 respectively) (8). The formula : $\alpha_3\beta_3$ was suggested.

Table 1 gives an enumeration of the fractions used as antigens ; sera induced by the original bacteria (n° 31) and by the L-Forms (n° 30) inhibit growth but in agar medium there is no visible precipitation line opposite ATPase. However, sera relating to the washed L-Forms membranes (n° 60) after solubilizing ATPase without magnesium gave a single precipitation line when tested against purified ATPase as well as inhibit growth.

After enzyme solubilization in a free Mg^{2+} buffer, a certain amount of ATPase remains bound to the membrane and is liable to produce more anti-ATPase antibodies, with the adjuvant effect of associated membrane compounds. Anti-crude ATPase serum (n° 32) and anti-purified ATPase serum (n° 28) (corresponding to peak 1) gave also a single precipitation line in Ouchterlony's plates and inhibit L-Forms growth. However, protein contaminants of ATPase during the solubilization process (identified in peak 2), are free of enzyme activity, and the corresponding serum (n° 29) does not have the two properties just described.

IMMUNSERUM OR CORRESPONDING GAMMA-GLOBULIN INHIBITION OF ATPase ACTIVITY

Optimum contact between the enzyme and antibodies was determined before-

SERA No.	IMMUNIZING ANTIGEN	GAMMA-GLOBULINS mg PROTEINS/ml	ATPase SPECIFIC ACTIVITY µmol Pi x min^{-1} x mg $proteins^{-1}$	INHIBITION PERCENTAGES
31	Proteus P 18	5,50	2,88	2,8 %
30	Proteus P 18 L-Forms	4,70	2,85	4,0 %
60	Washed L-Forms membranes	6,50	0,52	38,2 %
32	Crude ATPase	3,85	0,26	71,0 %
28	Purified ATPase (peak 1)	4,10	1,37	40,2 %
29	Associated proteins (peak 2)	4,95	2,98	0 %
Pool of rabbit sera before immunization		3,00	3,04	-
			enzyme alone : 2,61	

TABLE II :

INHIBITION OF ATPase ACTIVITY BY GAMMA-GLOBULINS. Inhibition was expressed as percentage of specific activity compared to a reference test (incubation of enzyme with non-immunized rabbits'gamma-globulins) and to control test (reaction mixture without gamma-globulins).

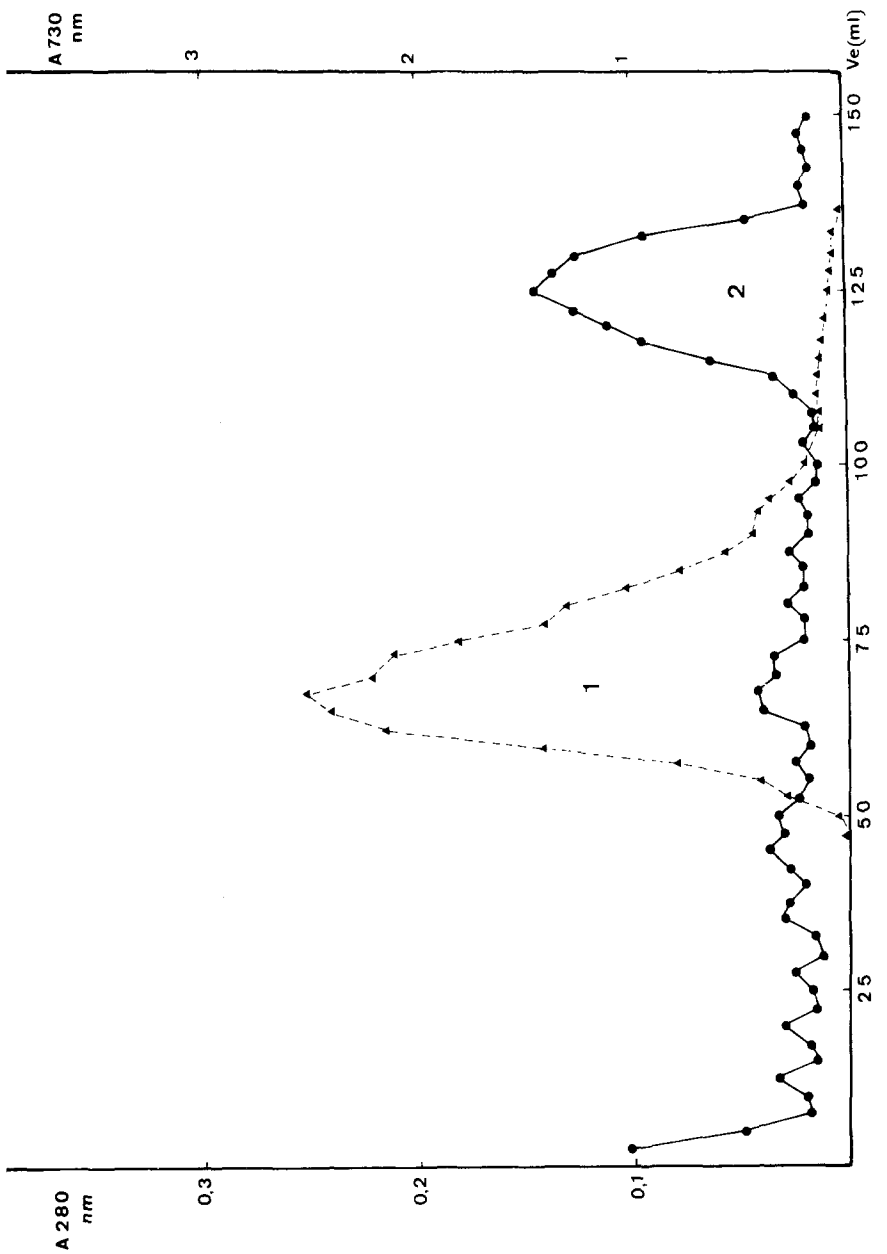

FIGURE I - Chromatography of solubilized ATPase on an AcA 3-4 Indubiose column (2 x 40 cm). Enzyme was centrifuged at 105,000 x g and elution was carried out with a 33 mM Tris HCl pH = 7.5, 0.75 mM $MgCl_2$ buffer (Vo = 45 ml, Ve = 122 ml) ●——● OD 280 nm (Proteins) ▲----▲ OD 730 nm (ATPase assay).

hand. A study of the inhibition or ATPase activity by anti-purified ATPase serum (n° 28) in terms of incubation time during a 120 minute period showed peak inhibition after a 10 minute contact. Yet, when serum concentration is such that 50 % enzyme activity is inhibited after 10 minutes, the addition of substrate simultaneously with antibody reduces inhibition to 46 %. In spite of this small drop in percentage, we found it more convenient to use a 10 minute preincubation period for following experiments.

A COMPARISON OF ATPase-INHIBITING CAPACITIES OF GAMMA-GLOBULINS EXTRACTED FROM VARIOUS IMMUNE SERUMS (Table II)

The gamma-globulins extracted from anti-Proteus serum (n° 31) and anti-Proteus-L-Forms (n° 30) have minor ATPase-inhibition capacity. Gamma-globulins from anti-Proteus-L-Form membranes (serum n° 60) are very competent in inhibiting ATPase (82.9 %) as are the gamma-globulins from the anti-crude ATPase serum (n° 32, 91,2 %) and anti-purified ATPase serum (n° 28, 55 %). Equal volumes of the different gamma-globulins solutions extracted under strictly identical conditions, were used for this first comparison.

By expressing inhibition percentage considering protein concentration of each gamma-globulin solution, we estimated the true efficiency of these antibody effects on ATPase. Considering the amount of injected antigen (table I) and the resulting inhibition percentage (table II) we established that peak I of the elution diagram (fig. 1), corresponding to the purified enzyme, induces antibodies with the highest inhibition capacity (n° 28). Later on, gamma-globulins extracted from this serum were used for our kinetic studies.

It must be pointed out that the sera giving precipitation lines in agar against ATPase (n° 60, 32, 28) show the best inhibition activity.

ATPase INHIBITION IN TERMS OF ANTIBODY CONCENTRATION

Inhibition of ATPase activity in terms of increasing concentrations of gamma-globulins from serum n° 28 is reported in fig. 2. Although there is total inhibition for high concentrations, inhibition displays a steep climb for gamma-globulin concentrations up to 1 mg and total blocking occurs only when gamma-globulin concentration is 2.5 times higher. Our results can be compared to those obtained by Whiteside and Salton (11) with anti-ATPase antibodies from Micrococcus lysodeikticus.

THE KINETICS OF INHIBITION BY VARIOUS ANTIBODY CONCENTRATIONS IN TERMS OF SUBSTRATE CONCENTRATION

The diagram of the inverse of the enzyme reaction rate (I/V) at varying antibody concentrations in terms of increasing substrate concentrations ($\frac{I}{ATP}$) is shown on fig. 3, and displays a series of parallel lines.

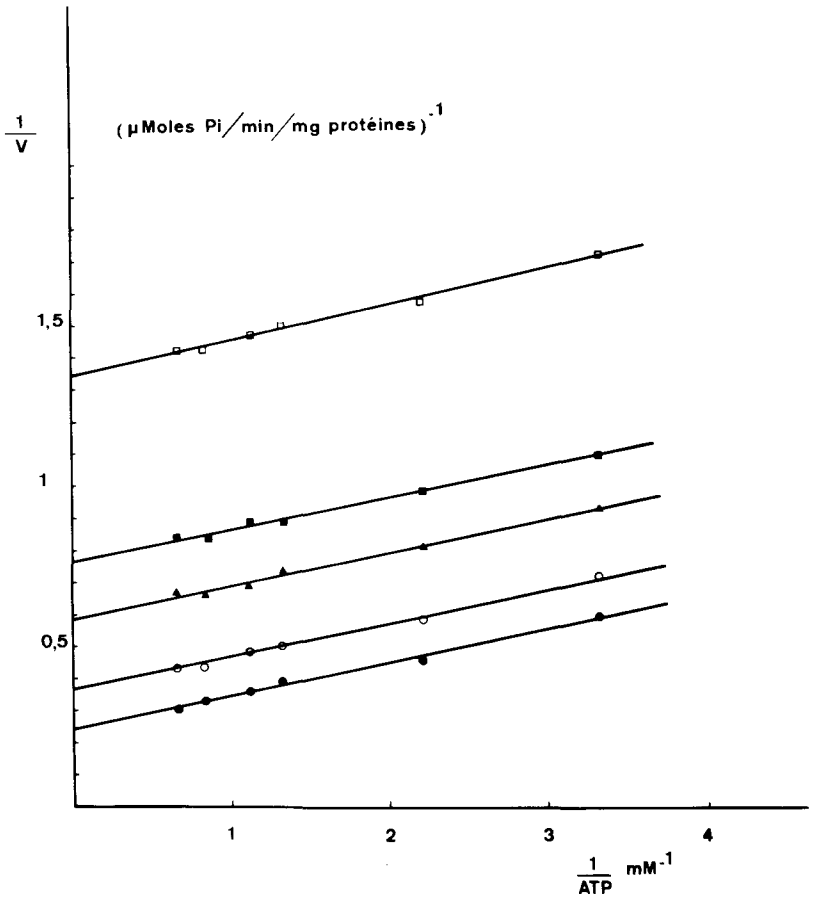

FIGURE 3 - Lineweaver-Burk plots of ATPase inhibition by gamma-globulins (n° 28).
●——● Zero gamma-globulins.
○——○ 200 µg gamma-globulins giving a 28 % inhibition.
▲——▲ 400 µg gamma-globulins giving a 50 % inhibition.
■——■ 500 µg gamma-globulins giving a 62.5 % inhibition.
□——□ 620 µg gamma-globulins giving a 75 % inhibition.

immunesera with a better inhibiting performance, but this fact could be due to non-negligible adjuvant activity of the associated components.

Inhibition of a specifically membrane-bound and easily soluble enzyme can explain the effect of these growth-interfering antibodies. Though we still have an unclear picture of ATPase functions, in bacteria (8), it is conceivable that impeding ATP as an energy-donor should imply metabolic perturbations.

Using anti-purified ATPase gamma-globulins conjugated to ferritin we were able to observe at a preliminary stage, that labeled antibodies were attached to the surface of the L-Forms. OPPENHEIM and SALTON (9), SALTON and Coll. (10) have shown that anti-ATPase antibodies from Micrococcus lysodeikticus react with the internal membrane surface. This confirms the fact that anti-ATPase antibodies are

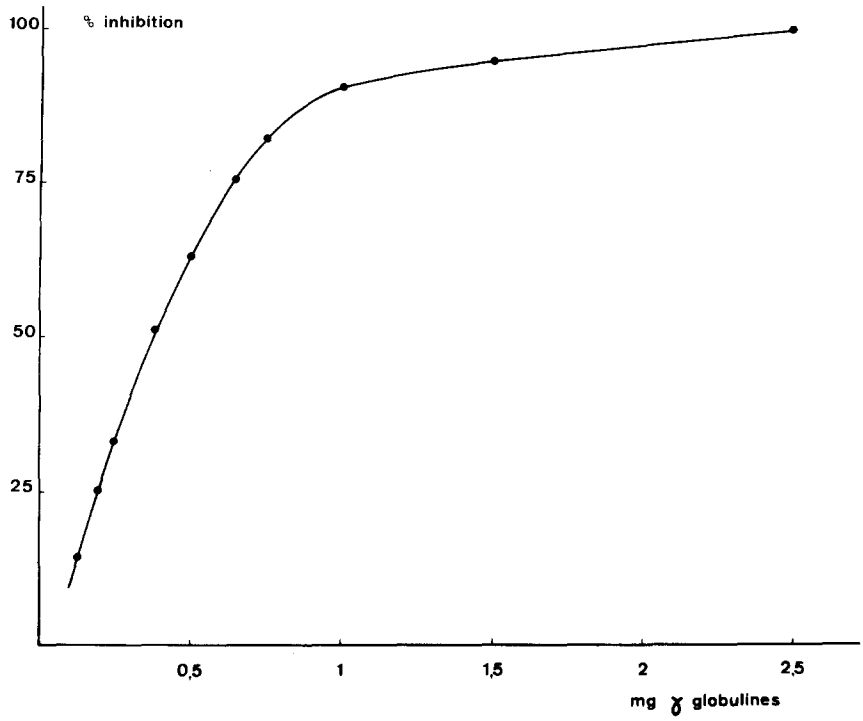

FIGURE 2 - Inhibition of solubilized ATPase by specific gamma-globulins (n° 28). Each measure was done for the same amount of enzyme protein = I2.5 µg. The enzyme and antibody mixture was preincubated for IO minutes before substrate (ATP) addition. Gamma-globulin from serum n° 28 (before immunization) was used as a reference.

This representation of inhibiting antibody effect calls to mind an uncompetitive inhibition.

KINETICS OF INHIBITION BY GAMMA-GLOBULINS AND BY ADP IN TERMS OF SUBSTRATE CONCENTRATION

Fig. 4 shows LINEWEAVER-BURK plots of inhibition by ADP, by gamma-globulins and by a blending of both. The Km for ATP without inhibitor is 0.42 mM. ADP is a competitive inhibitor for ATPase, modifying Km with a $1 + \frac{I}{KI}$ factor.

I expresses ADP concentration and Ki expresses the dissociation coefficient for ADP : its value is 0.65 mM. Simultaneous addition of both ADP and antibodies leads to a stronger inhibition than that caused by either effector (fig. 4). Evidently, as shown by inhibition kinetics their fixation sites on ATPase are distinct

DISCUSSION

A study of purified ATPase shows that this enzyme is liable to induce antibodies that inhibit L-Form growth ; whole membranes, and the crude enzyme yield

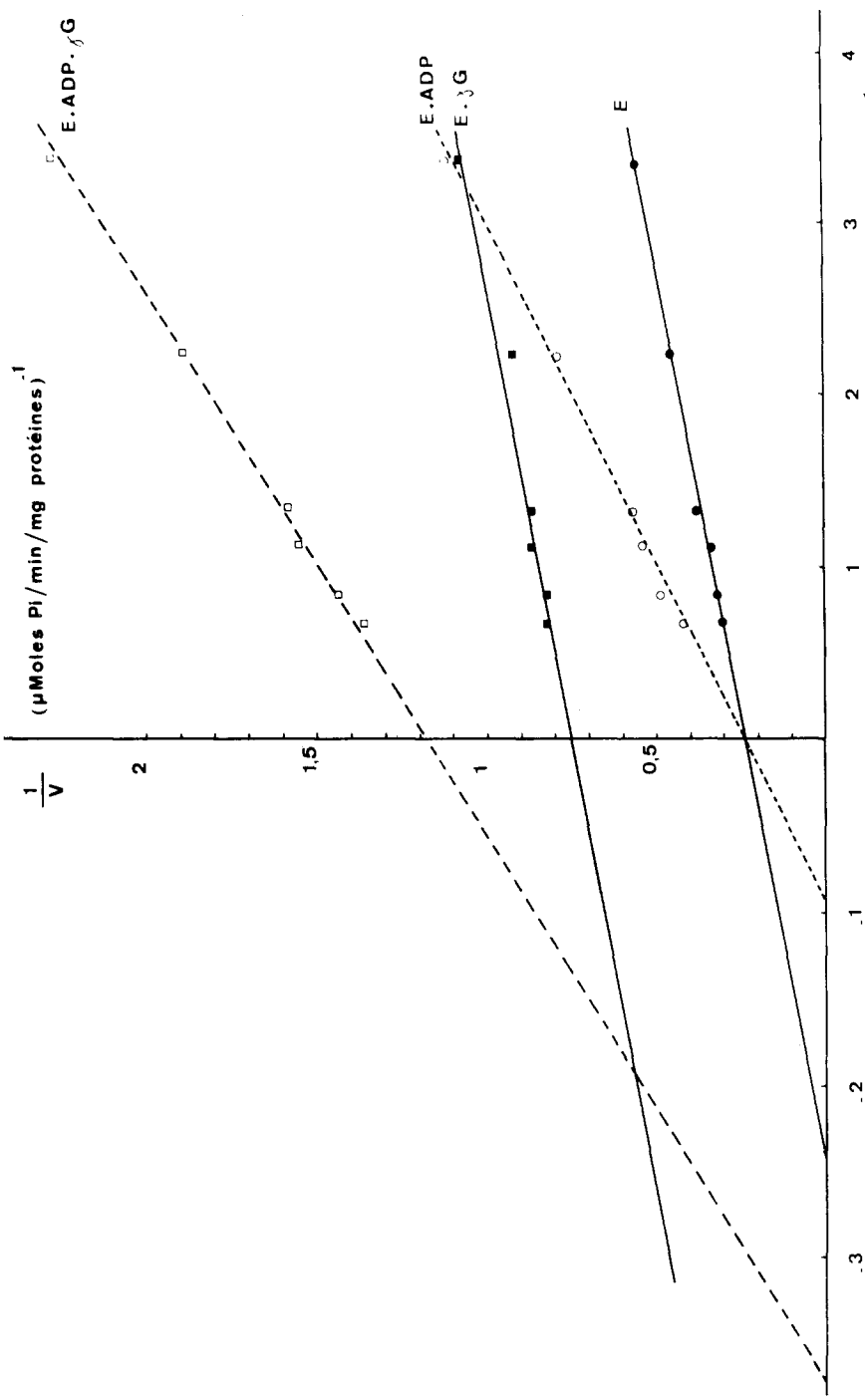

FIGURE 4 - Lineweaver-Burk plots of ATPase inhibition by gamma-globulins (n° 28) and ADP.

- ●——● ATPase alone.
- ○- - -○ ADP (0.75 mM).
- ■——■ 500 µg gamma-globulins.
- □- - -□ ADP (0.75 mM) + 500 µg gamma-globulins.

able to block membrane-bound enzyme activity because in the case of L-Forms, loose peptidoglycan structure of the altered cell wall makes antibody fixation easier.

Several authors have obtained anti-ATPase antibodies to gram (+) (10, 11) and gram (-) bacteria (4). They have found an inhibition of enzyme activity, but few kinetic studies have been carried out. Our results lead to certain conclusions about the kinetics registered. Inhibition percentage for a given concentration in gamma-globulins was independant of the substrate concentration. With different specific activities for the enzyme, the inhibition percentage was the same for a given concentration of antibodies. Whiteside and Salton (11) found that anti-ATPase antibodies acted as a non competitive inhibitor. In contrast, we find an uncommon inhibition which is indicated by parallel lines obtained with plots of 1/V against 1/S. This inhibition is of an uncompetitive type. Antibodies may not affect the catalytic site but may block enzyme reaction at the ESI stage by interfering with antigenic sites of the enzyme. We may suppose that these sites are adjacent to the active "site" or that a slight indirect change in enzyme structure may create disappearing or blocking of the active site. The phenomenon of enzyme protection by substrate described by Cinader (3) for a few enzymes and by Salton and Whiteside for ATPase, was not observed here. Furthermore, Proteus-L-Forms-ATPase retains antigenic capacity even though storage in the cold causes it to lose enzymatic activity.

To completely elucidate the mechanism by which these antibodies react with and relate to the enzyme, it would be interesting to induce antibodies against subunits α and β.

REFERENCES

I - ABRAMS, A. (I965) J. Biol. Chem. 240, 3675-368I.
2 - BAGINSKY, E.S., FOA, P.P., ZAK, B. (I967) Clin. Chim. Acta I5, I55-I58.
3 - CINADER, B. (I967) Antibodies to biologically active molecules pp. 85-I37, Pergamon Press, Ltd.
4 - HANSON, R.L., KENNEDY, E.P. (I973) J. Bacteriol. II4, 772-78I.
5 - HAROLD, F.M. (I974) Ann. New York Sci. 227, 297- 3II.
6 - MONTEIL, H., SCHREIBER, B. (I973) Ann. Microbiol. (Inst. Pasteur) I24 A, I03-205.
7 - MONTEIL, H., SCHOUN, J., GUINARD, M. (I974) Eur. J. Biochem. 4I, 525-532.
8 - MONTEIL, H., ROUSSEL, G., BOULOUIS, D. (I975) Biochim. Biophys. Acta (accepted for publication).
9 - OPPENHEIM, J.D., SALTON, M.R.J. (I973) Biochim. Biophys. Acta 298, 297-322.
IO - SALTON, M.R.J., SCHOR, M.T., NG, M.H. (I972) Biochim. Biophys. Acta 290, 408-4I3.
II - WHITESIDE, T.L., SALTON, M.R.J. (I970) Biochemistry 9, 3034-3040.