GROWTH ON ARTIFICIAL MEDIUM OF AN AGENT ASSOCIATED WITH ATYPICAL PNEUMONIA AND ITS IDENTIFICATION AS A PPLO

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Recent volunteer and controlled epidemiologic field studies have provided evidence which firmly associates the agent first recovered by Eaton in 1944 with lower respiratory tract illness of man.[1–3] A serologic response to the Eaton agent occurs in approximately 90 per cent of pneumonia illnesses in which cold agglutinins develop during convalescence as well as in a significant but variable proportion of cold agglutinin–negative pneumonias.[2, 4] The development of pneumonia and other forms of respiratory disease following the administration of tissue culture–grown Eaton agent to volunteers and the demonstration that naturally acquired antibody offered protection against such illness supports the contention that the agent is a respiratory tract pathogen.[5]

For many years, the agent was tentatively classified as a virus. The large size of the agent (180–250 mμ) and its sensitivity to streptomycin and various tetracycline derivatives, however, posed some difficulty with such a classification.[6–8] Recently, Marmion and Goodburn were able to visualize small cocco-bacillary bodies on the mucous layer covering the bronchial epithelium of the Eaton agent infected chick embryo.[9] The distribution of these bodies corresponded with the localization of Eaton agent as visualized by the fluorescent antibody technique. These workers also demonstrated that the Eaton agent was inhibited by an organic gold salt. Clyde visualized extracellular "colony-like" structures in stained preparations of infected tissue culture; these structures corresponded with the areas of specific immunofluorescence.[10] Both groups of workers suggested the possibility that the Eaton agent may be a "pleuropneumonia-like organism" (PPLO) rather than a virus. Cultivation of the organism in cell-free media, however, was not achieved.[11]

Stimulated by the findings of Marmion and Goodburn, we attempted cultivation of the Eaton agent on an agar medium incorporating 2.5 per cent yeast extract and 20 per cent horse serum. The present report will describe the successful growth of the agent on agar and its identification as a PPLO.

Materials and Methods.—Media: Agar plates, measuring 5 cm in diameter, were prepared with 7 parts Difco PPLO agar, 2 parts uninactivated horse serum and 1 part 25% yeast extract (prepared from active Baker's yeast and stored at − 20°C). The agar was prepared in 70-ml quantities and sterilized by autoclaving. After cooling to about 45°C, the agar was supplemented with the yeast extract and horse serum. In one passage series, antibiotics were not employed, while 500 units of penicillin/ml was added to the medium in a parallel series. The plates were generally used the same day they were prepared.

Cultivation: Agar plates inoculated with Eaton agent were incubated at 36°C. Initially, infected tissue culture fluid was streaked onto the agar. Subsequently, passages were initiated by rubbing a small block of agar, measuring 1–2 cm^2 and representing approximately one twentieth to one tenth of the volume of the agar on a plate, over the surface of a fresh plate.

Fluorescent antibody techniques: The method employed for the staining of Eaton agent antigen in infected chick embryo lung sections has been described previously.[2] Briefly, chick embryos

were inoculated by the amniotic route at 13 days; the lungs were harvested 6 days later and quick-frozen in an alcohol-CO_2 bath. The lungs were sectioned in the frozen state and the sections fixed in acetone for 10 min at room temperature. Serial twofold dilutions of human or rabbit serum were prepared in a diluent consisting of 1:10 normal guinea pig serum. Serum dilutions were then incubated on the lung sections for 30 min at 37°C. The sections were then washed in 3 changes of phosphate-buffered saline (pH 7.2), and horse antihuman globulin or sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate was then added. After 30-min incubation at 37°C and 3 washes in buffered saline, the sections were covered with buffered glycerol (pH 7.0) and viewed by ultraviolet microscopy using a Corning 5970 exciter filter and a Wratten 2B barrier filter.

Colonies from agar plates were stained by a method described previously.[12] A block of agar containing many colonies was applied to a slide, so that the agar surface exposed to the air during incubation was next to the glass. The slide with the agar block facing downward was then immersed in an inclined position in a 250-ml beaker of distilled water heated to 80°C. The water was rapidly heated to 85°C, during which time the agar block slowly slid off the glass slide which was then quickly rinsed in a beaker of distilled water at 90°C. Following this treatment a large number of colonies relatively free of agar were found adherent to the glass slide. These were then fixed with acetone for 10 min at room temperature and stained with either human or rabbit serum and the appropriate antiglobulin conjugate as described above.

Acute and convalescent phase sera from a patient with pneumonia were always tested simultaneously with consecutive chick embryo lung sections and with colonies from the same agar plate.

Sera: Acute and convalescent sera were available from patients with pneumonia who developed a rise in fluorescent stainable antibody for Eaton agent. These serum pairs were collected from 1955 to 1961 and from 4 different localities.[2, 4, 13] Eaton agent was recovered in embryonated eggs or in monkey kidney tissue culture from the 5 individuals in the group whose acute phase throat swab specimen was so tested.[2, 13]

A rabbit antiserum prepared against the Mac strain of Eaton agent was kindly supplied by W. Clyde. This antiserum was produced by repeated inoculations of rabbits with an infected chick embryo lung suspension.[10]

Eaton agent: The FH strain was originally supplied through the kindness of C. Liu, who recovered it in embryonated eggs from a student with atypical pneumonia.[1] After 3 egg passages in our laboratory, the strain was grown in chick embryo entodermal cell cultures and then subsequently in monkey kidney tissue culture.[14]

PPLO: A human oral strain, recovered from a Naval recruit with pneumonia, was kindly supplied by Y. Crawford. The H-110 human genital strain and the T5 tissue culture strain were supplied through the kindness of H. Morton. The avian strains were kindly supplied by J. Fabricant. The other strains were maintained in the laboratory.

Results.—Growth on agar: The isolation of the Eaton agent and the subsequent passage series were initiated by one of us (L. H.) from a frozen pool of third monkey kidney tissue culture passage fluid which contained 10^4 egg infectious doses per 0.1 ml.[14] This material was tested five times on agar plates, and on each occasion 5–10 colonies were observed on the sixth to seventh day. On two occasions, subsequent passage was unsuccessful, while in two other instances, a limited passage series was interrupted by bacterial contamination. With the fifth attempt, a successful transfer series was initiated which is now in its thirteenth passage.

During the first and second passage, colonies were not observed until the sixth or seventh day of incubation. Subsequently, colonies appeared more rapidly, and by the fifth passage, they were recognizable by the third day following inoculation.

Agar from the ninth passage was ground in nutrient broth (10 ml per plate) and titrated in embryonated eggs employing the fluorescent antibody technique. This material was found to contain a total of 10^5 egg infectious doses per plate.

Eggs infected with dilutions of this agar exhibited the characteristic distribution of Eaton agent immunofluorescence; i.e., staining was limited to the area of the bronchial epithelium. In addition, tests with acute and convalescent serum pairs from patients with Eaton pneumonia indicated that the immunofluorescence was specific. Since each passage of the agent on agar represented a 1:10 to 1:20 dilution, the total dilution of the original tissue culture material at the ninth passage was 10^{14} or greater. This indicated that an organism having the antigenic properties of Eaton agent had replicated on an artificial agar medium.

Properties of the agent on agar: The colonies formed on agar were granular with the center embedded in the agar. Occasionally, colonies having a "fried egg" appearance were seen, i.e., a dense center with a less dense periphery. Most colonies, however, exhibited a homogeneous granularity. A typical colony of the sixth passage at six days' indubation is shown in Figure 1. The embedded portion

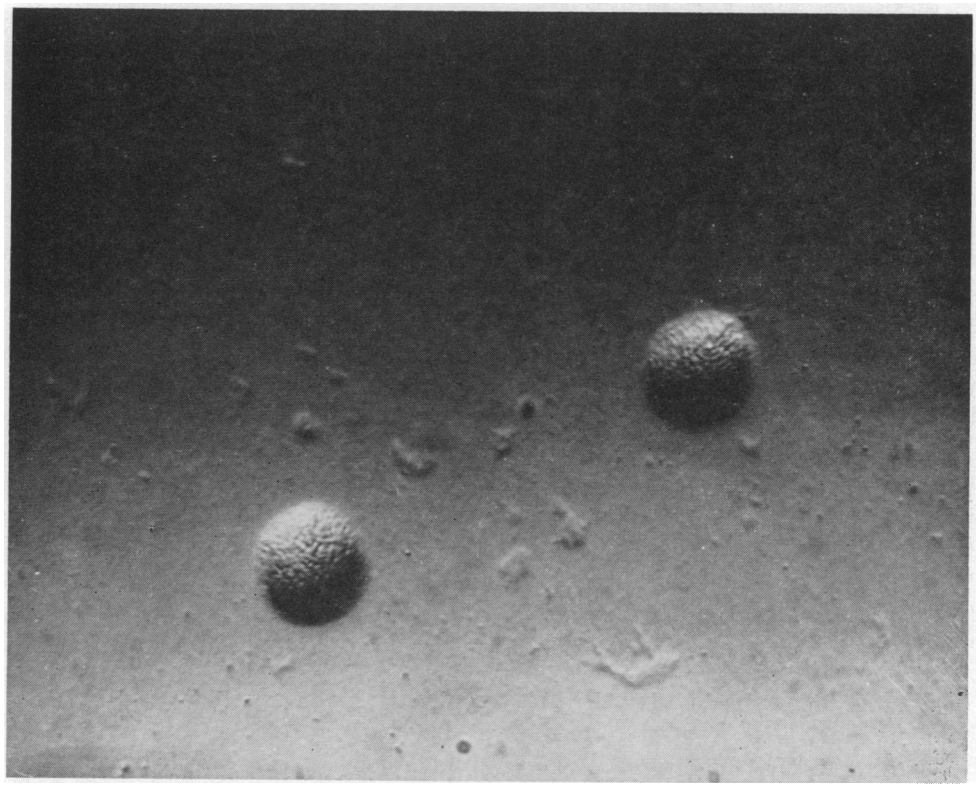

FIG. 1.—PPLO colonies (× 600).

of the colony could not be removed from the agar surface with a loop. When recognizable, colonies measured approximately 10 microns in diameter, and at maturation, they were 50 to 100 microns. When the Dienes stain was applied to the colonies (Fig. 2), many blue densely staining granules were seen.[15] The colonies did not decolorize this stain on incubation.

When horse serum was omitted from the agar medium, growth did not occur. Yeast extract was also essential for the growth of the organism on agar.

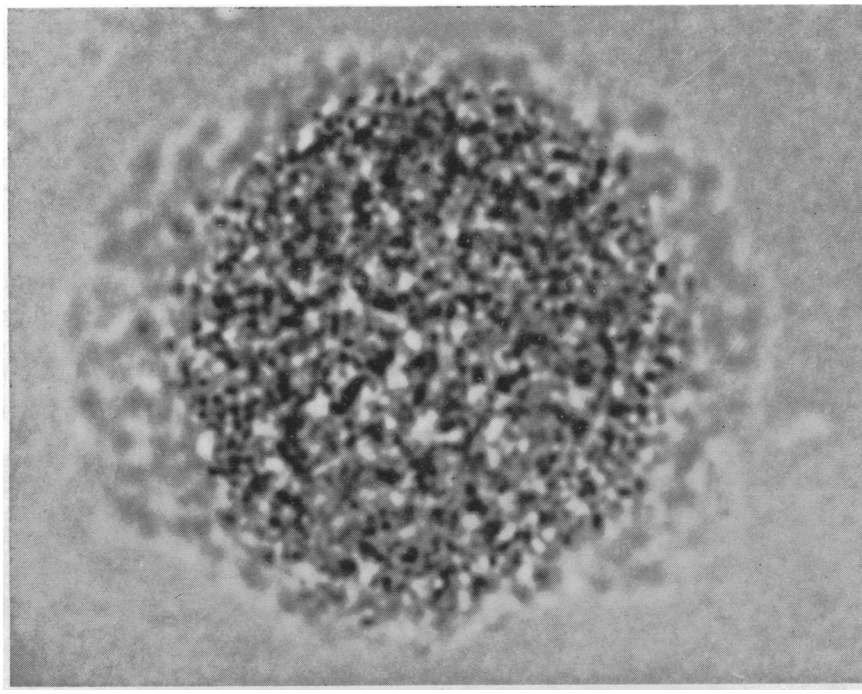

FIG. 2.—PPLO colony stained with Dienes stain (× 2100).

Colonies from the antibiotic-free and from the penicillin-treated passage series were morphologically indistinguishable. When tested by immunofluorescence, as described below, colonies from the two series gave identical staining reactions.

Identification of the colonies formed on agar: Colonies growing on agar plates were transferred to glass slides and then tested with acute and convalescent serum from patients who developed a rise in antibody to the Eaton agent or to adenovirus, para influenza virus, or Q fever. As shown in Table 1, the 10 patients with Eaton pneumonia who developed an antibody rise for the agent when tested with Eaton-infected chick embryo sections also showed similar but generally less extensive increments of antibody when the colonies from agar plates were examined by immunofluorescence. The failure of acute phase serum to react with the colonies and the intense fluorescence observed with convalescent serum are shown in Figures 3 and 4. These tests were performed with colonies from the seventh and ninth passages. Similar results were observed when colonies from the thirteenth passage were tested with acute and convalescent serum pairs. The diagnosis of Eaton infection in five of the pneumonia patients shown in Table 1 was confirmed by recovery of the agent in eggs or tissue culture from specimens collected during the acute phase of illness.

The parallelism in staining reactions observed with Eaton-infected chick embryo sections and the colonies grown on agar indicates that the latter are antigenically similar to the egg-propagated agent. A spurious relationship of the chick enbryo agent and the colonies which grow on plates seems improbable since the paired sera shown in Table 1 were derived from Eaton pneumonia patients

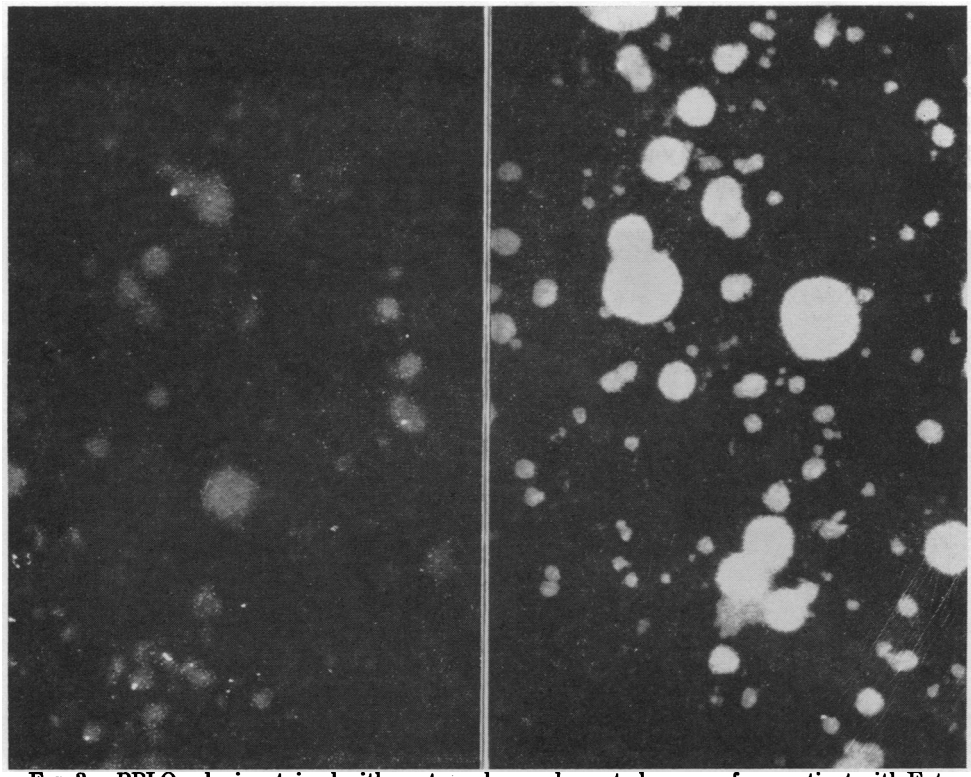

FIG. 3.—PPLO colonies stained with acute and convalescent phase sera from patient with Eaton pneumonia. Eaton agent recovered in tissue culture from this patient.
Left: Acute phase serum 1:10. *Right:* Convalescent phase serum 1:40.

TABLE 1

RELATION OF PPLO COLONIES FROM AGAR TO EATON AGENT

Diagnoses					Reciprocal of Fluorescent Staining Titer of Serum Tested with:			
					Eaton-infected Chick Embryo		PPLO Colonies from Agar*	
Clinical	Serologic	Year	Location	Isolation of Eaton agent	Acute	Conval.	Acute	Conval.
Pneumonia	Eaton	1955	D. of Col.	N.T.†	20	80 or >	10	80
"	"	1956	Italy	N.T.	< 10	80 or >	< 10	80 or >
"	"	1958	D. of Col.	Pos.‡	< 10	80 or >	< 10	40
"	"	1959	S. Car.	Pos.	< 10	80 or >	< 10	20
"	"	"	"	"	< 10	80 or >	< 10	80
"	"	"	"	"	< 10	80 or >	< 10	80 or >
"	"	"	"	N.T.	< 10	80	< 10	40
"	"	"	Illinois	N.T.	< 10	80 or >	< 10	20
"	"	1961	"	N.T.	< 10	80 or >	< 10	40
"	"	"	D. of Col.	Pos.	< 10	80 or >	< 10	40
Pneumonia	Adenovirus & Q fever	1960	S. Car.	N.T.	< 10	< 10	< 10	< 10
"	Adenovirus	"	"	N.T.	< 10	< 10	< 10	< 10
"	"	"	"	N.T.	< 10	< 10	< 10	< 10
"	Para influ. 3	"	"	N.T.	< 10	< 10	< 10	< 10
Upper respir. illness	Para influ. 1	1958	Maryland	N.T.	< 10	< 10	< 10	< 10
"	Para influ. 3	"		N.T.	N.T.	< 10	N.T.	< 10

* Colonies from seventh–ninth agar passage.
† Not tested.
‡ Eaton agent recovered from throat swab specimen collected during acute phase of illness.
Note: Acute and convalescent phase serum pair always titered with chick embryo sections and colonies from agar in same test by indirect fluorescent antibody method. Persons with adenovirus, para influenza virus, or Q fever infection developed a high level of homologous CF antibody during convalescence.

whose illness occurred in four different localities and in different years. Lower-staining titers were generally obtained when sera were tested with colonies from agar. Possibly, partial denaturation of antigen occurred at 80° to 85°C, the temperature required to separate the colonies from the agar. There was no evidence for antigenic heterogeneity among the colonies from agar, since fluorescent staining of every colony was observed in positive preparations.

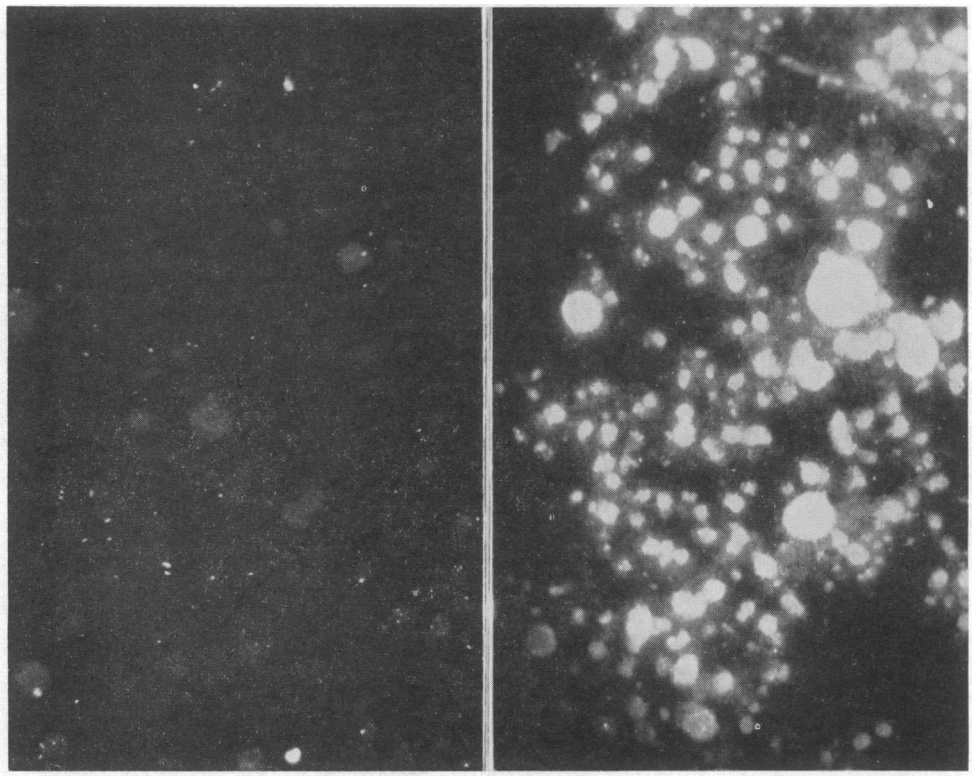

FIG. 4.—PPLO colonies stained with acute and convalescent phase sera from patient with Eaton pneumonia.
Left: Acute phase 1:10. *Right:* Convalescent phase 1:20.

As shown in Table 1, patients with respiratory disease associated with adenovirus, para influenza virus, or Q fever infection failed to develop antibody to the chick embryo-propagated Eaton agent and were similarly negative when tested with the agar-grown colonies.

Additional evidence that the agar colonies represented Eaton agent was provided when these colonies were tested with a rabbit antiserum prepared against the prototype Mac strain propagated in chick embryos. As shown in Table 2, this rabbit serum reacted with the colonies but failed to stain human genital, bovine, rat, or sewage strains of PPLO.

Relation of Eaton agent to other PPLO: The relation of Eaton agent to other PPLO was investigated in a more extensive fashion with a potent convalescent serum from a patient with Eaton pneumonia. As shown in Table 2, the Eaton agent was antigenically distinct from three human oral and four human genital

TABLE 2

RELATION OF EATON AGENT TO OTHER PPLO

PPLO		Reciprocal of Fluorescent Staining Titer with Indicated Serum*	
Source	Strain	Hyperimmune Eaton rabbit	Convalescent phase human pneumonia
Human	Eaton	40	80 to 160
Human oral	Illinois 1961†	N.T.§	< 10
	Maryland 1961 *a*‡	"	< 10
	Maryland 1961 *b*‡	"	< 10
Human genital	Campo	< 20	< 10
	39	< 20	< 10
	48	< 20	< 10
	H-110	N.T.	< 10
Bovine genital	B-15	< 20	< 10
Rat	JR-3(L4)	< 20	< 10
Avian	NTF	N.T.	< 10
	K86-B	"	< 10
Tissue culture	T5	"	< 10
	L cell	"	< 10
	HEp-2	"	< 10
	Human intestine	"	< 10
	Rabbit kidney	"	< 10
Sewage	Laidlaw	< 20	< 10

* Indirect fluorescent antibody method was employed.
† Recovered from a naval recruit with pneumonia.
‡ Recovered from a volunteer given Eaton agent.
§ Not tested.

strains of PPLO as well as PPLO strains derived from cows, rats, birds, sewage, and various tissue culture cell lines.

Discussion.—When the tissue culture-adapted FH strain of Eaton agent was inoculated onto horse serum agar plates, colonies developed which could be passaged in series on this artificial antibiotic-free medium. The morphologic and staining properties of the colonies as well as their requirement for serum identified the organism as a member of the genus *Mycoplasma* (PPLO). The PPLO colonies were identified as Eaton agent by immunofluorescent tests with acute and convalescent phase sera from patients with Eaton pneumonia and with a hyperimmune Eaton rabbit antiserum. Thus, the present findings indicate that the Eaton organism is a PPLO. Isolation and successful passage of the agent in an antibiotic-free agar medium indicates that it is probably not an L form of a bacterium.

The properties previously defined for the Eaton agent—size 180 to 250 mμ, sensitivity to tetracyclines and organic gold salts, and the occurrence of coccobacillary bodies on the infected chick embryo bronchial epithelium—are compatible with the contention that the organism is a PPLO.[6-9] The beneficial effect of demethychlortetracycline on the course of Eaton pneumonia is also consistent with this contention, since it is known that PPLO are sensitive to tetracyclines.[8, 16, 17]

Although previously the nature of the Eaton agent was not understood, it was possible to elucidate its role in human respiratory disease. Recent studies have shown that the Eaton agent is associated with at least 90 per cent of cold agglutinin–positive pneumonia.[1, 2, 4, 8] In addition, the agent has been implicated in a variable proportion of cold agglutinin–negative lower respiratory tract illness, the exact proportion varying with the particular population under investigation.[2, 4, 18] In one large Marine recruit population, the Eaton agent was associated with 51 per

cent of 530 pneumonias occurring over a 16-month period.[2, 8] The finding that the Eaton agent is a PPLO provides the first demonstration that an organism of this type is etiologically associated with human respiratory disease. It is of interest that the prototype PPLO, *Mycoplasma mycoides*, is responsible for a virulent and often fatal pneumonia in cattle.[19]

The association of one variety of PPLO with human respiratory disease suggests that PPLO other than Eaton agent may be capable of producing respiratory disease in man. The observation that demethylchlortetracycline had a beneficial effect in a large group of etiologically undiagnosed pneumonia illnesses is compatible with this possibility.[8] It is clear that attempts to recover PPLO should be included in any systematic investigation of the presently unexplained segment of human respiratory disease.

Many questions remain unanswered regarding the Eaton agent and its natural history. Growth of the organism on artificial medium may facilitate studies designed to answer such questions. Once the optimum conditions for growth on agar have been defined, it is possible that the recovery of the agent and its identification by immunofluorescence could be achieved within a few days, thus providing a rapid method for diagnosis of infection. The growth of Eaton agent on artificial medium should stimulate efforts to prepare inactivated vaccines and to search for attenuated variants which might be used for immunoprophylaxis. Antigenic preparations, either living or dead, should ultimately prove effective since antibody has been shown to protect against illness under experimental and natural conditions of infection.[2, 5]

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*HETEROGENEITY OF RABBIT ANTIBODY AND ITS SUBUNITS**

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It has been shown by Porter[1, 2] that hydrolysis of rabbit antibody by papain yields one inactive and two active fractions which are separable on carboxymethyl cellulose. The active fractions, designated I and II, are univalent[3–5] and are very similar to one another in molecular weight (~50,000), amino acid composition, and antigenic characteristics. Porter's findings were of great significance since they showed that the antibody consists of well-defined subunits and established many of the properties of the fragments. They have stimulated many subsequent investigations of the subunits of antibodies.

Some time ago it was noted that the amount of protein obtained as fraction I varies gradually with the pH of the 0.01 *M* acetate buffer used for its elution, increasing from about 15 per cent of the total at pH 5.0 to approximately 50 per cent at pH 5.6; the sum of I and II was fairly constant (about 60 per cent of the total). Approximately equal amounts of I and II were obtained at pH 5.4 with the carboxymethyl cellulose used. These findings appeared somewhat inconsistent with the hypothesis that fractions I and II represented two fragments of the molecule.

Recently Cebra *et al.*[6] reported that fractions I and II each contain 0.5 mole of *N*-terminal alanine, available for reaction with dinitrofluorobenzene, per mole of