the same as that observed in the present study but that the number of local tumors induced was considerably higher. It is difficult to compare this investigation with the current study in detail since there were differences in dosages and other aspects of technic. Nevertheless, it appears to be consistently the case that newborn mice differ from adults in having an enhanced response of the lymphoid tissues and a diminished response to local sarcoma formation by chemical carcinogens.

The mechanism of enhancement of these lymphomas having a viral component by a chemical carcinogen is obviously of basic interest but is beyond the scope of the present investigation.

Summary. Subcutaneous injection of 100 μg of 7,12-dimethylbenz(a)anthracene in 0.05 ml tri-n-caprylin in newborn AKR mice considerably shortened the latent period of malignant lymphomas. All malignant lymphomas observed in the treated group before the time of appearance of the first lymphoma in the controls were of the stem-cell type.

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Growth of Eaton PPLO in Broth and Preparation of Complement Fixing Antigen. (27681)

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Although the Eaton agent was first recognized in 1944, its natural history and relative importance in human lower respiratory tract disease are not fully understood(1) primarily because of technical difficulties involving propagation and identification of the organism and quantitation of antibody. Since 1957 the standard system for antibody assay has been the indirect fluorescent antibody test in which frozen sections of infected chick embryo lung serve as the source of antigen(2). The preparation of such frozen sections is slow and cumbersome, hence many laboratories have been reluctant to initiate diagnostic or epidemiologic studies of Eaton infection.

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One of the objectives of this laboratory has been the development of a simple but sensitive serologic test for infection with the Eaton agent. New impetus was provided by the recent demonstration that the Eaton agent is a PPLO (genus Mycoplasma) and can be cultivated on an artificial agar medium(3). Initially it was hoped that PPLO colonies transferred from agar plates to glass slides could be substituted for the chick embryo lung as antigen in the fluorescent antibody test. Comparative tests indicated, however, that serum titers were generally lower with the former source of antigen(3). Artificially propagated Eaton PPLO was also examined as a possible source of complement fixing (CF) antigen for serodiagnosis. This effort was successful and is reported here.

Materials and methods. PPLO. The FH strain of Eaton PPLO was used. It was originally recovered in embryonated eggs by Liu from a patient with atypical pneumonia, adapted to tissue culture in this laboratory, and subsequently grown on an agar medium (2,3,4).

Medium. Agar plates were prepared with 7 parts Difco PPLO agar, 2 parts unheated horse serum and 1 part 25% fresh yeast extract(3). This was supplemented with penicillin (500 units/ml), amphotericin (5 μg/ml) and thallium acetate (final concentration of 1:2000). Broth medium was similarly prepared except that Difco PPLO broth was substituted for PPLO agar.

Cultivation and titration. Inoculated agar plates or broth suspensions were incubated aerobically at 35°C. The quantity of infectious material present in a given preparation was estimated by inoculating 0.1 ml onto a 5-cm diameter agar plate and counting the number of PPLO colonies present after 10 to 14 days of incubation. When high titers were anticipated decimal dilutions of the test material were prepared in PPLO broth and inoculated onto agar plates.

Treatment of infected broth suspensions. These were concentrated by centrifugation in the Model L Spinco ultracentrifuge. Following centrifugation at 10,000 or 40,000 rpm sediments were resuspended to 1/50th-1/100th the original volume in veronal buffered saline pH 7.2. All preparations were heated at 56°C for 30 minutes before being tested for complement fixing activity.

CF technic. The micro CF technic used was that adapted by Sever from the procedure described by Takatsy(5,6) and is essentially the same as that described previously from this laboratory except for a decrease in volume of the various reagents(7). Two full units of complement were used and the serum-antigen mixtures were incubated at 4°C for 18 hours before addition of sensitized sheep erythrocytes. The end point was considered the highest serum dilution at which 75% or more of the sheep red cells remained unhemolyzed.

Sera. Acute and convalescent sera were collected from Marine recruits with pneumonia, febrile respiratory illness, or afebrile respiratory disease as well as from control individuals free of respiratory illness. The Marine recruit population and the design of our epidemiologic studies at the Parris Island Recruit Training Center have been described (8). The serum specimens were tested shortly after collection by the indirect immunofluorescent technic using infected chick embryo lung sections(2,8). The sera were stored at –20°C for 18 to 24 months before being tested by the CF technic.

Results. Growth in broth. Starting with an 8th agar plate passage of the Eaton agent, it was possible successfully to transfer the organism 10 times at 7-10-day intervals in PPLO broth medium. The agent was diluted $10^{-1.3}$ at each passage so that the final dilution at the 10th transfer was 10^{-13}. 10^4 colony forming units per ml were present in the 10th passage fluid, indicating that propagation had occurred.

Before a CF antigen was sought the growth pattern of Eaton PPLO in broth was studied, to determine the time when maximum quantities of PPLO were present in broth. In Experiment 1, $10^{1.2}$ colony forming units of 7th broth passage material were added per ml of broth. Maximal growth occurred by the 9th day when $10^{5.0}$ colony forming units per ml were present (Fig. 1). A significant quantity of viable PPLO was still present after 22 days of incubation. In Exp. 2 a larger inocu-

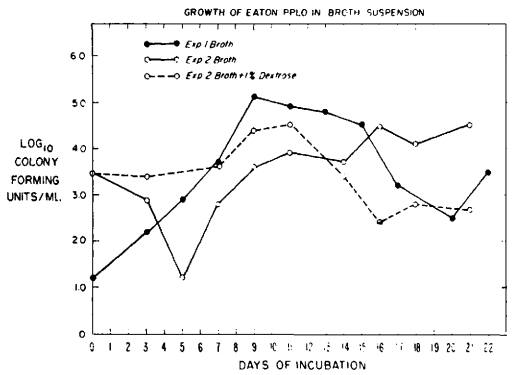

FIG. 1. Growth of Eaton PPLO in broth suspension.

lum was used (final concentration = $10^{3.5}$ colony forming units per ml). Two types of medium were tested in this experiment—the standard broth and standard broth supplemented with 1% dextrose. The agent reached the same titer in both media; however, the maximal level was attained earlier (9-10 days) in the broth supplemented with dextrose than in the unsupplemented medium (16-21 days). The significance of the difference observed for growth in the standard broth in Exp. 1 and 2 is not clear. It is clear, however, that Eaton PPLO grows slowly in broth compared to other human PPLO which generally reach maximal levels within 1-3 days(9,10). The slow growth of Eaton PPLO suggested that CF antigen might best be sought after the 8th day of incubation.

Properties of CF antigen. Centrifugation studies indicated that specific antigen was sedimented at 10,000 rpm for 30 minutes. When infected broth was concentrated 100 times at this speed the sediment had an antigen titer of 1:128. An equal or greater quantity of antigen was also of smaller size or lesser density. This material was sedimented from the 10,000 rpm supernatant fluid by centrifugation at 40,000 rpm; the titer of the 100 × concentrated sediment was 1:128. Centrifugation at 40,000 rpm for 30 minutes to one hour did not sediment all of this antigen since an equal quantity was recovered after a second centrifugation at 40,000 rpm for 1 hour. It appeared, therefore, that Eaton antigen(s) was associated with various sized particles, the smallest of which was sedimented with difficulty.

The time course of antigen production in broth was studied. The quantity of antigen recovered after 100 × concentration was the same (titer of 1:64-1:128) from harvests taken at 8, 12, or 16 days. In addition, antigen sedimentable at 10,000 rpm and that requiring 40,000 rpm for recovery were present in approximately equal quantities throughout this period.

Fifty- to 100-fold concentrates of Eaton infected broth material were anti-complementary to a dilution of 1:16 to 1:32. This meant that only a 4- to 8-fold difference existed between the titer of anti-complementary effect and that of specific antigen. It was possible to reduce this anti-complementary activity by treatment of the broth concentrate with 0.5% phenol for 4 days at 37°. Following such treatment the antigen was anti-complementary at a dilution of 1:4 to 1:8 but not at 1:16. In addition phenol treatment resulted in a 4-fold increase in specific antigenic activity.

The foregoing information was utilized for preparation of an antigen which was studied for its specificity and sensitivity in relation to the chick embryo lung section fluorescent antibody system. This CF antigen was prepared as follows: an inoculum which gave a final concentration of $10^{1.7}$ colony forming units per ml was added to PPLO broth containing 2.5% yeast extract, 1% dextrose, and 20% unheated horse serum. After 16 days incubation at 35°C, phenol was added to a final concentration of 0.5% and the mixture was incubated for an additional 4 days at 35°C. The broth was then centrifuged at 10,000 rpm for 30 minutes. The sediment was suspended in 1/50th the original volume in veronal buffered saline pH 7.2. The supernatant fluid was centrifuged at 40,000 rpm for 30 minutes and the resulting sediment suspended in 1/50th the original volume in veronal buffered saline pH 7.2. The sediment and supernatant fluid concentrates were pooled and the combined material had an antigen titer of 1:320, while it was not anti-complementary at 1:10. The antigen was used at a dilution of 1:10.

Specificity and sensitivity of CF antigen. The specificity and sensitivity of CF antigen prepared from broth culture was investigated using paired sera from Marine recruits with and without immunofluorescent evidence of Eaton infection. Representative CF reactions are shown in Table I. Thirty-four of 42 fluorescent antibody positive recruits developed a rise in CF antibody (Table II). A CF response did not appear to be related to the severity of illness associated with Eaton infection. CF antibody developed in a high proportion of asymptomatic or mild infections as well as those associated with fever and/or pneumonia. Ten of the recruits developed a 4-fold rise, 11 an 8-fold rise, while 13 developed a 16-fold or greater rise in CF antibody. In only one instance was CF antibody detected in the acute phase serum. This specimen did not contain fluorescent stainable antibody at a dilution of 1:10. CF antibody was not detected at a dilution of 1:8 in the second or convalescent serum specimen of 39 fluorescent antibody negative recruits. Approximately one half of these individuals had pneumonia. These findings suggested that

TABLE I. Representative Reactions of CF Antigen with Paired Sera from Marine Recruits with or without Immunofluorescent Evidence of Eaton Infection.

		Reciprocal of antibody titer			
		Fluorescence*		CF†	
Diagnoses	Recruit	Acute	Conval.	Acute	Conval.
Pneumonia	1	<10	40	<8	16
	2	<10	160 or>	<8	64
Febrile respir.	3	<10	40	<8	32
	4	<10	160 or>	<4	8
Afebrile respir.	5	<10	160	<4	16
No respir. illness	6	<10	80	<8	32
Pneumonia	7	—	<20	—	<8
	8	—	<20	—	<8
	9	—	<20	—	<8
No respir. illness	10	—	<20	—	<8
	11	—	<20	—	<8

* Performed with sections of Eaton infected chick embryo lung.

† Performed with 32 units of Eaton PPLO antigen, *i.e.*, a 1:10 dilution of a pool containing a 50-fold concentrate of broth sediment (obtained after centrifugation at 10,000 rpm), and a 50-fold concentrate of supernatant fluid (obtained by centrifuging 10,000 rpm supernate at 40,000 rpm).

TABLE II. Comparison of Immunofluorescence and Complement Fixation for Serodiagnoses of Eaton Infection.

Category by immunofluorescence	Diagnoses	Reciprocal of fluorescent antibody titer* Acute	Conval.	No. in group	No. with 4 × or > rise in CF antibody†
Eaton positive					
	Pneumonia	<10	20	1	0
		<10	40	9	8
		<10	80 or>	14	14
	Febrile respir. illness	<10	20	1	0
		<10	40	3	2
		<10–20	80 or>	3	2
	Afebrile respir. illness	<10–20	80 or>	5	4
	No respir. illness	<10	40	1	0
		<10–20	80 or>	5	4
	Total			42	34 (81%)
Eaton negative					
	Pneumonia	—	<20	19	0
	No respir. illness	—	<20	20	0
	Total			39	0

* Performed with sections of infected chick embryo lung.

† Convalescent serums tested at 1:8 dilution; if fixation of complement occurred then dilutions of acute phase serum and convalescent phase serum were tested simultaneously.

Note: This test was performed with 32 units of antigen – see footnote Table I.

the CF antigen is a specific and moderately sensitive reagent for serodiagnosis of Eaton infection.

Discussion. The slow growth of Eaton PPLO is in contrast to the more rapid replication of other human PPLO strains recovered from oropharyngeal or genital sources (9,10). Growth of Eaton PPLO was minimal on the 2nd and 3rd day of incubation at a time when non-Eaton PPLO reach peak titer in broth suspension. Maximal levels of Eaton PPLO were obtained in broth suspension on the 9th to 16th day of incubation. Previously a difference in colonial morphology had been noted between Eaton and non-Eaton PPLO recovered from the human oropharnyx(11). The former produced homogeneously granular colonies, while the latter produced "fried egg" or "nippled" colonies. It remains to be determined whether the slower growth rate and unusual morphological appearance of Eaton PPLO reflects an essential biologic property of the organism or our failure to provide an optimal medium for cultivation.

Independently, Somerson, Freundt, and Edward have recently demonstrated that the Eaton PPLO metabolizes dextrose(12,13,14). More rapid growth of the organism in a medium supplemented with dextrose (Fig. 1) is consistent with these findings and suggests that ordinary PPLO broth does not contain an optimal level of dextrose for growth of this PPLO.

The biophysical properties of the antigen reactive in the CF test are of interest. A considerable proportion of this material is smaller or less dense than the organism, and does not sediment at 10,000 rpm. What is the nature of the soluble antigen? Does it represent a degradation product of disrupted PPLO, or is it produced in excess in a soluble form during replication?

Although infected broth concentrate contained an appreciable quantity of antigen (titer 1:64-1:128) such material was not suitable for serodiagnosis because of anti-complementary effects (titer 1:16-1:32). Phenol treatment of the antigen reduced the anti-complementary activity and permitted production of an antigen which could be used at a dilution which contained 32 units. In addition, phenol treatment of the antigen enhanced antigenic potency. Similar findings have been reported for the CF antigen of lymphogranuloma venereum(15). Phenol treated Eaton antigen was highly specific. The serum of 39 fluorescent antibody negative individuals did not fix complement with the antigen, whereas, 34 of 42 recruits with immunofluorescent evidence of Eaton infection developed a rise in CF antibody during convalescence.

Relatively simple laboratory tools are now available for the study of Eaton infection in various ecologic settings. It was recently shown that naturally occurring strains of this PPLO can be recovered on an artificial agar medium from a high proportion of infected individuals(11). The ease of direct isolation of the organism on agar plus the availability of a moderately sensitive CF antigen should expedite efforts to define the role of Eaton PPLO in both acute and chronic respiratory illness.

Summary. Eaton PPLO grew in broth medium supplemented with 20% unheated horse serum and 2.5% yeast extract. Growth was slow and maximal levels were attained on the 9th to 16th day of incubation. A specific CF antigen was prepared from infected broth suspension by concentration (50 ×) and treatment with 0.5% phenol. CF antigen was estimated to be 80% as sensitive for serodiagnoses of Eaton infection as immunofluorescence with chick embryo lung sections.

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Calcium Absorption in the Rat in Relation to Excessive Vitamin D and Cortisone. (27682)

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Our initial studies(1), measuring active transport of calcium across the intestinal wall of the rat by an *in vitro* intestinal sac technic, indicated that excessive Vit. D increased active transport in all segments studied, but cortisone decreased this transport in the duodenal segment only. This technic would be most helpful in investigation of factors affecting calcium absorption if the results obtained by this *in vitro* technic correlated with those obtained by studying calcium absorption in the intact animal. It was considered important therefore to extend our initial *in vitro* experiments, to study *in vivo* calcium absorption under the same experimental conditions, and to compare the results of the two procedures, in an attempt to determine: 1) whether excessive amounts of Vit. D will increase intestinal calcium absorption *in vivo* above that induced by physiologic amounts, 2) whether cortisone will inhibit calcium absorption *in vivo* in presence of either physiologic or excessive amounts of Vit. D, 3) whether changes in intestinal active transport of calcium determined *in vitro* can be used as an accurate index of calcium absorption *in vivo*.

Materials and methods. Male Sprague-Dawley rats weighing 100-130 g were treated with daily doses of 20,000 units of Vit. D* by stomach tube, or 2.5 mg of cortisone acetate† subcutaneously, or a combination of both drugs. A control group received cottonseed oil and saline, which were the vehicles for the Vit. D and cortisone respectively. During treatment, the rats were maintained on distilled water and a nutritionally complete diet containing a physiologic amount (148 USP units/100 g diet) of Vit. D.

Calcium metabolic balance studies were carried out from the 2nd through the 5th day of the treatment period. On the 6th day blood was obtained by orbital sinus puncture, the rats were killed by decapitation and a 3-cm segment of duodenum, jejunum, and ileum was quickly removed and prepared for *in vitro* active transport studies as previously described(1). In some of the animals, serial segments of small intestine (beginning at the pylorus) were studied for active transport. Calcium was extracted from the feces with trichloroacetic acid as described by Harrison and Harrison(2). The calcium content of serum, stool extract, and urine was determined by a modification of the method of Kingsley and Schaffert(3).

Using the above methods, a comparison of the effects of equal doses of cortisone and corticosterone on net calcium absorption, serum calcium level and duodenal active calcium transport was carried out, each treatment group consisting of 9 animals.

Results. In vivo net calcium absorption

* Kindly furnished by Brewer and Co., Inc., Worcester, Mass.

† Kindly furnished by Sharp and Dohme, Division of Merck and Co., Inc., Philadelphia, Pa.