

were checked culturally and gave the same reactions as were obtained in 1935, 1936, 1937, and 1938. The reason for duplication of cultures was the desire to check the work as carried on. The ampules were assigned by code number to Mr. Nozaki who did not know at the time the work was done that any specimens were duplicates. Actually the type 2's were run as numbers 1, 7, and 48 and the type 8's as numbers 34, 35, and 49. In the experiment in question, 34 different coliform types were included, and it was only by chance that these two (T2 and T8) were run in triplicate. Obviously colicine determinations were not being made by us at the time these cultures were originally studied prior to 1940.

The production upon first cultivation from the desiccated state of colicine active against *S. paratyphenteriae* Flexner type III (Stock "Z") in the case of three ampules each of two strains of *E. coli* desiccated in April and in May, 1938, a period of 15 years, is reported. It is believed that these data offer evidence as to the intrinsic nature of colicine production by certain coliform bacteria, particularly since there was not only a significant time lapse, but also because the cultures were repeatedly tested and subcultured following their isolation from feces and were far removed from any contact with heterologous bacteria.

CHICK EMBRYO EXTRACT, AN ENRICHMENT FOR CERTAIN STRAINS OF PLEUROPNEUMONIALIKE ORGANISMS ISOLATED FROM MAN¹

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Pleuropneumonia-like organisms (PPLO) from animals require the presence of some body fluid in the culture medium for growth *in vitro*. A variety of substances has been observed to serve satisfactorily as supplements. These substances are blood serum, ascitic fluid, hemoglobin (Salaman *et al.*, Brit. J. Venereal Diseases, **22**, 47, 1946), serum fractions A and B (Smith and Morton, J. Bact., **61**, 395, 1951; Arch. Biochem. Biophys., **38**, 23, 1952), and lipid extracts of egg yolk (Edward and Fitzgerald, J. Gen. Microbiol., **5**, 576, 1951). In addition, some of the P (parasitic) strains, when freshly isolated from the bovine genital tract, require the presence of hog gastric mucin or desoxyribonucleic acid (Edward and Fitzgerald, Vet. Record, **64**, 395, 1952). Virulence tests in animals with pleuropneumonia-like organisms isolated from man usually have not yielded positive results. A few investigators have reported intracellular

TABLE 1
The enrichment activity of chick embryo extract in relation to concentration and stability to heat and ether extraction using the Campo strain as the test organism

SUBSTANCE	GROSS ACTIVITY	APPROXIMATE YIELD (μG CELLULAR N/ML)
Embryo extract, 5% ..	++++	140
Embryo extract, 1% ..	++++	
Heated embryo extract, 1%	++++	100
Supernatant of heated embryo extract, 1%.	++++	
Sediment of heated embryo extract, 1%.	+	not measurable
Embryo extract, ether extracted, 1%	++	10
Control—no enrichments	—	0
Control—bacto-serum fraction A, 1%	++++	280

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pleuropneumonia-like organisms in cells from the urethra. Cultivation of the pleuropneumonia-like organisms in tissue culture might contribute valuable information on the parasitic nature of

these organisms. These studies are concerned with the possibility of using chick embryo extract, an important nutrient in tissue cultures, as an enrichment for the growth of pleuropneumonia-like organisms from man.

An extract was prepared by forcing 9 day old chick embryos through a narrow mesh stainless steel screen, mixing this material with an equal volume of Hank's solution, allowing the mixture to remain at room temperature for 30 minutes, and then clarifying by centrifugation. The extract was kept frozen until used. Its activity was checked by adding various amounts to "bacto-PPLO broth". The product that resulted from heating the extract at 100 C for 20 minutes and the product remaining after extracting three times with ether also were tested for activity.

Gross activity was determined by streaking a standard loopful of 3 day old broth cultures containing the above enrichments onto plates of "bacto-PPLO agar" (Morton, Smith, and Leberman, *Am. J. Syphilis, Gonorrhoea, Venereal*

Diseases, **35**, 361, 1951). The plates were incubated aerobically at 37 C for 3 days and then read by microscopic examination.

Total yield of growth (μg cellular N per ml) was determined by centrifuging (10,000 rpm for 15 min) 6 day old broth cultures (80 ml) containing the various enrichments. The sedimented growth was resuspended in 5 ml of 0.9 per cent NaCl solution, and the turbidity was determined in a Klett-Summerson photoelectric colorimeter (filter no. 42). This turbidity was equated to μg cellular N using a previously standardized curve.

As may be seen from table 1, extract of 9 day old chick embryos can be substituted for a variety of enrichments used for growing pleuropneumonia-like organisms from man. This growth promoting substance is heat stable and partially stable to ether treatment. The embryo extract also had enrichment activity for 4 other strains of pleuropneumonia-like organisms in our collection.

MUTANTS OF THE AZOTOBACTER UNABLE TO USE N_2 ¹

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Biochemical mutants have been used for the demonstration of metabolic pathways in several organisms, but they have not yet been applied extensively in studies of nitrogen fixation by the azotobacter because of the difficulty in isolating nonfixing strains. Using the technique of penicillin screening (Lederberg, *Meth. Med. Research*, **3**, 5, 1950) to concentrate any spontaneous nonfixing mutants, we have obtained numerous isolates of *Azotobacter agile*, strain 4.4, and *Azotobacter vinelandii*, strain 0, which cannot assimilate molecular N_2 .

With the azotobacter the parent culture is cultivated initially in a medium containing fixed nitrogen to permit multiplication of any naturally occurring mutants. The final culture is washed in $\text{m}/15$ phosphate buffer, pH 7.0, and inoculated into Burk's nitrogen-free medium (Wilson and Knight, *Experiments in bacterial*

physiology, Burgess Publishing Co., Minneapolis, 1952) at an initial concentration of 10^6 to 10^7 cells per ml. This medium is incubated at 30 C on a rotary shaker, four hours for *A. agile* and ten hours for *A. vinelandii*; then penicillin G is added at a concentration of 300 to 700 units per ml. Cells able to grow under these conditions are killed by the antibiotic whereas any nonfixing strains present will not be destroyed since they cannot grow. The culture is diluted and plated at varying times to Burk's medium containing 300 ppm ammonium-nitrogen. Plates which have well separated colonies are replicated to similar plates containing nitrogen-free or 300 ppm ammonium-nitrogen media, according to the method of Lederberg and Lederberg (*J. Bact.*, **63**, 399, 1952). The mates of those colonies on the replicated N-free media which did not grow or grew abnormally slowly were selected from the fixed nitrogen media and further tested in liquid culture for their ability to use molecular N_2 . Essentially the same method could

¹ On request the authors will furnish a multi-graphed summary that supplies the details of this study.