host antibodies on repeated immunization of guinea pigs, these were present in such low titers that on simple dilution the antisera would show specific activity only. The practical value of some of the antisera prepared for this study has been proved by using them in CF typing of enterovirus isolates(8).

On the other hand, the data presented show that, besides the host activity, the specific activity of fluorocarbon-treated antigens should be tested before using them for immunization. From ECHO types 15-19 no antisera were available during time of immunization. Hence, the lack of specific virus antibodies in ECHO type 19 antiserum may be caused by lack of specific virus activity in fluorocarbon-treated immunization antigen, which could not be tested.

Since each virus group differs in susceptibility to fluorocarbon treatment(4), the fluorocarbon technic can not be adapted directly to preparation of antisera of other viruses, but if adaptable it might be of value.

Summary. It was found that ECHO and poliomyelitis virus antisera without host CF antibodies could be prepared in guinea pigs by immunizing the animals with fluorocarbon-treated cell culture grown virus. Although in repeated immunization of guinea pigs 3 out of 12 fluorocarbon-treated antigens with no host CF activity produced host antibodies in titers of 1:32 and 1:64, specific virus titers were 16 and 32 times higher. It was also found that, besides the host activity, the specific virus activity of fluorocarbon-treated antigens should be tested before using them for immunization.

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1. Halonen, P., Huebner, R. J., Turner, H. C., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 530.

2. Manson, L. A., Rothstein, E. L., Rake, G. W., *Science,* 1957 v125, 546.

3. Hummeler, K., Hamparian, V. V., *ibid.,* 1957, v125, 547.

4. Hamparian, V. V., Muller, F., Hummeler, K., *J. Immunol.,* 1958, v80, 468.

5. Comm. on ECHO viruses, Nat. Foundation for Infantile Paralysis: *Science,* 1955, v122, 1187.

6. Comm. on Enteroviruses, Nat. Foundation for Infantile Paralysis: *Am. J. Pub. Hlth.,* 1957, v47, 1556.

7. Archetti, J., Weston, J., Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 265.

8. Halonen, P., Rosen, L., Huebner, R. J., unpublished data.

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Concentrated Culture of Gonococci in Clear Liquid Medium.* (26005)

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The common practise is to grow *Neisseria gonorrhoeae* on solid media, although the organism can be cultivated successfully on liquid semi-defined(1,2) and undefined media (3). These must usually be enriched with blood, starch, charcoal and similar material, however, and the resulting cloudiness complicates the observation of growth. An important growth-limiting characteristic of the gonococcus is its sensitivity to certain amino acids, fatty acids and possibly other toxic materials that are carried with ordinary medium constituents(4), including agar(5). Adsorptive removal of such inhibitory factors apparently accounts for the necessity of adding various enrichment materials. These might be spatially separated from the actual growth environment and still function, provided that diffusional access to the enrichment material is provided. A simple biphasic flask system (6) was so employed and allowed attainment of gonococcal growth in a water-clear men-

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struum and to a population density several times higher than usual.

Methods. The biphasic flask system(6) consisted simply of a thick layer of medium solidified with 2% agar and overlaid with a thin layer of liquid medium. Most of the experiments were done with 100 ml of agar and 25 ml of supernatant broth in a 250-ml Erlenmeyer flask. This method superficially resembles but in principle differs from the double-medium technic of Castaneda(7), which facilitates transfer and detection of colonies on an agar surface.

The medium used was Difco's dextrose-starch formulation consisting of 1.5% proteose peptone No. 3, .2% dextrose, 1% soluble starch, .5% NaCl, .3% $Na_2HPO_4 \cdot 2H_2O$, and 2% gelatin. The final pH was 7.3. The broth was clarified by adding Celite #512 (Johns-Mansville Co.) and filtering while hot through a pad of the same material. Agar was added at a concentration of 1% for direct use and 2% for use as the underlay in the biphasic system.

Seven freshly-isolated strains of culturally proven *Neisseria gonorrhoeae* were obtained from the State Bacteriological Laboratory in Stockholm and were maintained by serial transfer on agar medium. Experimental cultures were inoculated with approximately 1% by volume from a 48- or 72-hour broth culture, but the number of viable cells inoculated was found difficult to standardize. Consequently, the time course of growth could not be compared directly between experiments. Humidified air containing 10% carbon dioxide by volume was maintained over the cultures by connecting them with a reservoir. Since little change in volume of the gas phase was observed, it also was possible simply to stopper tightly the flasks after flushing with the CO_2-air mixture. Incubation was at 37°C on a rotary shaker, which was operated at 100-150 rpm in a radius of 1¾ inches. Cultural purity was checked by Gram staining and streaking on peptone agar.

Growth was measured in nephelometer with a linear decimal scale to 100. Direct counts of formalinized and diluted samples were made by phase microscopy using a conventional counting chamber. Estimates were made of numbers of cells in clumps, and the assumption was made that half of the apparent single cells were doubles. However, degree of multiplicity varied between different methods of cultivation so that at best the method only provided estimates of actual numbers of single cells. The data given represent minimum estimates.

The results of growth in the biphasic system were compared to results in a control flask containing the same total volume of medium. Concentration index expresses how many times more concentrated the organisms are per milliliter in the liquid layer of the biphasic culture than in the control broth culture. Yield index represents the ratio of total number of cells per flask in the biphasic system to total number in the broth control.

Results. A qualitative comparison of growth of 7 strains of *N. gonorrhoeae* first was made in shaken broth flasks, shaken biphasic flasks, static broth tubes, static biphasic tubes, and static agar-slant tubes. Complete dextrose-starch medium with appropriate addition of agar was used in each case. Some growth occurred under all of the conditions, but it was readily apparent that the most rapid, dense and homogeneous liquid growth occurred in the shaken biphasic flasks. Growth of the strains varied from light to very heavy final populations. Typical quantitative results with a representative strain grown in shaken biphasic flasks are given in the first line of data in Table I. Somewhat higher cell density but lower efficiency were obtained using 50 ml of agar overlaid with 12.5 ml of broth; *e.g.* 82 nephelos units, 5.1 x 10^9 single cells per ml, a concentration index of 2.2, and a yield index of 0.44 were obtained after 72 hr incubation.

Since the large molecular weight materials in dextrose-starch broth probably function as adsorptive and neutralizing materials rather than as nutrients, it seemed likely that incorporating them in the agar layer of the biphasic system should provide the same function and that the broth overlay consequently could be simplified. Accordingly, a series of biphasic flasks was made in which components of dextrose-starch broth were omitted from the broth overlay and the layers were allowed to

TABLE I. Growth of *N. gonorrhoeae* Strain 861 in Biphasic System with Simplified Overlay Broths.

Composition of overlay	System	24 hr Nephelos units of 1-11 dil	48 hr Nephelos units of 1-11 dil	Nephelos units of 1-11 dil	Total cells per ml in billions	Yield index	Concentration index
Dextrose-starch broth	Biphasic	28	34	64	3.3	.79	3.9
	Control	14	18	22	.84		
Idem, less starch	Biphasic	43	56	66	2.6	.57	2.9
	Control	13	14	15	.91		
" , less gelatin	Biphasic	41	49	67	3.2	.58	2.9
	Control	12	16	19	1.1		
" , less gelatin & starch	Biphasic	31	31	34	1.4	.38	1.9
	Control	10	11	12	.73		
Distilled water only	Biphasic	18	34	50	2.2		

Underlay in all cases consisted of 100 ml of complete dextrose-starch medium with 2% agar. The 125 ml of medium in control flasks had the same composition as the 25 ml of overlay in respective biphasic flasks. A water blank read 5 and a 1-11 dilution of complete broth read 11 nephelos units; turbidity data are not corrected for these blanks.

equilibrate on the shaker for 24 hours before inoculation. These biphasic flasks together with broth controls of the same composition as the overlay media then were inoculated and incubated on the shaker. Representative results of such experiments are given in Table I. More rapid multiplication was seen in all of the biphasic flasks, although subsequent studies with other bacteria have indicated that rate differences measured nephelometrically may only reflect cell size differences. By 72 hours, excellent and fairly comparable growth had occurred in all of the simplified biphasic overlay broths, representing population densities at least 2 to 4 times greater than broth controls although at some expense of total yields.

The comparison in Table I of the complete with simplified overlay media is not entirely representative, since in confirming experiments the complete medium gave the greatest density at all times of sampling. Either starch or gelatin, but not both, could be omitted from the biphasic overlay broth without appreciably affecting final population density. Remarkably, when water was added over complete dextrose-starch agar and allowed to equilibrate, the gonococcal growth attainable was only a little below that in complete medium. This lower yield partly reflects the reduced total amount of nutrient in the water/agar system. Even so, the growth attained in this clear and almost colorless menstruum was more than twice the density of that in control flasks with complex media.

Discussion and summary. The results first demonstrate the applicability and usefulness of an agar/liquid flask system to obtain gonococcal growth several times more dense than normal in a representative liquid medium. Counts as high as 5 x 10^9 cells per ml and turbidity similar to that normally seen with staphylococci were attained.

Second and perhaps more significant, the results illustrate a principle by which the enrichment materials normally incorporated into gonococcal media can be separated from the immediate growth environment. Under the conditions existing in a biphasic flask, even a clear water extract of the complete medium supported luxuriant growth of gonococci. Such medium simplification in principle should be applicable to other fastidious organisms.

1. Gould, R. G., Kane, L. W., Mueller, J. H., *J. Bact.*, 1944, v47, 287.

2. Griffin, P. J., Racker, E., *ibid.*, 1956, v71, 717.

3. Wilson, G. S., Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, 4th ed., The Williams and Wilkins Co., 1957, p634.

4. McLeod, J. W., Wheatley, M. B., Phelon, H. V., *Brit. J. Exp. Path.*, 1927, v8, 25.

5. Ley, H. L., Jr., Mueller, J. H., *J. Bact.*, 1946, v52, 453.

6. Tyrrell, E. A., MacDonald, R. E., Gerhardt, P., *ibid.*, 1958, v75, 1.

7. Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 114.

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