of interference between virus inocula given at distant sites(9). However, the lack of effectiveness of mumps vaccine in older mice may make the role of interferon unlikely.

From these considerations it seems likely that viral multiplication was partially suppressed by interference, and was further inhibited as the immune mechanism developed. This interpretation takes into account the observation that vaccinated animals developed encephalitis, though less severely than unvaccinated controls, and further that there was evidence of recovery between the 16-18 day, when according to Overman(3,4) mice have fully developed antibody-forming mechanisms. The possible sparing effects of other myxoviruses, as well as unrelated viruses will be studied.

Summary. A striking degree of protection from fatal mumps encephalitis is afforded mice if they are injected intravenously with mumps vaccine when less than 4 hours old. A less marked but very significant degree of protection is also afforded by influenza vaccine used in this same way, but none at all using the formalin diluent alone. With advancing age above the four-hour group this sparing effect diminishes. These results may be due to combined effects of viral interference and antibody production.

1. Overman, J. R., Kilham, L., *J. Immunol.,* 1953, v71, 352.
2. Overman, J. R., *ibid.,* 1954, v73, 244.
3. ———, *ibid.,* 1954, v73, 249.
4. Wacker, W. B., Lipton, M. M., Steigman, A. J., *Fed. Proc.,* 1961, v20, 265.
5. Schlesinger, R. W., In: *The Viruses,* vol. 3, p157-194, Academic Press, New York, 1959.
6. Overman, J. R., Peers, J. H., Kilham, L., *Arch. Path.,* 1953, v55, 457.
7. Isaacs, A., Hitchcock, G., *Lancet,* 1960, ii, 69.
8. Hitchcock, G., Porterfield, J. S., *Virology,* 1961, v13, 363.
9. Grossberg, S. E., Hood, E. W., Wagner, R. R., *J. Immunol.,* 1962, v88, 1.

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Alteration of Bile Salts by Bacteria.* (27391)

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A variety of studies of the effects of microorganisms on sterols and steroids has been made. Talalay(1) has reviewed many of these, including certain studies of bile acids. The use of aerobic conditions in which hydroxyl groupings were converted to ketones has been the most commonly reported experimental technic. The influence of bile salts on growth of microorganisms has also been studied extensively, particularly in working out methods for isolation of bile salt resistant organisms(2).

In the whole animal the bile salts present in the intestinal tract undoubtedly account, in part at least, for the paucity of microorganisms in the small intestine and the character of the microflora in the large intestine. The microorganisms of the large intestine modify the chemical nature of the bile acids and cholesterol carried from the upper gastrointestinal tract. The most prominent bile acid in the feces of animals(3) and man(4) is often deoxycholic acid. It has been demonstrated that cholic acid in the intact mammalian organism is reduced to deoxycholic (3α, 12α-dihydroxycholanic) acid(5,6,7,8), although keto-bile acid derivatives of cholic acid have also been found in feces(6,9). Bergstrom(10) considers that deoxycholate

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exists in mammalian bile and feces only as a product of bacterial action. Norman and associates have studied bile acid metabolism in bacterial cultures, as well as in rats infected with single organisms(11,12), and have observed the hydrolysis of conjugated bile acids and formation of keto bile acids, although the conversion of cholic to deoxycholic acid does not appear to have been demonstrated in cultures of pure organisms.

This report describes the isolation of organisms which could be grown aerobically or anaerobically in bile salt-containing media. A series of cultures of bacteria commonly found in intestinal contents (obtained from a commercial source) were also evaluated. The chemical transformations which occurred were determined. An increased growth response and capacity to convert cholic acid in acute trials to the analogues with ketone groupings was demonstrated in 3 species of bacteria which were serially transferred in cholate-containing media. The capacity of mixtures of rat cecal organisms and of one apparent single colony isolated from rat cecum to convert cholate to deoxycholate *in vitro* was demonstrated. Conversion by the apparently pure culture was obtained in only 2 instances.

Methods. The media used for aerobic and anaerobic cultures were respectively modifications of Marcus and Talalay's(13) medium (including 1% Difco yeast) and a thioglycollate medium. Some of the anaerobic incubations were carried out in Talalay media in a nitrogen atmosphere. Agar slants and plates were prepared from the same media by addition of 2% Bacto-agar. All anaerobic preparations were stored in desiccators that were repeatedly exhausted and filled with high grade (oxygen-free) nitrogen. Cholic acid neutralized to pH 7.4 was added to media to final concentrations of 0.01%, 0.1%, 0.2%, 0.4 and 1.0%. Other bile acids were also used in media at the 2 highest dilutions. Routine inoculations were carried out in 5 cc of media in screw cap culture tubes with teflon-lined caps (which were suitable for subsequent bile acid extractions). Acute aerobic growth studies were carried out in 250 ml Erlenmeyer flasks containing 50 ml of media and equipped with special aerators connected by manifold to an air line. The incubation temperature used was usually 38°.

All samples used for bile acid measurements were immediately treated with 0.5 ml of 2N KOH and autoclaved for 1 hour at 125°C. This procedure resulted in nearly complete clearing of the medium. The medium was then acidified with concentrated HCl to the methyl orange end-point, and extracted with three 8-ml aliquots of diethyl ether. The ether was then washed once with one ml of distilled water. This subnatant layer was separated with a Pasteur pipette; the ether was evaporated under a stream of nitrogen, and the residue dissolved in an appropriate volume of ether from which aliquots were taken for analysis.

Assay of test bile acids in incubation media was performed using the reactions of Minibeck(14) at 390 mμ for cholic acid and of Eriksson and Sjovall(15) at 385 mμ for dihydroxycholanic acids. Identification of the conversion products was made using separations based on the procedures of Sjovall (16) and of Mosbach *et al.*(17) or by gas-liquid chromatography. The following radiolabelled bile acids were used: cholic acid-24-C^{14} at either 2.0 or 4.5 μc/mg, and deoxycholic acid-24-C^{14} at 3.5 μc/mg(18). Both acids appeared to be radio-chromatographically pure (Sjovall(16) and Mosbach *et al.*(17)) and to have satisfactory absorption spectra (Eriksson and Sjovall(15) and Wysocki *et al.*(19)).

Results. Growth of various bacteria in cholate media. Initially 12 aerobic species and 2 anaerobic species were evaluated for their ability to grow in cholate-containing media and on cholate-containing agar plates. Three of these: *E. coli, Proteus vulgaris* and *Streptococcus faecalis,* grew particularly well aerobically in media containing 0.1% cholate and could be adapted to grow at 0.4% cholate. Therefore, most subsequent work under aerobic conditions on the effect of bile salts on growth of pure cultures and of bacteria in the modification of bile salts was carried out on these 3 species. Neither of 2 species of *Clostridia* (sporogenes or butyricum) grew well initially in cholate-containing thioglycollate media, although they were

adapted by serial transfer to grow at 0.01% cholate and eventually at 0.1% cholate.

Effect of serial transfer in cholate media. Fig. 1 demonstrates the growth of a culture of *E. coli* (as measured by optical density at 600 mμ) which had been serially cultured in cholate-free media and of a culture that had been through 5 transfers in an otherwise identical medium containing 0.2% cholate. Regardless of concentration the cholate "adapted" culture grew more rapidly than the unadapted culture in the aerated system. In the test system containing cholate at 0.4% the adapted organisms grew at a substantial rate whereas growth of the unadapted culture was almost completely inhibited.

The disappearance (modification) of cholate (as evidenced by reduction of optical density at 390 mμ with the reagent of Minibeck) from these same cultures is illustrated in Fig. 2. With the conditions of aeration of the media used the Minibeck reaction of cholate in uninoculated media was not altered. The extent of removal of cholate from the media paralleled the growth of the culture. When cholate-24-C^{14} was included in the growth media containing unlabelled cholate, the bile acid fractions recovered from the media contained all of the label added.

A single transfer of adapted cultures to cholate-free media and of unadapted cultures to cholate-containing media prior to the standard test procedure resulted in significant loss and gain respectively of tolerance to the test media and of capacity to alter the cholate of the media. The adaptation phenomenon seen with *E. coli* was also observed in cultures of *Proteus vulgaris* and *Streptococcus faecalis,* the only differences from the above studies being the more rapid growth of *Proteus vulgaris* and the slower growth of *Streptococcus faecalis* (compared to *E. coli*).

Specificity of cholic acid structure in the adaptation phenomenon. Deoxycholate and hyodeoxycholate were used in the test media to test whether the adaptation phenomenon was specific for cholate. There was a suggestion that the cholate adapted organism grew better in the 0.01% deoxycholate test media than did the unadapted organism. Deoxycholate appeared to be highly disruptive of cell structure as indicated by decreased optical density after transfer of a standard inoculum to medium containing the 0.1% test concentration of deoxycholate. Similar results were also obtained for hyodeoxycholate. At the 0.01% concentration both of the dihydroxy bile salts appeared to be rapidly modified, as followed by the pertinent spectrophotometric measurements described by Eriksson and Sjovall(15) (60°-60′ in 65% H_2SO_4; 385 mμ). The products of deoxycholate from the incubation media have not been definitely identified. When deoxycholate-24-C^{14} was included in incubation media, all of the radioactivity was recovered in the bile acid fractions after the 180-minute incubations.

Effect of added fatty acids to cholate-containing media. Fig. 3 and 4 illustrate growth curves and modification of cholate by unadapted *E. coli* when tested in the 0.4% cholate media supplemented with palmitate or linoleate at 0, 10, or 100 μg/ml. Both fatty acids appeared partially to overcome the growth inhibition of cholate and to accelerate the rate of modification of cholate. Palmitic acid had a greater influence than linoleic acid. There was also a stimulatory effect of the added fatty acids in cultures of adapted *E. coli,* as well, although as in previous experiments rates of growth and cholate alteration of the adapted cultures were greater than those for the unadapted cultures regardless of composition of the medium. In the tests of added fatty acids to bile salt-free media (optimum growth media) there was no significant effect on growth of *E. coli* except that at the higher concentration (100 μg/ml) linoleic acid was inhibitory.

Demonstration of a DPN-linked dehydrogenase from E. coli. Cell-free extracts from cultures of *E. coli* were recovered either from alumina pastes and centrifugation or by ultrasonic fragmentation, centrifugation, and quick freezing of the supernatant in liquid nitrogen. Both preparations were active. The enzyme in pH 7.2 phosphate buffer plus cholate (100 μg/ml) and DPN (100 μg/ml) could be demonstrated to be active both from reduction of DPN as measured by optical density at 340 mμ and in the disappearance of cholic acid (as measured by the Minibeck re-

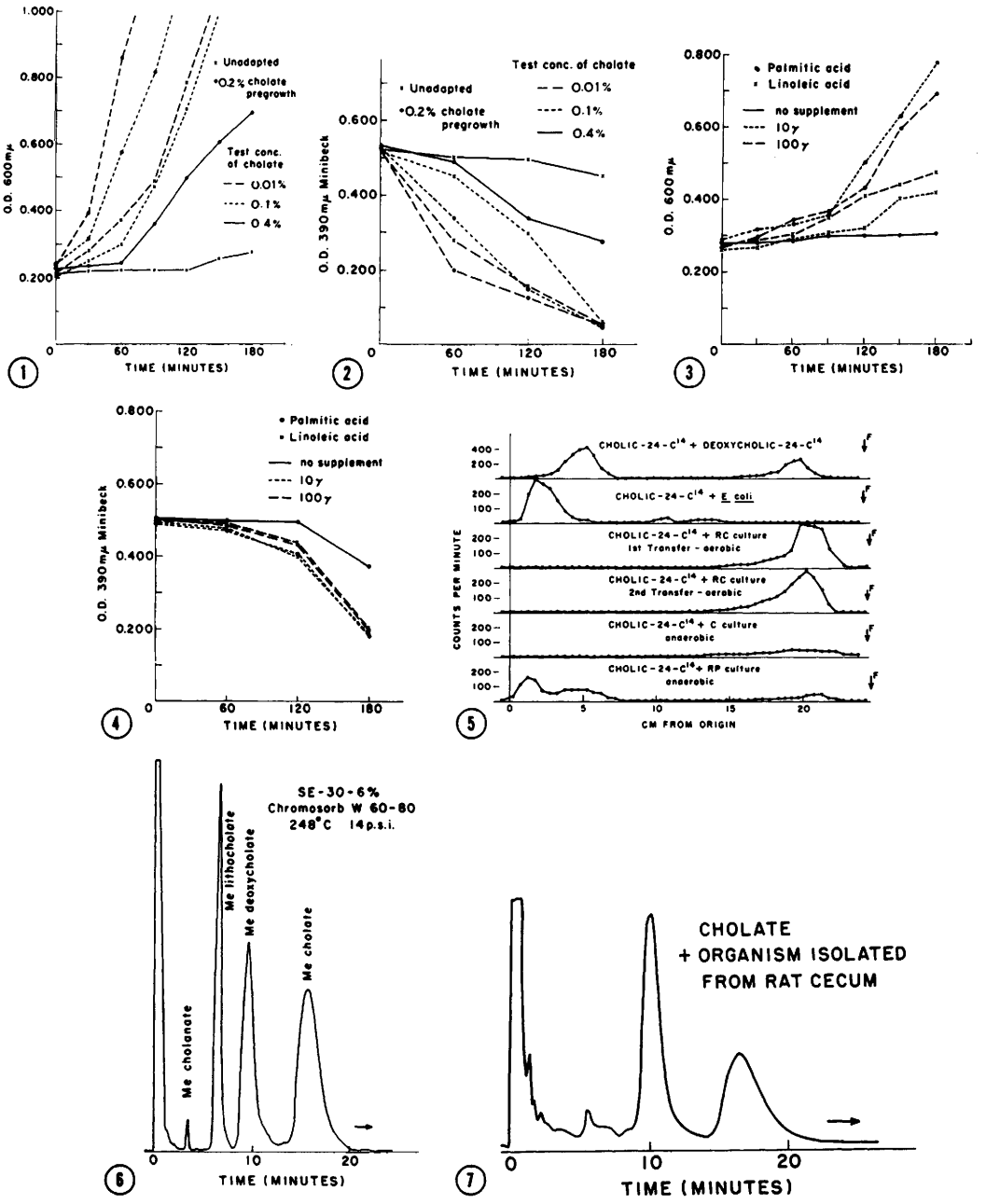

FIG. 1. Growth of adapted and unadapted cultures of *Escherichia coli* in cholate-containing media. Adapted indicates that the organism had been transferred 5 times in media containing 0.2% Na cholate. The media during tests contained one of 3 concentrations of sodium cholate. Test media were maintained at 38° and continuously aerated.

FIG. 2. Alteration of cholate, as measured by optical density at 390 mμ using the reagent of Minibeck(17), in the study described in Fig. 1. Rates of alteration of cholate by adapted and unadapted cultures of *Escherichia coli* were tested in 3 concentrations of cholate.

FIG. 3. Effect of supplements (10 μg or 100 μg/ml media) of linoleic acid or palmitic acid (neutralized to pH 7.4) in media containing 0.4% cholate on growth of *Escherichia coli* (unadapted). The spectrophotometer readings for the medium unsupplemented with fatty acids did not increase during 180-min. incubation period (as solid line indicates).

FIG. 4. Rates of alteration of cholate by unadapted cultures of *Escherichia coli* (as meas-

action at 390 mμ). Typical enzyme concentration and substrate concentration curves were obtained. Regardless of enzyme concentration, concentrations of cholate greater than 150 μg/ml were strikingly inhibitory. Removal of either of the 3 essential components: enzyme, DPN, or cholate resulted in complete inactivity by the criteria of DPN reduction or cholate alteration. These observations are reminiscent of the findings of Hayaishi *et al.* (20) using *E. freundii.*

Nature of the cholate alteration products in E. coli *cultures.* In agreement with prior observations of Norman and Bergman(12), the cholate conversion products of *E. coli* (determined both by paper chromatography (Fig. 5) and gas liquid chromatography) were largely 7-ketodeoxycholate.

Conversion of cholic acid to deoxycholic acid by mixed organisms and one apparently pure culture isolated from the rat cecum. The ceca of rats or Cebus monkeys were opened using sterile technics, and a loop of contents was transferred either to media or to sterile saline, followed by immediate inoculation into aerobic and anaerobic media containing either no cholate, labelled cholate at 75 μg/5 ml of media, 0.01% or 0.1% unlabelled cholate. 0.1% Cholate agar plates were also streaked and transferred to 38° incubators or to nitrogen filled desiccators in incubators. At the end of 24 hours second transfers of all cultures were made to identical media and homogeneity was again evaluated by streaking agar plates. In certain cases single colonies were transferred from plates to cholate media and cholate agar slants and then carried, apparently as homogeneous cultures, in serial transfer through cholate-containing media.

Fig. 5 also illustrates the paper radiochromatographs of bile salts isolated from inoculated media containing a trace dose of cholate-24-C^{14}. Culture RC (a mixture) from a rat cecum converted the trace of labelled cholic acid almost quantitatively to deoxycholic acid on the first 3 transfers. The organism or organisms lost almost all of this capacity at the fourth transfer. One organism isolated as an apparently pure culture from an agar plate from first transfer media also converted cholic to deoxycholic acid. No organisms either from cholate agar plates or from media containing traces of radiocholate or unlabelled cholate at 0.01% or 0.1% have in these experiments been able to perform the cholate to deoxycholate conversion after more than 3 transfers. One of the most significant factors which appeared to influence the success of the conversion of cholic to deoxycholic acid by the microorganisms studied was the size of the inoculum, a large inoculum giving much more consistent conversion of cholic to deoxycholic acid.

Fig. 6 and 7 illustrate the separation of model mixtures of methyl esters of bile salts, and the methylated bile acid fraction from a 0.1% cholate incubation mixture which had been inoculated with the organism isolated from a sample of rat cecum contents. The rat cecal organism had converted approximately one-half of the cholate present to deoxycholate. The relative retention time of the methyl deoxycholate was identical to that of authentic methyl deoxycholate in a system which was capable of partially separating the 3, 12-dihydroxy (deoxycholic) from the 3, 6-(hyodeoxycholic) and 3, 7-(chenodeoxycholic) dihydroxycholanic acids. Similarly, the formoxylated mixture contained a peak with retention time equal to that of formoxy-

ured by reaction of Minibeck(17)) in media containing 0.4% cholate and supplements (10 μg or 100 μg/ml media) of palmitic or linoleic acid (neutralized to pH 7.4).

FIG. 5. Paper chromatographic separation of cholic acid-24-C^{14} and deoxycholic acid-24-C^{14} and bile acid fractions from several different inoculations into media containing cholic acid-24-C^{14} (neutralized to pH 7.4). Chromatographic separation was that of Sjovall(19). Stationary phase was 70% acetic acid; moving phase was 60 isopropyl ether: 40 heptane. Support was Whatman 3 MM paper. Radioactivity peak in the cholic-24-C^{14} + *E. coli* chromatograph has an Rf consistent with 7-ketodeoxycholic-24-C^{14} acid. Designations RC, C, and RP refer to mixed cultures from rat cecum.

FIG. 6. Separation by gas-liquid chromatography of a model mixture of methyl esters of cholanic, lithocholic, deoxycholic, and cholic acids.

FIG. 7. Gas-liquid chromatographic separation of the methylated bile acid fraction from media which contained 0.10% cholate and had been inoculated with an organism from rat cecum.

lated methyl deoxycholate.

Characterization of one organism (single colony) which converted cholic to deoxycholic acid. One bacterium which was isolated as a pure organism by streaking a loop of rat cecal contents (diluted with saline) on 0.1% cholate agar was able almost quantitatively to convert a trace (75 μg) of labelled cholate to labelled deoxycholate on two first transfers to liquid media. It was characterized as follows: a short gram negative non-motile rod which tended to appear on stained slides as pairs and tetrads. Colonies which appeared on cholate-agar were one mm in diameter, translucent, and non-spreading. The bacterium formed acid and gas on lactose, glucose, maltose, sucrose, and salicine media. It was acid-forming in Russell's double sugar. The organism was indol negative, methyl red positive, Voges-Proskauer negative and citrate positive.

Discussion. Our results were in agreement with many of the findings of Norman and Bergman(12). The differences were as follows: 24-hour cultures of cecal inocula in media containing 0.1% and 0.01% cholate or 15 μg cholate-24-C^{14} per ml resulted in quantitative conversion to deoxycholic for the labelled material and the 0.01% cholic acid and about 50% conversion of the 0.1% cholic acid to deoxycholic acid in 24 hours. Incubations were carried out in screw cap culture tubes which may have resulted in relative anaerobiosis. In certain instances the capability of converting cholic acid to deoxycholic acid was carried through 3 transfers from rat cecum, *i.e.*, 2 serial 24-hour transfers by pipette from the original 24-hour growth of the culture inoculated from the cecum. One organism isolated on 0.1% cholate agar was shown to convert cholic to deoxycholic acid on 2 different first transfers to cholate-C^{14} containing 0.1% cholate media, but this conversion was not demonstrated subsequently. Another significant difference from the report of Norman and Bergman(12) was the observation that deoxycholic, when incubated with a strain of *E. coli* in the aerated system, was altered as evidenced by reduction of its absorbance at 385 mμ after heating at 60° in 65% H_2SO_4.

The ability of one apparently single species of organism isolated on cholate agar to convert cholic acid to deoxycholic acid (even though it was impossible to maintain this capacity on subsequent transfer through liquid media, and morphologically similar colonies on agar apparently lost or never had this capacity) suggests that the difficulty in reproducing this biologically common reaction results primarily in defining the proper composition of media and incubation conditions. The relative inconsistency with which we could demonstrate and sustain this reaction on subsequent transfer of organisms suggests that optimum conditions have still not been defined. Subsequent to the submission of this manuscript cultures (probably mixed) of cecal organisms have been maintained through 9 transfers in 0.01% cholate-Talalay media with complete conversion of cholate to deoxycholate in 48 hours. The reaction occurred most readily in a nitrogen atmosphere but also occurred in screw cap culture tubes in air.

Summary. The ability of a variety of common intestinal microrganisms to grow in bile salt-containing media and to alter the structure of those bile salts was studied. Strains of *Escherichia coli, Proteus vulgaris,* and *Streptococcus faecalis* were adapted by serial transfer in cholate-containing media to grow more rapidly in high concentrations of cholate and to alter the composition of the cholate more rapidly than unadapted cultures. *E. coli* was also shown to alter the structure of deoxycholate. Low concentrations of linoleic or palmitic acids when added to cholate-containing media partially overcame the inhibition of growth and cholate-altering capacity of *E. coli.* 7-Ketodeoxycholate was the principal metabolite of cholate in continuously aerated cultures of *E. coli.* The conditions used for transfer of mixed cultures and the isolation of one apparently pure culture of organisms from rat cecum which was capable on 2 first transfers, but not subsequently, of converting cholic acid to deoxycholic acid were described.

1. Talalay, P., *Physiol. Rev.*, 1957, v37, 362.

2. Stacey, M., Webb, M., *Proc. Roy. Soc.* (London) 1947, v134, 523.

3. Abell, L. L., Mosbach, E. H., Kendall, F. E., *J. Biol. Chem.*, 1956, v220, 527.
4. Carey, C. B., Jr., Watson, C. J., *ibid.*, 1955, v216, 847.
5. Lindstedt, S., *Arkiv. Kemi,* 1957, v11, 145.
6. Norman, A., Sjovall, J., *J. Biol. Chem.*, 1958, v233, 872.
7. Portman, O. W., Murphy, P., *Arch. Biochem. Biophys.*, 1958, v76, 367.
8. Portman, O. W., *ibid.*, 1958, v78, 125.
9. Schmidt, L. H., Hughes, H. B., *J. Biol. Chem.*, 1942, v143, 771.
10. Bergstrom, S., Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols, Little, Brown & Co., Boston, 1959, p185.
11. Gustafsson, B. E., Norman, A., Sjovall, J., *Arch. Biochem. Biophys.*, 1960, v91, 93.
12. Norman, A., Bergman, S., *Acta Chem. Scand.*, 1960, v14, 1781.
13. Marcus, P. I., Talalay, P., *J. Biol. Chem.*, 1956, v218, 661.
14. Minibeck, H., *Biochem. J.*, 1938, v297, 29.
15. Eriksson, S., Sjovall, J., *Arkiv. Kemi,* 1955, v8, 303.
16. Sjovall, J., *ibid.*, 1955, v8, 317.
17. Mosbach, E. H., Zomzely, C., Kendall, F. E., *Arch. Biochem. Biophys.*, 1954, v48, 95.
18. Bergstrom, S., Rottenberg, M., Voltz, J., *Acta Chem. Scand.*, 1953, v7, 489.
19. Wysocki, A. P., Portman, O. W., Mann, G. V., *Arch. Biochem. Biophys.*, 1955, v59, 213.
man, E. F., *ibid.*, 1955, v56, 554.

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Excretion of Vacuolating SV-40 Virus (Papova Virus Group) After Ingestion as a Contaminant of Oral Poliovaccine.* (27392)

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The vacuolating SV-40 virus(1), the most recently discovered member of the papova virus group(2), became the object of immediate and widespread interest when it was discovered as a viable contaminant of killed and live virus vaccines grown in rhesus and cynomolgus kidney cell cultures(1,3,4). It appears that the virus has been injected as a live contaminant of formalinized polio- and adenovaccines into hundreds of thousands, if not millions, of persons, and that it has been fed in active form together with live poliovaccine to groups equally as large.

American(1) and British(5) investigators reported that 2 to 3 injections of some formalinized adeno- and poliovaccines were sufficient to produce vacuolating virus antibodies in man, though whether from simultaneous injection of living or inactivated vacuolating virus could not be established retrospectively. Intranasal administration of vacuolating virus has been found to lead to viral multiplication and antibody formation(6). In this instance the vacuolating virus was present as a live contaminant of another virus preparation being studied in human volunteers. It should be emphasized that in spite of the large numbers of persons injected or fed the virus, not a single human illness has been attributed to this agent.

Studies on the isolation of vacuolating virus from children who had inadvertently received contaminated lots of oral poliovaccine are reported here. Some negative studies on such individuals have been reported, but they have been very few in number(5,7).

Materials and methods. Tissue cultures. Monkey kidney (MK) monolayer cultures were grown and maintained in our lactalbumin medium, as described previously(8). Cultures were grown from African green monkeys (*Cercopithecus aethiops*). The animals were all tested for SV-40 antibody and only those free of antibody were selected for culturing. In all experiments a number of uninoculated cultures were observed to insure that vacuolating virus was not a spontaneous contaminant of the test cultures.

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