Segment 8 had a slightly greater mean activity than segment 7, but this difference was not statistically supported.

Discussion. The monophosphoesterase activity gradient along the small intestine does not exhibit the necessary characteristics to be the direct rate limiting component for the active uptake of glucose. The maximal absorption of glucose in the upper half of the small intestine was accompanied by an exponential decrease in phosphatase activity. Preliminary studies utilizing various concentrations of phosphate ester assured that at all times during the thirty-minute incubation period the velocity of the reaction would remain constant. The decrease in the enzyme activity observed in the present experiments (see figure) may therefore be attributed to a decrease in the concentration of enzyme rather than to possible substrate deficiency. As these areas of decreased monophosphate esterase show no corresponding decrease in glucose absorption it follows that the relationship between the two processes is not a direct one. This observation is in agreement with the report of Crane and Krane, which has shown that the absorption of glucose analogues incapable of phosphorylation is similar to the absorption of D-glucose(3). Obviously this study does not rule out the possibility that certain of the monophosphoesterases may be involved in the hydrolysis of high energy phosphate compounds indirectly involved in the energy requiring "uphill" transport of glucose by the intestinal mucosa(1).

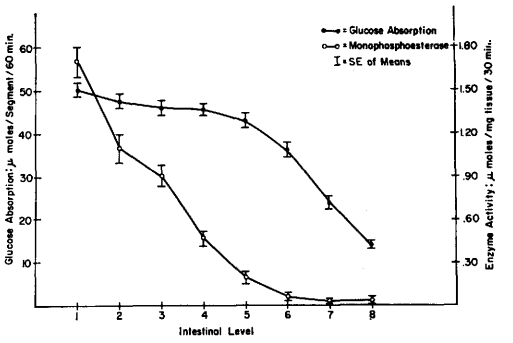

FIG. 1. Glucose and monophosphoesterase activity at eight levels of rat intestine.

Conclusion. The dissimilarity of the glucose absorption rate and concentration of monophosphoesterase along the rat small intestine indicates that no direct relationship exists. This would tend to support the finding of Crane and Krane in regard to the phosphorylation of glucose in its absorption and subsequent transport by the intestinal mucosa.

1. Baker, R. D., Searle, G. W., Nunn, A. S., *Am. J. Physiol.*, 1961, v200, 301.

2. Crane, R. K., Wilson, T. H., *J. Appl. Physiol.*, 1958, v12, 145.

3. Crane, R. K., Krane, S. M., *Biochim. Biophys. Acta*, 1956, v20, 568.

Received May 29, 1963. P.S.E.B.M., 1963, v114.

Identification of Coxsackie and ECHO Virus Isolates with Fluorescent Antibody. (28614)

M. H. HATCH (Introduced by C. C. Shepard)
Communicable Disease Center, Public Health Service, U. S. Dept. of HEW, Atlanta, Ga.

A fluorescent antibody (FA) method for identification of poliovirus isolates has been described(1). This paper presents data concerning the use of FA for identification of certain Coxsackie and ECHO virus isolates. A preliminary report of these results has been given(2).

Materials and methods. The experimental approach was to study a series of stool specimens from which enteroviruses had previously been isolated and identified by routine methods involving neutralization tests. The 70 specimens studied were selected to contain in most instances one of the 6 enteroviruses against which fluorescent antibodies had been prepared. Fourteen stools contain-

ing other enteroviruses and one negative stool were also included. Specimens were given to us as stool suspensions under code number and processed without knowledge of the earlier results. The stool suspensions were prepared by standard methods in Hanks' balanced salt solution with penicillin and streptomycin added to final concentrations of 4000 units/ml and 2000 μg/ml, respectively. Leighton tubes of rhesus monkey kidney cells were inoculated with 0.1 ml of the suspensions and incubated at 37°C. Sufficient tubes, generally 6, were inoculated with each suspension to permit more than one staining with FA if necessary. As soon as a 1 to 2+ cytopathic effect (CPE) was observed, coverslips were removed from the tubes, air-dried and fixed in acetone at room temperature for 10 minutes. They were either stained at once or stored at −20°C until stained. Uninoculated monkey kidney cells and cells infected with known Coxsackie B2, B4, B5, ECHO 4, 6 or 9 viruses were included as controls with each group of unknowns. All stools which produced a toxic reaction or which failed to produce CPE in first passage were passed a second time. Rarely a third passage was necessary to obtain definite CPE.

Rabbit antisera were used for preparation of all fluorescent antibodies. Antisera for Coxsackie B2 (strain #50207, New York State Collection) and B5 (strain Faulkner) were prepared at the Communicable Disease Center by a series of 3 intraperitoneal and 3 intravenous injections of virus grown in rhesus monkey kidney cells. Neutralization titers of these antisera were 1:50 and 1:1000, respectively, when tested against 100 TCD_{50}/ml virus. Antisera for Coxsackie B4 (strain Powers), ECHO 4 (strain Pesascek), ECHO 6 (strain D'Amori) and ECHO 9 (strain Hill) were purchased from Microbiological Associates, Inc. Neutralization titers of these antisera in our hands were 1:400, 1:10, >1:1000, and >1:1000, respectively. Globulins were separated from the antisera and labeled with fluorescein isothiocyanate as previously described(1). Before use, conjugates were absorbed twice with monkey liver powder(3) and twice with packed, washed rhesus monkey kidney cells harvested by scraping from monolayers. The direct method of FA staining was employed. Conjugates diluted 1:2 with buffered saline were applied to coverslips broken into thirds with a diamond point. Throughout most of the study, if the first set of coverslips examined was FA negative, a second set, harvested at the same time, was stained. All preparations were observed with dark field illumination from a 200 watt mercury vapor bulb using a Corning 5970 or 5840 exciter filter. A Wratten 2B gelatin filter was placed in the microscope ocular. Results of FA method were compared to those obtained previously with the routine neutralization method.

The monkey kidney cells were grown in a medium of Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate, 2.0% calf serum, 200 units/ml penicillin and 100 μg/ml streptomycin. Maintenance medium consisted of Earle's balanced salt solution with 0.5% lactalbumin hydrolysate and antibiotics. Buffered saline used throughout was made up of 0.14 M NaCl, 0.01 M NaH_2PO_4, with the final pH adjusted to 7.0-7.2 by addition of 40% NaOH solution.

Results. In preliminary experiments, conjugates were studied by various methods to establish specificity of staining. Several cross staining experiments were performed in which each conjugate was used to stain coverslip preparations of cells infected with known strains of Coxsackie B2, B4, B5, ECHO 4, 6, or 9. In each case, only the homologous system showed specific staining. Cells infected with the 6 viruses were also harvested at various stages of CPE. The number of cells showing staining increased as CPE increased to about the 2+ stage. However, as previously observed with the polioviruses(1), coverslips harvested at a 3+ or greater CPE did not stain well. Lastly, antibody inhibition tests were performed by the one-step method(4). When a mixture of equal parts of undiluted, unlabeled homologous antiserum and undiluted, homologous conjugate was used to stain infected coverslips, staining was markedly inhibited. Significant inhibition of staining did not occur when unlabeled nor-

TABLE I. Comparison of Fluorescent Antibody and Routine Procedures in Identification of Coxsackie and ECHO Virus Isolates.

Enterovirus type in stool	No. by routine procedure	No. by fluorescent antibody procedure
Coxsackie B2	9	9
" B4	8	7
" B5	13	13
ECHO 4	6	7*
" 6	8	8
" 9	11	8*
Other†	15	0‡

* Two ECHO 9 isolates were not typed by FA; one ECHO 9 was incorrectly typed as ECHO 4. See *Discussion* in text.

† Three Coxsackie B3, 1 Coxsackie A9, 1 ECHO 7, 1 ECHO 11, 2 ECHO 14, 3 Polio 1, 3 Polio 3, 1 negative.

‡ Fluorescent antibody results negative with the 6 conjugates used.

mal rabbit serum was mixed with homologous conjugate.

Results of the comparison of fluorescent antibody and routine procedures with the 70 stool specimens are shown in Table I. FA method correctly typed most of the enteroviruses present in the stools. One Coxsackie B4 and 2 ECHO 9 isolates were not identified. Although these 3 unknowns produced a definite CPE in tissue culture, staining was not observed with any of the 6 conjugates. It should be pointed out that only one set of coverslips was used and only a single staining performed for each of these unknowns. In a fourth case, a positive staining reaction was recorded with the ECHO 4 conjugate where the isolate was actually ECHO 9. Fourteen stools containing viruses other than the 6 tested for and one negative stool, all processed by the FA method, gave negative results with the 6 conjugates. Thus, there were 3 failures and one incorrect typing in the 70 specimens examined.

Table II shows the number of days after inoculation of tissue culture at which unknowns were identified by the FA method. Thirty-one were identified in first passage; 16 required a second passage because of either toxicity or indefinite CPE in first passage. For 2 specimens, a third passage was necessary. In the majority of cases (34 out of 49), the unknowns were identified within 3 days after inoculation of the tissue culture passage in which definite CPE was observed. All unknowns were identified within 7 days after inoculation irrespective of the passage involved. The routine method required 1, 2 or 3 passages to establish presence of virus just as did FA method. In addition, the routine method required 2 to 3 weeks after recognition of the presence of virus to determine its identity. It can be seen, therefore, that FA identification was much more rapid than the routine method.

Discussion. In the early part of the study, only one set of coverslips was stained for each unknown. The 3 failures to identify isolates occurred at this stage. When repeat stains were carried out on 2 of these 3 failures, correct typing was made. Thereafter, a second set of coverslips was stained if the first set was FA negative. No further failures to type occurred after this became routine practice. It seems likely, therefore, that unaccounted variability between different coverslips was responsible for the early failures. In the course of the study, 39 unknowns were identified by one staining. Ten required a second staining, and, in 2 cases a third stain was made to confirm previous tentative FA identification. Accordingly, staining of one or, in some cases, two sets of coverslips would appear sufficient either to identify the isolate or to establish its non-identity with the 6 viruses studied. No instance was encountered in which 2 sets of coverslips were FA negative and a third set positive. In the 2 cases where 3 stains were made before final identification, both first and second stains yielded strongly presumptive typing.

TABLE II. Days After Inoculation on Which Unknowns Were Identified by Fluorescent Antibody Method.

Tissue culture passage	No. identified on day							Total
	1	2	3	4	5	6	7	
First	7	7	4	6	3	1	3	31
Second	4	7	4			1		16
Third			1		1			2
Total	11	14	9	6	4	2	3	49*

* Total includes only those specimens for which correct, positive identification was made by FA. There were 2 correct positives for which time data were not available.

Since there were no failures to type unknown viruses by the FA technic after it became routine practice to stain a second set of coverslips if the first set was negative, the FA method would appear to be as sensitive as the neutralization technic used. This would be true, however, only if repeat stains are carried out on those unknowns negative on first staining.

In the instance where FA gave an incorrect typing, a specimen found to contain ECHO 9 by the routine neutralization method was recorded as ECHO 4 on the basis of FA results. After the results were decoded, the coverslips stained with ECHO 9 conjugate were reexamined and a few faintly stained cells seen. Repeat stains showed staining with both ECHO 4 and ECHO 9 conjugates. It was also found that some, but not all, uninfected control coverslips from this lot of monkey kidney cells stained with the ECHO 4 conjugate. Subsequently, a few other lots of monkey kidney cells were encountered in which uninfected controls showed a similar type of staining with the ECHO 4 conjugate. Absorption of the conjugate with packed cells (about 0.1 ml cells per 0.5 ml conjugate) from some of these lots removed its staining reactivity with uninfected cells. No further mistypings occurred after this absorption. These findings suggest 2 explanations for the observations with the ECHO 4 conjugate: (1) ECHO 4 virus may be antigenically related to a simian virus found in some batches of monkey kidney cells, or (2) the monkey kidney cells in which the ECHO 4 virus was grown for immunizing purposes (Microbiological Associates, ECHO 4 antiserum, Lot #286) may have contained a simian virus so that antibodies were produced to it as well as to ECHO 4. Similar problems may be encountered in FA work with other viruses grown in monkey kidney cells. Accordingly, wherever possible, it would seem desirable to grow either the immunizing antigen or the viruses to be stained in tissue culture cells other than those prepared from monkey kidney.

The data on length of time required for identification of the unknowns by FA clearly show the greater rapidity of this technic compared to the routine method. Frequently, FA gave very rapid results. Eighteen specimens positive in first tissue culture passage were identified within 3 days after inoculation. Even where more than one tissue culture passage was necessary for recognition of the presence of virus, FA retained the same general time advantage, since the necessity for repeat passages also applied to routine method.

The results reported suggest the possible use of fluorescent antibody as a screening procedure in the identification of the 3 Coxsackie and 3 ECHO viruses studied, with routine methods being applied to those agents which fail to type with the 6 conjugates. Conjugates could undoubtedly be prepared for other Coxsackie and ECHO viruses for use in typing. Indeed, based on the present results and those previously described for the polioviruses(1), it seems feasible to set up an FA screening method for any of the cytopathogenic enteroviruses desired. Preliminary reports have also appeared concerning the use of combinations of antiserum pools(5) for identification of enteroviruses in tissue culture by the direct (6) and indirect(7) FA technics. Further trials are indicated to determine the general applicability of all these FA methods to laboratory identification of enteroviruses. The advantages of such procedures as far as relative rapidity and elimination of cumbersome neutralization tests are obvious.

Summary. A direct fluorescent antibody staining method for laboratory identification of 3 Coxsackie and 3 ECHO viruses isolated from stool specimens is reported. In the method, monkey kidney tissue cultures inoculated with stool suspensions are stained when they show early cytopathic effect. Seventy stools were studied as unknowns by the fluorescent antibody method. Fifty-five of these had previously been found to contain 1 of the 6 viruses by routine procedures involving neutralization tests. Fifty-one were correctly identified by the fluorescent antibody method. Of the 4 failures, 3 were found negative and 1 was incorrectly typed. Fifteen specimens shown not to contain any of the 6 viruses by the routine method were negative by fluorescent antibody. The reasons for the 4 failures

with fluorescent antibody were investigated and modifications introduced into the method that should make it as sensitive and specific as the procedure using neutralization tests. Identification of the viruses was made much more rapidly with fluorescent antibody.

The author wishes to express appreciation to Mrs. Anna Hall for supplying prepared stool suspensions and to Mrs. Sarah Hall for providing Coxsackie B2 and B5 antisera. The excellent technical assistance of Mrs. Frances Touchon is also gratefully acknowledged.

1. Hatch, M. H., Kalter, S. S., Ajello, G. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 1.
2. Hatch, M. H., *Bact. Proc.*, 1962, 137.
3. Coons, A. H., Leduc, E. H., Connolly, J. M., *J. Exp. Med.*, 1955, v102, 49.
4. Goldman M., *ibid.*, 1957, v105, 557.
5. Lim, K. A., Benyesh-Melnick, M., *J. Immunol.*, 1960, v84, 309.
6. Shaw, E. D., Newton, A., Powell, A. W., Friday, C. J., *Virology*, 1961, v15, 208.
7. Page, R. H., Stulberg, C. S., *Bact. Proc.*, 1962, 137.

Received July 24, 1963. P.S.E.B.M., 1963, v114.

Absence of a Renal Effect from Two Substituted Propanediols: Meprobamate and Mebutamate. (28615)

FRANK E. GREENE (Introduced by T. H. Maren)
Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville

Substituted propanediols are widely used in medicine as skeletal muscle relaxants and psychotropic agents. Recently, mebutamate (2-methyl-2 sec-butyl-1,3-propanediol dicarbamate) was introduced as an antihypertensive agent. Published data indicate that the primary site of action of this drug is the central nervous system(1): however, urine electrolyte excretion was not studied, and the possibility of renal participation in this hypotensive effect was not excluded.

Perhaps the most commonly used drug of this group is meprobamate, (2-methyl-2-n-propyl 1,3-propanediol dicarbamate). This drug has also been reported to have weak hypotensive activity(2). It was the purpose of this investigation to determine if a renal action, either on electrolytes or hemodynamics, could be demonstrated for these compounds in the dog. In these experiments renal plasma flow was only moderately depressed by mebutamate and electrolyte excretion was not affected by either drug.

Materials and methods. Conscious, trained, beagles of both sexes, raised in these laboratories were used. Food, but not water, was withheld for 24 hours prior to the experiment. All animals were given 20 ml/kg of tap water by stomach tube at start of experiment. Thirty minutes later the bladder was drained and washed with distilled water and this material was discarded. The subsequent urine samples were collected by a catheter which drained directly into cylinders containing toluene. The bladder was then washed twice with 10 ml of distilled water and this rinse was added to the urine sample. Further details of experiments are given in Table I.

Blood samples were taken in heparin-rinsed syringes at the mid-point of each urine collection period.

Drugs were supplied as the pure powder (Wallace Laboratories, Cranbury, N. J.) and given intravenously in a 20% solution of propylene glycol in saline. Concentration was adjusted so that each dose was given as 1 ml of the vehicle per kg of body weight. Due to the limited solubility of these compounds, the solutions of higher concentrations were warmed prior to administration to prevent precipitation.

Glomerular filtration rate (GFR) was measured by the clearance of inulin using the direct resorcinol method without alkali treatment, according to Schreiner's modification of the method of Roe, Epstein and Goldstein(3).