hr) are of comparable magnitude while the disappearance constants differ by a factor of 3 (.00273 and .000907 min.$^{-1}$). The disappearance constant for potassium is roughly of the same magnitude as that of sodium, but a comparison of the exchange rates shows that the number of milliequivalents of sodium exchanged per minute is 35 times greater than the number of milliequivalents of potassium. The ratio of these exchange rates is about the same as the ratio of their respective concentrations in the amniotic fluid.

Summary. The exchange rate of sodium to and from the amniotic fluid was determined by the simultaneous application of sodium-22 and sodium-24. This exchange rate, in opposite directions, was found to be 15 and 16 mEq. of sodium per hour, respectively. The transfer rate of hydrogen, sodium, and potassium from the amniotic fluid to the maternal system was determined by the simultaneous application of tracers for each of these elements. The disappearance constants for sodium and potassium were found to be nearly equal, indicating that the same percentage of these elements is exchanged per unit of time, while that for deuterium was found to be 5 times greater. The exchange rates were calculated as 31.9 moles of water, .014 mole of sodium, and .00041 mole of potassium per hour. It is concluded that the water and electrolytes of the amniotic fluid are in dynamic equilibrium with maternal plasma, each exchanging at its own characteristic rate.

1. Plentl, A. A., and Hutchinson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 681.

2. Sheppard, C. W., and Householder, A. S., *J. Applied Physics,* 1951, v22, 510.

3. Neslen, E. D., Hutchinson, D. L., Hallet, R. L., and Plentl, A. A., *Obs. and Gyn.,* 1954, v3, June.

4. Vosburgh, G. J., Flexner, L. F., Cowie, D. B., Hellman, L. M., Procter, N. K., and Wilde, W. S., *Am. J. Obs. and Gyn.,* 1948, v56, 1156.

Received May 6, 1954. P.S.E.B.M., 1954, v86.

Propagation of Salivary Gland Virus of the Mouse in Tissue Cultures.* (21123)

MARGARET G. SMITH. (With the technical assistance of Betty Jean Worthey.)

From the Department of Pathology, Washington University School of Medicine.

The presence of greatly enlarged cells containing large intranuclear inclusions in the salivary glands of several species of rodents has been shown to be associated with a transmissible filterable virus(1-3). In each case the virus has proved to be entirely species-specific. In most species of animals basophilic cytoplasmic inclusions also occur in the cytoplasm of many of the enlarged cells, but in the experimental infections they appear later than the intranuclear inclusions(4). They are seen only occasionally in the salivary glands of infected mice. Intracellular inclusions resembling those occurring in the salivary glands of rodents have been observed in the salivary glands in 10 to 30% of autopsies of infants and young children(5-8) regardless of the cause of death and are present in other organs, as well as the salivary glands, in cases of inclusion disease of infancy. Transmission of the etiologic agent of the human disease to experimental animals has not been reported. The only reported attempt to propagate any of the salivary gland viruses of rodents in tissue cultures is that of Andrews(9). He tried to cultivate the salivary gland virus of the guinea pig in surviving tissue in a modified Maitland medium. Intranuclear inclusions appeared in mononuclear cells in the interstitial tissue in cultures of guinea pig testis and less constantly in cultures of guinea pig ovary, brain, or salivary glands. The results of all attempts to subculture the virus were negative. No inclusions were found in the

* This investigation was supported by a research grant (PHS E-158) from the National Microbiological Institute, of the National Institutes of Health, Public Health Service.

subcultures, and inclusions did not occur in the salivary glands of young guinea pigs inoculated with the subcultures.

No adequate serologic tests for the identification of the salivary gland viruses have been developed. With the exception of 2 investigations dealing with the guinea pig virus there have been no serologic studies of any of the salivary gland viruses. Kuttner(1) reported that the sera of chronically infected guinea pigs did not prevent infection in guinea pigs inoculated with the serum-virus mixtures. Andrews(9), although he was unable to propagate the guinea pig virus in tissue cultures, showed that the sera of chronically infected guinea pigs was effective in preventing the production of the inclusions which occurred in cultures inoculated with the guinea pig virus. The large intranuclear inclusions occurring in greatly enlarged cells in the salivary glands of animals and young children are readily differentiated from the intranuclear inclusions occurring in other virus infections and have been considered as definitely diagnostic of such infections(1,3,10). The demonstration of these characteristic cytologic changes in the tissues has been the only means available for recognizing infection caused by any of the salivary gland viruses. Propagation of the salivary gland viruses in tissue cultures may facilitate the development of serologic tests for these viruses and offer a method for the study of the human salivary gland virus disease.

In the present study propagation of the murine salivary gland virus in cultures derived from mouse tissue was undertaken as a model for future attempts to isolate the human virus. This report is concerned with the isolation and propagation of the mouse salivary gland virus.

Material and methods. Tissue cultures. Mouse embryonic tissue was cultured in roller tubes (20 x 100 mm). Mouse embryos were obtained during the third week of gestation. The head was discarded and the remainder of the embryo was minced with scissors. Fifteen to 20 fragments were embedded in a thin layer of clotted chicken plasma on the lower two-thirds of the roller tubes. The nutrient media employed consisted of Hanks' balanced salt solution and ox serum ultrafiltrate in the proportion of 3:1, chick embryonic extract (10%) and human ascitic fluid (5%). Fifty units of penicillin and 50 μg of streptomycin per ml of media were added. The cultures were incubated at 35°C, and the nutrient fluids were changed at intervals of 2 or 3 days. The cultures were usually maintained for 8 to 14 days before the virus was inoculated. At this time the cultures consisted largely of spindle cells, presumably fibroblasts. A small amount of epithelial-like growth persisted in some cultures. For histological examination the cultures were fixed in the tubes with Bouin's solution and stained in the tubes with hematoxylin and eosin. In a few instances fragments of the cultures were removed from the tubes before fixation and placed in Bouin's solution. After fixation a number of fragments were embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin.

Origin of virus and inoculation of cultures. The mice used in these experiments were from a colony in which the latent salivary gland virus does not seem to be present. This colony was isolated from other mice and will be referred to as the uninfected colony. Several adult mice obtained from the Wernse Laboratory of Cancer Research at Washington University were shown to have the salivary gland virus infection as indicated by the demonstration of small numbers of the characteristic large intranuclear inclusions in cells of the salivary glands. Portions of the salivary glands of 3 of these mice in which inclusions were demonstrated were pooled and used to pass the virus to young adult mice of the uninfected colony. Many of the characteristic intranuclear inclusions were demonstrated in sections of the salivary glands of passage mice killed 2 weeks following the subcutaneous inoculation of 0.2 ml of a 10% suspension of the salivary gland material. The virus has been maintained in young adult mice from the uninfected colony by serial subcutaneous passages with salivary gland material at intervals of 2 to 3 weeks over a period of several years.

Salivary gland material from mice infected 2 weeks previously by subcutaneous inocula-

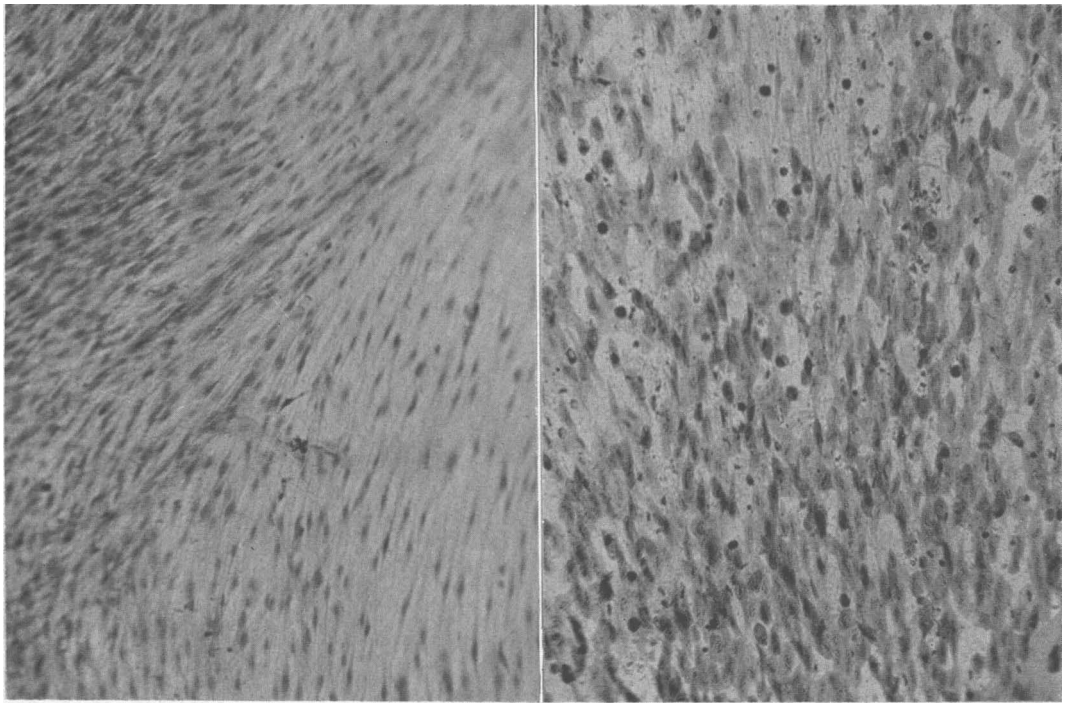

Fig. 1 Fig. 2

FIG. 1. Control uninoculated culture of mouse embryonic tissue. Fixed in culture tube, hematoxylin-eosin stain. × 90.

FIG. 2. Culture of mouse embryonic tissue 9 days following inoculation with mouse salivary gland virus. Fixed in culture tube, hematoxylin-eosin stain. × 90.

tion of the virus was used for the inoculation of the tissue cultures. Recently infected mice were used as the source of virus in order to have material of a relatively high viral titer. The salivary glands of 3 mice were ground and diluted 1:10 by weight in Hanks' solution. After centrifugation the supernatant fluid was further diluted 1:10 and a portion was filtered by means of the Swinny adaptor. For inoculation of the tissue cultures the filtrate was added to fluid culture media in the desired dilutions and 2 ml of the inoculated media placed on each culture when a change of nutrient fluid was made. Usually the final dilution of the infective salivary gland material in the inoculated culture media was 10^{-4}.

Titration of virus. The salivary gland material from recently infected animals was often infective for mice 3 to 5 weeks of age in dilution as great as 10^{-8}. Therefore the filtered infective salivary gland material used as the inoculum for cultures was titrated in mice in 10-fold dilutions to 10^{-9} and the pooled nutrient fluids removed from cultures after periods of incubation were titrated in 10-fold dilutions to 10^{-7}. Three mice, 3 to 5 weeks of age, from the uninfected colony were inoculated intraperitoneally with 0.2 ml amounts of each serial 10-fold dilution. The microscopic demonstration of the characteristic intranuclear inclusions in the salivary glands of these mice when killed 2 weeks following intraperitoneal inoculation was used as the criterion for infection with the virus. Three to 5 sections from each animal cut to include as great an area of salivary gland tissue as possible were examined. The salivary glands of 3 to 5 uninoculated control mice were examined for each titration. The greatest dilution of the infective material which produced inclusions in the salivary glands of 2 of the 3 test mice was considered as the end point of the titration. This method was used in preference to one dependent upon death of one-week-old mice, because the smallest amount of virus which will uniformly cause the death of one-

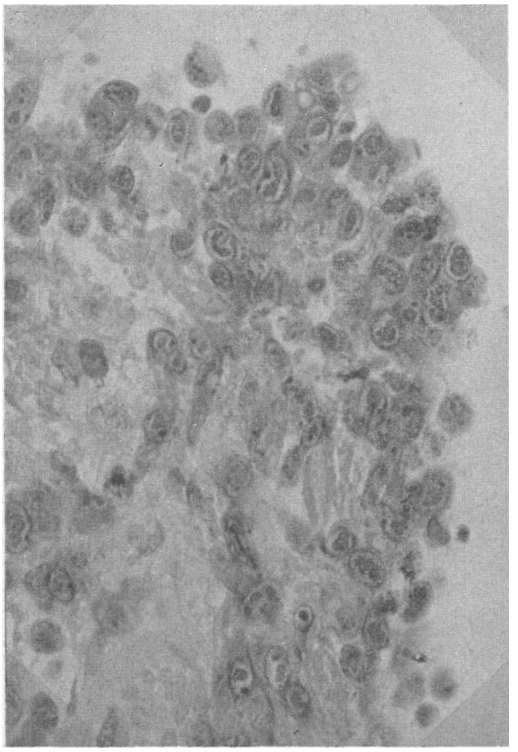

FIG. 3. Section of fragment from culture inoculated with mouse salivary gland virus. Large intranuclear inclusions are shown. Hematoxylin-eosin stain. × 400.

week-old mice within 2 weeks is considerably greater than the infective dose of virus for mice 3 to 5 weeks of age as indicated by the occurrence of inclusions in the salivary glands.

Experimental results. Cytologic changes occurred in tissue cultures when virus was added to the cultures by replacement of the nutrient media with 2.0 ml of media containing the infective salivary gland material in dilution of 10^{-4}. In 3 experiments when the infective material in this dilution was added to 3 or 4 culture tubes focal cytologic lesions occurred in all tubes on the 6th or 7th day. Small groups of cells, which were large, rounded and refractile, contrasted sharply with the surrounding spindle cells. In the next few days the focal lesions increased in number and enlarged by the involvement of adjacent cells in the process. Within 3 to 5 days almost every cell in the culture became affected (Fig. 1 and 2). Some cells began to degenerate within 2 or 3 days after the lesions appeared, but a few of the large refractile cells persisted into the 4th week after inoculation of the cultures.

Such changes never appeared in control uninoculated cultures nor in cultures inoculated with materials prepared from salivary glands of mice from the uninfected colony, which did not show inclusions when examined microscopically.

In cultures that were fixed and stained in the tubes 9 days after inoculation a large intranuclear inclusion was observed in almost every intact large cell and an occasional cell contained 2 or 3 nuclei each having an inclusion. The intranuclear inclusions tended to be somewhat basophilic when stained with hematoxylin and eosin. The inclusion was separated from the nuclear membrane and the shape of the inclusion frequently corresponded closely to that of the nucleus (Fig. 3). In the sections of the fragments which had been embedded in paraffin the intranuclear inclusions appeared granular or, in some instances, consisted of coarse clumps of material. One or 2 small masses of chromatin material staining more deeply basophilic than the inclusions were present in most nuclei, usually attached to the nuclear membrane.

In 2 serial passages of the virus from cultures inoculated with salivary gland material, cytologic lesions appeared in all the subcultures and were of the same character as those observed in the original cultures.

TABLE I. Multiplication of Mouse Salivary Gland Virus in Tissue Cultures.

Day of incubation when nutrient fluid was titrated	No. of nutrient fluid changes before titration of fluid	Infective titer of pooled fluid from cultures*	Dilution of original sal. gl. material before fluids were titrated
2	0	10^{1}	10^{-4}
5	1	10^{3}	10^{-5}
7	2	10^{4}	10^{-6}
9	3	10^{4}	10^{-7}
12	4	10^{5}	10^{-8}
16	6	10^{3}	10^{-10}
19	7	10^{4}	10^{-11}

The estimated increase of virus after 19 days was in the order of 10^{9} times.

* Salivary gland material having an infective titer of 10^{0} was diluted to 10^{-4} in the nutrient fluid when the infection was initiated.

TABLE II. Multiplication of Mouse Salivary Gland Virus in Serial Tissue Cultures.

Passage	Days of incubation subcultures made		No. of nutrient fluid changes		Mouse infective titre of pooled fluid, end of incubation period		Dilution of original sal. gl. material, end of incubation period	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1*	Exp. 2†	Exp. 1	Exp. 2
I	7	14	1	5	10^5	10^5	10^{-5}	10^{-11}
II	6	9	1	3	10^6	10^5	10^{-8}	10^{-19}
III	7	12	1	4	10^5	10^4	10^{-11}	10^{-28}

Estimated increase of virus was in the order of 10^8 times in the 1st exp.; 10^{26} times in the 2nd exp.

* Salivary gland material having an infective titre of 10^8 was diluted to 10^{-4} in nutrient fluid when infection was initiated. Nutrient fluid placed on subcultures when they were inoculated was a dilution of 1:100 of pooled fluids from preceding cultures.

† Salivary gland material having an infective titre of 10^6 was diluted to 10^{-6} in nutrient fluid when infection was initiated. Nutrient fluid placed on subcultures when they were inoculated represented a dilution 10^{-5} of pooled fluids from preceding cultures.

Multiplication of virus. The salivary gland virus disease as characterized by the large intranuclear inclusions in enlarged cells of the salivary glands was reproduced in every instance in mice inoculated subcutaneously or intraperitoneally with the supernatant fluids withdrawn from original cultures or subcultures showing the cytologic changes. To demonstrate the extent of multiplication of the virus in tissue cultures, the infective titers of pooled supernatant fluids removed at intervals from one set of 3 cultures inoculated with the infective salivary gland material were determined by titrations in mice and compared with the infective titer of the inoculum. Considering each successive removal and replacement of the nutrient fluid as a further 10-fold dilution of the original inoculum, it is estimated that an increase in virus in the order of 10^9 times occurred during a 19-day period (Table I).

The extent of multiplication of the virus during 3 serial passages was also determined. Pooled fluids from 2 or 3 culture tubes were titrated in mice when the subcultures were made and in the case of the second subcultures at the end of the period for which the cultures were maintained. The results of the titrations together with the calculated dilution of the original inoculum during the course of the experiment indicated that the increase in virus during 3 serial passages over a period of 20 days was in the order of 10^8 times (Table II).

In a second experiment less virus was inoculated into both the original cultures and the subcultures in order to demonstrate multiplication of the virus more clearly. When the dilution of the infective salivary gland material effected during the experiment is calculated, it is estimated that the increase of virus over a period of 35 days during which 2 serial subcultures were made was in the order of 10^{26} times (Table II).

Summary and conclusions. Cytologic changes including large intranuclear inclusions were produced in cultures of mouse tissue inoculated with salivary gland material from mice infected with the salivary gland virus. They also occurred in 2 serial subcultures. The large intranuclear inclusions resembled those occurring in cells of the mouse salivary gland. Further evidence that the mouse salivary gland virus has been propagated in serial passages in cultures derived from mouse embryonic tissue is as follows. After 3 serial passages of the infective agent in the cultures the salivary gland virus disease, as characterized by specific intranuclear inclusions, has been reproduced in the salivary glands of mice by intraperitoneal inoculation of the supernatant fluids from the cultures. Also the infective titers of supernatant fluids withdrawn from cultures at intervals have been determined by titrations in mice. The results of the titrations, together with the calculated dilutions of the original inocula effected during the course of the experiments, demonstrated

significant increase in the virus in 3 serial passages in tissue culture.

1. Kuttner, A. G., *J. Exp. Med.*, 1927, v46, 935.
2. Kuttner, A. G., and Wang, G. H., *ibid.*, 1934, v60, 773.
3. McCordock, H. A., and Smith, M. G., *ibid.*, 1936, v63, 303.
4. Rosenbusch, C. T., and Lucus, A. M., *Am. J. Path.*, 1939, v15, 303.
5. Lowenstein, C., *Zentralbl. f. Allg. Path. u. path. Anat.*, 1907, v18, 513.
6. Farber, S., and Wolbach, S. G., *Am. J. Path.*, 1932, v8, 123.
7. McCordock, H. A., and Smith, M. G., *Am. J. Dis. Child.*, 1934, v47, 771.
8. Soewadji Prawirohardjo, *Nederl. Tydschr. V. geneesk.*, 1938, v82, 6218.
9. Andrews, C. H., *Brit. J. Exp. Path.*, 1930, v11, 23.
10. Pinkerton, H., *Am. J. Clin. Path.*, 1950, v20, 201.

Received May 10, 1954. P.S.E.B.M., 1954, v86.

Uric Acid in Two Patients with Wilson's Disease (Hepatolenticular Degeneration).* (21124)

CHARLES BISHOP, WALTER T. ZIMDAHL, AND JOHN H. TALBOTT.
(Introduced by Fred R. Griffith, Jr.)
From the Chronic Disease Research Institute and the Department of Medicine, University of Buffalo School of Medicine, Buffalo, N. Y.

In the course of a complete laboratory examination of a brother and sister both suffering from Wilson's disease, it was discovered that the serum urate levels in both individuals were unusually low (1-2 mg %), according to the colorimetric method(1). To elucidate the mechanism of this behavior it was decided to determine uric acid pool size and turnover rate by injecting N^{15} uric acid in the manner previously described(2).

The male subject was injected intravenously with 21.7 mg of N^{15} uric acid (14.81 atom % excess), and all urine was collected *ad libitum* for 5 days. From these results the pool size was calculated to be 442 mg and the turnover rate, 1.95 pools per day. The mean excretion for 4 days, determined by the isotope dilution method in the period just after the other measurements was 848 mg per day. The turnover was 442 x 1.95 = 862 mg and the per cent of the turnover excreted was 848/862 x 100 = 98%(4).

A similar study was performed on the female subject, using 19.5 mg of N^{15} uric acid. The pool size was calculated to be 304 mg and the turnover rate was 1.78 pools per day. The turnover was therefore 541 mg. The mean excretion for 8 days immediately following the test period was 391 mg and the per cent of the turnover that was excreted was 72%.

To test the possibility that the kidney tubules were completely inhibited in so far as their resorption of uric acid was concerned, the drug, Benemid, [p(di-n-propylsulfamyl) benzoic acid] was administered to the female subject and the uric acid injection was repeated. Sirota *et al.*(3) have demonstrated that this drug selectively inhibits tubular resorption of uric acid. The subject was given 3 × 0.5 g Benemid per day by mouth for 6 days and was then injected intravenously with 20.8 mg of isotopic uric acid. The pool size was determined to be 167 mg and the turnover rate 2.34 pools per day. The turnover was therefore 391 mg and the per cent turnover excreted was 86%, based on a mean excretion of 452 mg during 12 days on Benemid. The mean urinary excretion after Benemid was not statistically different from the control value, the P value for the t test being well above the 10% level.

After Benemid therapy was started on the female subject, her clinical condition became considerably worse. After cessation of the therapy she had not improved. Her present serum uric acid level is approximately 1 mg

* This study was supported in part by a grant-in-aid from the National Institutes of Health, Bethesda, Md.