readily isolated in the embryonate egg than in monkey kidney cell tissue culture.

1. Jensen, K. E., Hogan, R. B., *Publ. Health Rep.*, 1958, v73, 140.

2. Comm. on Standard Serological Procedures in Influenza Studies, *J. Immunol.*, 1950, v65, 347.

3. Jensen, K. E., Influenza, in Diagnostic Procedures for Virus and Rickettsial Diseases, *N. Y. Am. Public Health Assn.*, 1956, 241.

4. Jensen, K. E., Dunn, F. L., Robinson, R. Q., *Progress in Med. Virol.*, 1958, v1, 165.

5. Vogel, J., Shelokov, A., *Science*, 1957, v126, 358.

6. Henle, W., Lief, F. S., Fabiyi, A., *The Lancet*, April 19, 1958, 818.

7. Shelokov, A., Vogel, J., Chi, L., PROC. SOC. EXP., BIOL. AND MED., 1958, v97, 802.

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Factors Related to Psittacosis Virus (Strain 6BC) Growth. V. Folic Acid-like Factor in Infected Cells.* (24630)

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Inhibition of growth of the 6BC strain of psittacosis virus by sulfonamides and reversion of this action of sulfonamides in a competitive manner by p-aminobenzoic acid and pteroic acid and in noncompetitive manner by folic acid or citrovorum factor led Morgan to suggest(1) that the cell infected with psittacosis virus might be capable of synthesis of folic acid from precursors. If this is true, one would expect that folic acid content of viral-infected cells would be higher than that of noninfected cells, and studies were initiated to determine if it was possible to detect such net increase in folic or folic acid-like factors in viral-infected cells. L cells were chosen since these cells require folic acid(2) and they support growth of psittacosis virus when cultivated in a semi-synthetic medium(3). These experiments indicate that L cells infected with psittacosis virus contain increased amounts of folic acid or a folic acid-like factor.

Materials and methods. The mouse fibroblast cell line, strain L, obtained from Dr. Wilton R. Earle, Nat. Cancer Inst., Bethesda, was maintained in T flasks‡ in growth medium consisting of Earle's balanced salt solution; 0.06% yeast extract§; 0.18% lactalbumin hydrolysate‖; and 10% horse serum. Semi-synthetic medium (SSM) was a modification of medium described by Bader and Morgan(3) in which folic acid was omitted, concentration of p-aminobenzoic acid (PABA) was doubled, and 1% dialyzed horse serum was added. The egg-adapted strain of psittacosis virus (6BC) was used, as described previously(1,4). To minimize folic acid content of virus inocula, virus stock was prepared from virus grown in L cells which were maintained in SSM for 8 days before infection. After infection, daily microscopic observations of cells was carried out until a definite cytopathic effect of the virus was noted; then the flasks were frozen and thawed slowly to break up the cells and the entire culture harvested and stored in a dry-ice chest for use as stock virus which was diluted in SSM as needed. Sulfadiazine (SDZ) was used as a sterile solution of sodium salt. P-aminobenzoic acid (PABA) was dissolved in distilled water as the sodium salt and sterilized by filtration. Folic acid determinations were carried out according to the method adopted by Assn. of Official Agricultural Chemists(5), using *Streptococcus faecalis* as test organism (ATCC #8043). Chicken pancreas extract was used as source of glutamic acid conjugase. Bacterial growth was measured turbidimetrically with Bausch

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‡ Obtained from Kontes Glass Co., Vineland, N. J.

§ Obtained from Difco Labs., Detroit, Mich.

‖ Obtained from Nutritional Biochemicals Corp., Cleveland, O.

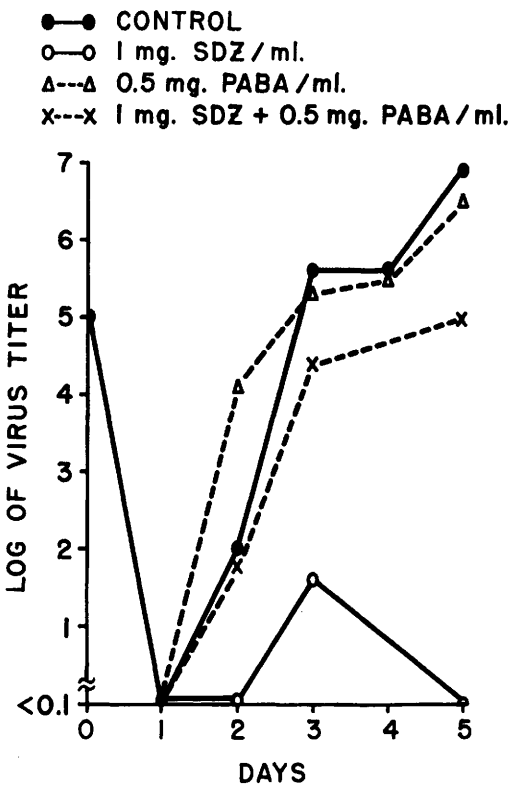

FIG. 1. Inhibition of growth of psittacosis virus in L cells by SDZ and its reversal by PABA.

and Lomb spectrophotometer at 5,000 Angstrom units.

Results. Inhibition of viral growth in L cells by SDZ and its reversion with PABA. Because of reported variability(6,7) in sulfonamide sensitivity *in vivo* of members of lymphogranuloma-psittacosis group of viruses, depending upon host used, it was of interest to determine if the 6BC strain used was sensitive to SDZ in L cells and if inhibition could be reversed by PABA.

L cells, cultivated in T-15 flasks in growth medium and infected with the virus, were divided into 4 groups and media containing compounds noted in Fig. 1 were then used in each flask. The fluids were removed daily for 5 days and titrated for virus content, and media containing respective drugs in the described concentrations were added to each flask. PABA (0.005 mg) was injected into each egg with the virus inoculum to counteract possible growth inhibition by SDZ carried over from tissue cultures(1,4). Results are shown in

TABLE I. Folic Acid Content of Infected and Noninfected L Cells.

Exp.	Flask	Virus titer of inoculum, LD_{50}/ml	Cytopathic effect, days	Folic acid (mμg) Total	Inoculum	Net
I	A	3.9	9	426.4	.0	426.4
	B	0	0	202.8	2	200.8
II	A	4.0	9	431.6	.0	431.6
	B	0	0	213.2	5	208.2

Fig. 1. Each curve represents the geometric mean of titers of 3 identical experiments. SDZ inhibits virus growth in L cells and PABA has the effect of reversing this inhibition. PABA alone had no significant effect on growth of virus.

Folic acid content of L cells infected with virus. When a uniform monolayer of L cells had covered the bottom of T-60 flasks, each flask was changed to the SSM, using 10 ml/flask. Cells were then maintained in this medium with complete renewal of fluids daily for 14 days to deplete them of folic acid. Two flasks were chosen and flask A was inoculated with living virus, while flask B received a similar quantity of heated virus. Folic acid assays were made of media containing the virus inoculum and only barely detectible quantities of folic acid were found in the heated virus. On the following day, the fluids were removed, stored at –40°C, and replaced with SSM. During remainder of experiment, depending on pH of cultures, the media on both flasks were removed, stored, and replaced with SSM. On the day the infected flask began to exhibit cell degeneration due to cytopathogenic effect of the virus, both flasks were frozen and thawed, and the entire contents of each flask were harvested and pooled with the respective fluids taken off on previous days, concentrated by lyophilization, and assayed for folic acid with results shown in Table I.

These data demonstrate that materials from the cultures infected with psittacosis virus contain twice as much folic acid as the uninfected cell cultures.

Discussion. Susceptibility of viruses in the lymphogranuloma venereum-psittacosis group to sulfonamides seems to indicate that these agents either possess enzyme systems of their

own or are capable of inducing formation of such new systems essential for viral replication in host cells following infection. Folic acid is essential for replication of psittacosis virus, as indicated by viral growth inhibition by sulfonamides(1) or folic acid analogues(8) in chick embryo tissue *in vivo* or *in vitro* in amounts not toxic for host or its isolated cells. It appears that quantities of folic acid available in the cells are not adequate for growth of psittacosis virus and that synthesis of this essential factor takes place in the infected cell. The fact that cells depleted of folic acid for 14 days still support viral growth, further emphasizes this probable folic acid synthesis.

Increase in folic acid in L cells infected with psittacosis virus lends further support to the suggestion that such infected cells are capable of folic acid synthesis(1). Since folic acid has been shown to be a vitamin requirement for L cells(2), it is unlikely that these cells possess such synthetic capacity, but rather that this property is due to an enzyme or enzyme system acquired as a result of virus infection. Whether this synthetic activity is part of the intact virus particle itself or can be demonstrated only in the infected host cell remains to be determined.

Recent evidence indicates that folic acid or allied compounds are specific constituents of the psittacosis group(9), and the analyses on virus inocula showed some folic acid in the heated preparations. Furthermore, direct evidence has been presented that viruses in this group do possess enzymatic activity with the demonstration by Allen and Bovarnick(10) of reduced diphosphopyridine nucleotide cytochrome c reductase activity in purified preparations of meningopneumonitis virus, though other investigators(11,12) have failed to demonstrate enzymatic activity for viruses of this group. However, as Cohen(13) has pointed out, random testing for enzymes in viruses has little chance of success unless experiments are designed to test for those metabolic systems which may be related to the particular pattern and site of development of a given virus. Sulfonamide sensitivity of psittacosis virus (6BC) presents a guide to such a system, *i.e.*, folic acid synthesis, though the enzymatic mechanism remains unknown.

Summary. Growth of psittacosis virus (6BC) in L cells *in vitro* was inhibited by sulfadiazine and this inhibition was reversed by addtion of PABA. L cell tissue cultures infected with the virus contained 2 or more times the amount of folic acid in noninfected cultures. The possibility of introduction into the cell or induction in the cell by the virus of enzyme or enzyme system, making the infected cell capable of folic acid synthesis, is discussed.

1. Morgan, H. R., *J. Exp. Med.*, 1948, v88, 285.
2. Eagle, H., *ibid.*, 1955, v102, 595.
3. Bader, J. P., Morgan, H. R., *ibid.*, 1958, v108, 617.
4. Golub, O. J., *J. Immunol.*, 1948, v59, 9.
5. *Methods of Vitamin Assay,* Assn. of Vitamin Chem., Interscience Publishers, N. Y., 1951.
6. Eaton, M. D., Hanford, V. L., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 63.
7. Wiseman, R. W., Meiklejohn, G., Lackman, D. B., Wagner, J. C., Beveridge, G. W., *J. Immunol.*, 1946, v54, 9.
8. Morgan, H. R., *J. Exp. Med.*, 1952, v95, 269.
9. Colon, J. I., Moulder, J. W., *J. Inf. Dis.*, 1958, v103, 109.
10. Allen, E. G., Bovarnick, M. R., *J. Exp. Med.*, 1957, v105, 539.
11. Moulder, J. W., *Bact. Rev.*, 1954, v18, 170.
12. Perrin, J., *J. Gen. Microbiol.*, 1952, v6, 143.
13. Cohen, S. S., *Advances in Virus Research,* Academic Press, N. Y., 1955.

Received November 10, 1958. P.S.E.B.M., 1959, v100.