repeatedly in this laboratory and in several others, insofar as laboratory animals are concerned. In such a case certain military, diplomatic, Peace Corps, industrial and other groups exposed in areas with endemic group B arboviruses might well utilize such a vaccine together with others in group B for the postulated breadth of at least partial protection against members of group B spread widely throughout the world. Furthermore, since JBE and SLE can apparently be prepared so easily by this means, a number of other group B viruses can probably be handled in the same way, especially those of the same subgroup, including those of Murray Valley encephalitis, West Nile, Ilheus, and bat salivary gland.

Summary. The large plaque variant of the P-15 strain of SLE virus which grows to a titer of 10^8 to 10^9 $TCID_{50}$/0.1 ml in HKC was inactivated by formalin and tested for antigenicity. The virus was harvested in medium 199 containing 2% H.Al., and was readily and apparently predictably inactivated by 1:4000 formalin at 37°C. Loss by filtration prior to activation was in the expected range of 0.3 log. The inactivated preparation when tested for the minimal immunogenic dose (MID) in mice gave a low value, indicative of excellent immunogenicity. The MID for a preparation with a preinactivation titer of $10^{-7.3}$/0.1 ml was 0.0079 ml, whereas that with a titer of $10^{-8.7}$/0.1 ml furnished an MID of <0.0015 ml. The latter vaccine elicited an unexpectedly good response in guinea pigs in an antigen extinction test. The protection the mice developed from such vaccine exceeded that of an earlier mouse brain St. Louis virus vaccine and that of a recently developed HKC JBE vaccine.

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Effect of L-Cystine and Sulfated Polysaccharides on Replication of Echovirus Type 32 in Monkey Kidney Cells.* (31457)

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In studies with recently isolated and characterized echovirus type 32 attempts to produce plaques by methods utilized were unsuccessful(1,2). Branche(1) noted that plaques could be produced when $NaHCO_3$ was incorporated into the overlay medium. This method was tested in our laboratory; plaques less

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than 0.5 mm in diameter were formed in monkey kidney cells.

Described herein is an efficient plaque assay method for echovirus type 32 by which plaques 3-4 mm in diameter are formed in 2 days. The effect of diethylaminoethyl (DEAE)-dextran on the plaque forming capacity of the virus and its dependence on L-cystine for replication are reported.

Materials and methods. Cells. Primary rhesus *(Macaca mulatta)* monkey kidney (PRMK) cells were grown in high cystine altered Eagle's maintenance ($hcAE_M$) medium (3) containing 4% calf serum, as previously described(4).

Virus and antiserum. Echovirus type 32 and antiserum were kindly supplied by Dr. J. L. Melnick, Baylor University College of Medicine.

DEAE-dextran and sodium dextran sulfate. DEAE-dextran and sodium dextran sulfate were obtained from Pharmacia, Uppsala, Sweden. Stock solution of dextran sulfate (DS) was prepared by dissolving appropriate concentration of sodium dextran sulfate to contain 4,000 μg/ml. DEAE-dextran was similarly prepared.

Plaque assay. Monolayers of PRMK cells were twice washed with PBS and inoculated with appropriate concentrations of virus. Following one hour adsorption at 37° plates were overlayered with 4-6 ml $hcAE_M$ medium or altered Eagle's maintenance (AE_M) medium containing 0.9% agar. Specified concentrations of DEAE-dextran or DS were incorporated into the medium. Plates were incubated at 36-37° in the humidified atmosphere of a continuous-flow incubator flushed with a mixture of 3% CO_2 and 97% air. In 3-5 days plaques were stained with agar overlay containing 0.01% neutral red.

Results. Effect of DEAE-dextran and plaque development period on plaque formation. Although echovirus type 32 fails to form visible plaques on primary monkey kidney cells when standard plaque techniques are used, the virus produces CPE in these cells comparable to that caused by other enteroviruses(1). Previous experiments had shown that certain echoviruses were inhibited to various degrees by sulfated polysaccharide in agar(5); therefore the effect of incorporation of DEAE-dextran into the agar overlay to counteract possible inhibition of echovirus type 32 by this compound was tested. A concentration of 100 μg/ml DEAE-dextran in the overlay resulted in formation of plaques 3-5 mm in diameter in $hcAE_M$ medium as early as 2 days post infection. The effect of various concentrations ranging from 0-200 μg/ml of DEAE-dextran on plaque diameter of this virus is shown in Table I. In the absence of DEAE-dextran no discernible plaques formed during the 7-day observation period. Plaques appeared on the 4th day in the presence of 12.5 μg/ml DEAE-dextran and on the 2nd day with 25 μg/ml and higher concentrations. Plaque diameters continued to increase during the 7-day period with each concentration tested.

TABLE I. Effects of Various Concentrations of DEAE-Dextran and Plaque Development Period on Plaque Diameter of Echovirus Type 32 in Monkey Kidney Cells.

Plaque development period (days)	Plaque diameter (mm) Conc of DEAE-dextran (μg/ml)						
	None	12.5	25	50	75	100	200
2	NV*	NV	1.0	2.5	3.0	4.0	4.0
3	NV	NV	1.5	3.0	3.5	4.5	4.5
4	NV	.5	1.8	4.0	4.0	5.5	5.5
5	NV	.8	2.0	5.0	5.0	6.0	6.0
6	NV	1.0	2.5	6.0	6.5	7.0	7.0
7	NV	1.5	3.0	7.0	7.5	8.5	9.0

* Not visible.

Effect of DS on plaque diameter and titer. Apparently plaque formation by echovirus type 32 was completely inhibited by sulfated polysaccharide occurring naturally in agar; hence the effect of a synthetic sulfated polysaccharide—DS—on the rate of virus synthesis in PRMK cells was tested. Concentrations of DS ranging from 0-200 μg/ml were tested. Incorporation of 100 μg/ml DS into the medium of PRMK cells infected with 1,000 PFU/ml resulted in marked inhibition of CPE as compared to that in controls without DS. Propagation curves of the virus were compared in standard $hcAE_M$ and $hcAE_M$ + 100 μg/ml DS. Results are shown in Fig. 1. Yields were 1-2 logs lower in the presence of DS; new progenies first appeared in 3-7 hours in standard medium as compared to 12

TABLE II. Effect of Various Concentrations of L-Cystine on Plaque Titer of Echovirus Type 32 in Monkey Kidney Cells.

	Plaque titer (PFU/ml)							
	Concentration of L-cystine (mM/ml)							
Exp No.	None	.025	.05	.10	.20	.40	.80	1.60
1	$<10^1$	1.6×10^5	1.5×10^6	3.5×10^6	4.0×10^6	3.7×10^6	3.1×10^6	1.5×10^6
2	$<10^1$	4.0×10^5	2.0×10^6	3.2×10^6	4.5×10^6	3.5×10^6	2.8×10^6	8.0×10^5
3	$<10^1$	2.5×10^5	1.8×10^6	3.4×10^6	6.5×10^6	4.0×10^6	3.2×10^6	2.0×10^6

TABLE III. Effect of L-Cystine and DEAE-Dextran on Plaque Production by Echovirus Type 32 in Monkey Kidney Cells.

	Exp #1		Exp #2		Exp #3	
Medium	Plaque diameter	Plaque titer	Plaque diameter	Plaque titer	Plaque diameter	Plaque titer
AE_M without cystine	0	$<10^1$	0	$<10^1$	0	$<10^1$
Idem + 100 μg/ml DEAE-dextran	0	$<10^1$	0	$<10^1$	0	$<10^1$
$hcAE_M$ (0.2 mM L-cystine)	0	$<10^1$	0	$<10^1$	0	$<10^1$
" + 100 μg/ml DEAE-dextran	5.0	$10^{6.5}$	6.5	$10^{7.2}$	7.5	10^7

hours in DS-containing medium.

Effect of L-cystine on plaque titer. Various degrees of cystine dependence have been demonstrated with certain echovirus (unpublished data). The effect of various concentrations of L-cystine in the maintenance medium on replication of echovirus type 32 as expressed by plaque titer is shown in Table II. Absolute cystine dependence for growth of the virus was indicated when no plaques were formed in the absence of L-cystine, although the medium contained 100 μg/ml DEAE-dextran. However, in concentrations as low as 0.025 mM L-cystine the virus multiplied; yield increased as the concentration of L-cystine was increased up to 0.2 mM. Higher concentrations up to 1.6 mM did not significantly increase plaque titers; in fact a decrease in titer was noted and plaques became turbid when medium contained 1.6 mM L-cystine.

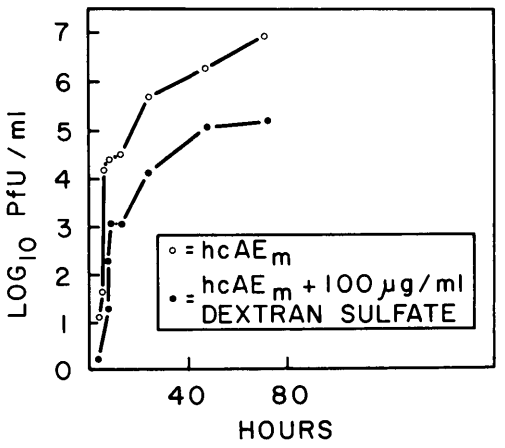

FIG. 1. Effect of a synthetic polysaccharide (dextran sulfate) on rate of synthesis of echovirus type 32 in primary monkey kidney cells.

Effect of a combination of DEAE-dextran and L-cystine on plaque diameter. The effects of DEAE-dextran and L-cystine on plaque formation were tested separately and together using the previously determined optimal concentrations of each compound. In one series of experiments AE_M medium containing no cystine was used; DEAE-dextran (100 μg/ml) was incorporated into half of the plates and omitted from the other portion. In another experiment $hcAE_M$ medium containing 0.2 mM L-cystine was used; half of the plates received DEAE-dextran and the others received only washed agar. Results in Table III indicate that echovirus type 32 required both L-cystine and DEAE-dextran for plaque formation in PRMK cells.

Separation of echovirus type 32 from echovirus type 29. The dependence of echovirus 32 on DEAE-dextran for plaque formation was a useful tool for separation of the virus from stocks which contained a mixture of echovirus types 29 and 32. Echovirus 29 formed plaques 5-7 mm in diameter on PRMK cells and was not significantly affected by 12.5-25 μg/ml concentrations of DEAE-dextran, which enabled echovirus type 32 to form plaques 0.8-1.5 mm in diameter in these

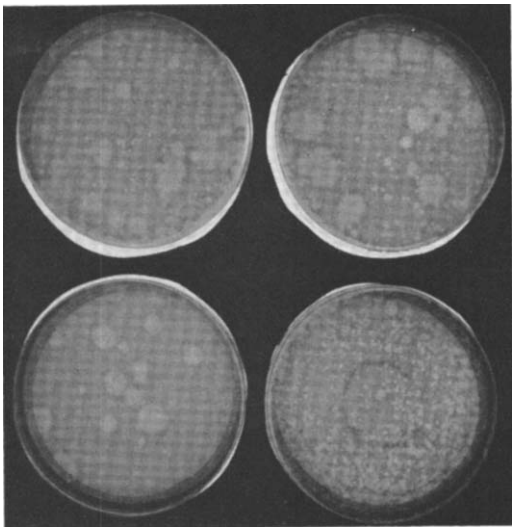

FIG. 2. Purification of echovirus type 32 from a contaminated stock of echovirus type 29 by incorporation of 25 μg/ml DEAE-dextran into the agar overlay medium.

cells. The marked difference in plaque sizes enabled selection and plaque purification of each virus. Fig. 2 shows the steps in purification by this method.

Discussion. While monkey kidney tissue culture cells support the growth of echovirus type 32 to the extent that cellular destruction is complete in about 5 days, apparently this virus possesses the distinctive character of requiring the presence of two factors not required by other known echoviruses for plaque formation in these cells. The phenomena by which DEAE-dextran and L-cystine enable this virus to form plaques are of course unrelated.

Inhibition by sulfated polysaccharides is theorized to be due to interaction between oppositely charged sites on the inhibitor and the virus which results in binding of the virus and prevention of its adsorption to the cells (6). On the other hand, L-cystine is required for intracellular synthesis of new virus (unpublished data). Results obtained indicate that echovirus type 32 possesses detailed characteristics which differ from other echoviruses in both its protein coat and process of replication.

Complete dependence of echovirus type 32 on both DEAE-dextran and L-cystine affords an opportunity for study of certain genetic phenomena with this virus. If various spontaneous and induced mutants could be obtained (*e.g.*, a virus dependent on only one of these compounds and possibly one that required neither DEAE-dextran nor L-cystine for plaque formation) they could be utilized in genetic recombination studies with another echovirus or picornavirus having biochemical response or other suitable mutants.

Summary. In order to form visible plaques on PRMK cells echovirus type 32 requires (1) the incorporation of DEAE-dextran into the agar overlay to counteract the inhibitory effect of sulfated polysaccharide occurring in agar and (2) the presence of L-cystine in the maintenance medium. A method employing a combination of these two compounds as described affords efficient plaque assay in PRMK cells.

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