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Anomalous Hemadsorption by Cell Cultures.* (30131)

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The adhesion of red blood cells to viral infected cell cultures was discovered by Vogel and Shelokov(1) and employed for identification of different types of viruses(1,2). Since then, hemadsorption has been utilized in a variety of ways to assay, identify and study viruses(3-7).

In the present report, a consistent hemadsorption by primary kidney cell cultures and stable lines of monkey kidney cell cultures not known to be infected with viruses is described. Critical requirements for this phenomenon were the presence of calcium ions or treatment of the red blood cells with periodate.

Material and methods. Fresh chicken red blood cells (RBC's) were obtained as a stabilized suspension from the Baltimore Biological Laboratory, Baltimore, Md. RBC's from other animals were collected fresh in citrated blood specimens. All RBC's were centrifuged immediately before use, washed twice in physiological saline (0.85%) and resuspended as a 5% suspension in physiological saline solution. For application to cell cultures, the RBC's were prepared in 0.2% suspension in buffer solutions.

Buffer solutions were prepared at pH 4.0 to 5.8 with 0.15 M acetate and at pH 6.9 to 9.3 with 0.04 M veronal containing 0.1 M NaCl.

Cell cultures were grown from freshly trypsinized tissues (primary cultures), subcultures of these (secondary cultures), or from stable lines of cells. For tests, the cultures were grown in Leighton tubes with a flat bottom area 5 × 1 cm. The medium for routine cell cultures has been described(8). Eagle's minimum medium(9) and Medium 199(10) also were used at times. Media routinely contained 5% unheated calf serum obtained from Baltimore Biological Laboratory. Other sera were from the same source.

BSC-1, a stable line of green monkey *(Cercopithecus aethiops)* kidney cells, was obtained from Microbiological Associates, Inc., Bethesda, Md. RK-13 cells were obtained from Dr. Hope Hopps, Bureau of Biological Standards, National Institute of Health. The LLC-MK 2 Rhesus monkey kidney derived stable line was obtained from the American Type Culture Collection Cell Repository, Rockville, Md.

For general treatment of cultures with RBC's, growth medium was removed; the cells were washed twice with physiological saline and overlaid with 1.5 ml RBC's in test solutions. After 30 minutes at room temperature (25°C), the cultures were agitated, the RBC suspension removed and the cultures washed twice with Earle's balanced salt solution. The hemadsorption was evaluated microscopically. Values were ascribed as (—) for absence of adhering RBC's, (±) for some scattered adherence and (+) to (+++++) for increasing amounts of RBC's up to apparent maximal adsorption. A complete

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TABLE I. Effect of Different Concentrations of Calcium Ions on Hemadsorption by BSC-1 and Primary GMK Cells.

Concentration of calcium ions* (mM)	BSC-1 cells		Primary GMK cells	
	Microscopic evaluation†	Protein of eluted RBC's (μg/tube)	Microscopic evaluation†	Protein of eluted RBC's (μg/tube)
0	—	33	—	25
.01	—	43	±	46
.05	±	40	+	59
.1	+	56	++	87
.5	++		++++	156
1	+++	97	++++	154
2	++++	180	++++	154
5	++++	184	++++	160

* Supplied by $CaCl_2$.

† — = no hemadsorption; ++++ = apparent maximal hemadsorption.

Adsorption of chicken RBC's by cultures in Leighton tubes was for 30 min at 25°C and pH 7.5.

coverage of the cell sheet with a monolayer of RBC's usually constituted the maximum. Hemadsorption was also estimated by determination of the protein of eluted RBC's by the procedure of Oyama and Eagle(11). Elution was accomplished by agitation for several minutes with 0.1 M phosphate buffer pH 6.0 in the absence of calcium and with or without the inclusion of 0.02 M sodium citrate.

Trypsinization of RBC's was by treatment of a 2.5% suspension of washed cells in 0.04 M veronal-NaCl buffer, pH 7.5 for 2 hours at 37°C with 0.1% Bacto-Difco 1:250 trypsin.

For periodate oxidation, a 0.5% suspension of washed RBC's in 0.001 M sodium metaperiodate in saline was incubated one hour at room temperature.

For enzymic treatment, cell cultures in Leighton tubes were washed twice in Earle's solution and incubated at 37°C at pH 7.4 with varying concentrations of 2 × crystallized trypsin, 2 × crystallized lysozyme (Worthington Biochemical Corp., Freehold, N. Y.), 3 × crystallized chymotrypsin (Mann Research Laboratories, New York) or a purified neuraminidase preparation (Merck, Sharp & Dohme, West Point, Pa.), in Earle's solution. Proteolytic activity was determined on casein when required(12).

Electron micrographs were prepared by the procedure of Howatson and Almeida(13).

Results. Initial observations showed that chicken RBC's in buffer solutions at pH 7 to 8 in the presence of calcium ions strongly adsorbed on BSC-1 and on primary green monkey kidney (GMK) cell cultures as shown in Fig. 1. Hemadsorption by BSC-1 occurred throughout the cell sheet. Localized areas of GMK were not reactive. Each of these examples was evaluated microscopically at ++++, however. Hemadsorption in the absence of calcium was negligible and was scored as —.

The effect of varying concentrations of calcium ion on the hemadsorption in veronal buffer, pH 7.5 is shown in Table I. Appraisals by microscopic examination and by protein determinations were concordant. Positive values for protein in the absence of observable hemadsorption were due to extracted protein and loosened cells and could be obtained with cultures not exposed to RBC's. In the absence of calcium ion, no hemadsorption occurred. Response was detectable at 0.05 mM and was maximal at 0.5 to 2 mM. Slight inhibition occurred at higher concentrations.

The effect of pH variation on hemadsorption in the presence of 2 mM calcium ion is shown in Table II. Hemolysis sometimes occurred at pH 4.0 to 5.0. Nuclei adhered to the cells in such instances. The cultured cells tended to agglomerate and loosen from the glass at pH's above 8.0. Maximal hemadsorption was obtained at 6.9 to 8.6.

Several cationic materials, Ba^{++}, Mg^{++}, salmine and spermine failed to replace the calcium ion requirement for hemadsorption,

although examined over the practicable pH range. Calcium ion did not promote adhesion to glass surfaces; however, salmine and spermine strongly supported adsorption to glass. Several heavy metal ions: Cu^{++}, Co^{++}, Fe^{+++}, Ni^{++}, and Zn^{++} caused a non-specific clumping of RBC's and adhesion to cultured cells of all types and to glass surfaces.

Temperature was not critical for hemad-

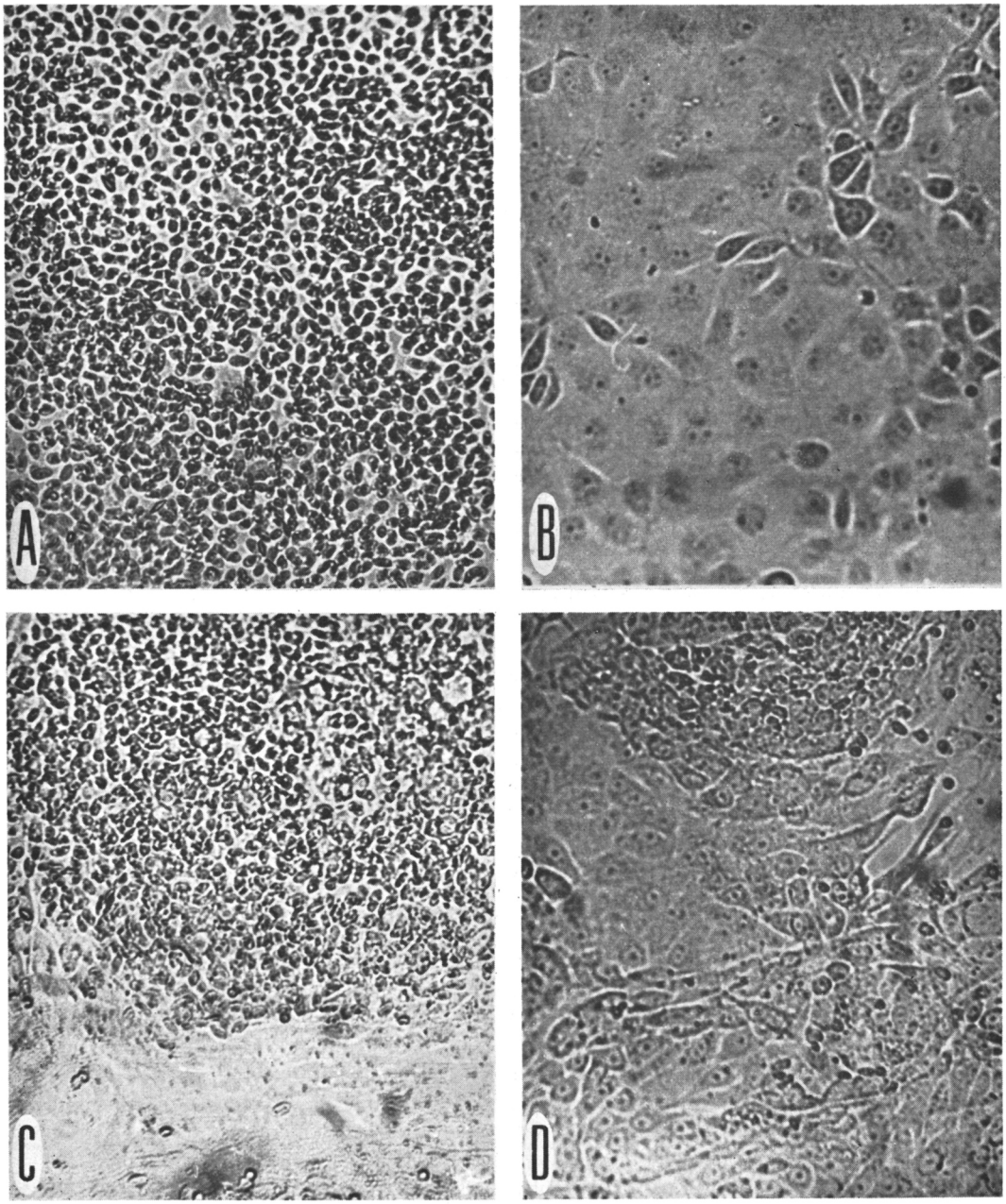

FIG. 1. Hemadsorption of chicken RBC's by cell cultures. RBC's, 0.2% suspension in 0.04 M veronal buffer, pH 7.5, containing 0.1 M NaCl, with and without 2 mM $CaCl_2$. 30 min at room temperature. 64×. A. BSC-1 cell culture, 2 mM $CaCl_2$. B. BSC-1 cell culture, no $CaCl_2$. C. Primary GMK cell culture, 2 mM $CaCl_2$. D. Primary GMK cell culture, no $CaCl_2$.

TABLE II. Effect of pH on Hemadsorption by BSC-1 and Primary GMK Cells.

pH	BSC-1 cells: Microscopic evaluation*	BSC-1 cells: Protein of eluted RBC's (μg/tube)	Primary GMK cells: Microscopic evaluation*	Primary GMK cells: Protein of eluted RBC's (μg/tube)
Acetate buffer				
5.4	++	164	++++	148
5.8	++++	224	++++	166
Veronal buffer				
6.9	++++	320	++++	230
7.5	++++	272	++++	252
8.0	++++	252	++++	280
8.6	++++	228	++++	244
9.3		146	++++	212

* — = no hemadsorption; ++++ = apparent maximal hemadsorption.
Adsorption of chicken RBC's by cultures in Leighton tubes was for 30 min at 25°C in presence of 2 mM calcium.

sorption. Parallel results at comparable rates were obtained at 4° and 25°C. The time required for hemadsorption by GMK cells at room temperature (25°) is represented by data in Table III. Hemadsorption was noted almost immediately. It increased linearly for about 10 minutes and attained maximal values at 20 to 60 minutes. Because of possible effects of prolonged incubation in saline on the cultured cells, 30 minutes of exposure was adopted in the present studies.

A variety of cultured cell lines was treated with RBC suspensions at pH 4.5 to 9.3 with results presented in Table IV. All cultures derived from monkey kidney cells strongly adsorbed the chicken RBC's. Rabbit kidney cells and the stable RK-13 derived from rabbit kidney exhibited areas of

TABLE III. Rate of Hemadsorption by Primary GMK Cells.

Hemadsorption period (min)	Hemadsorption: Microscopic evaluation*	Hemadsorption: Protein of eluted RBC's (μg/tube)
0	—	9
1	±	16
3	+	32
5	++	53
10	++++	106
15	++++	112
20	++++	119
30	++++	140
45	++++	153
60	++++	136

* — = no hemadsorption; ++++ = apparent maximal hemadsorption.
Adsorption of chicken RBC's was at pH 7.5 at 25°C in presence of 2 mM calcium.

TABLE IV. Hemadsorption by Different Cell Cultures.

Cell cultures	Hemadsorption*
Primary green monkey kidney	++++
Secondary " " "	++++
Primary " " testes	—
Primary rhesus monkey kidney	++++
BSC-1 (stable " ")	++++
MK-2 (" " ")	++++
Primary rabbit kidney	+
" " uterus	—
RK-13 (stable rabbit kidney)	++
KB	—
HeLa	—
S-15 (stable line human skin)	—
Primary human amnion	—
WISH (stable line human amnion)	—
Primary mouse kidney	—
Mouse strain L fibroblasts	—
Primary hamster kidney	—
" " embryo	—
Primary guinea pig kidney	—
" calf kidney	—
" chick embryo	—
Primary duck embryo	—

* — = no hemadsorption; ++++ = apparent maximal adsorption.
Adsorption of chicken RBC's was observed over pH range of 5 to 9, 30 min at 25°C in presence of 2 to 10 mM calcium.

hemadsorption. The remaining wide assortment of cell cultures including green monkey testicular, primary human embryo kidney, primary human amnion and stable line human cells exhibited negligible hemadsorption. The hemadsorption of primary rhesus monkey kidney followed the localized topological pattern of the primary GMK. Hemadsorption of secondary GMK and MK-2 was distributed in a general manner throughout the cultures. The hemadsorption of rabbit kidney

TABLE V. Hemadsorption of Metaperiodate-Treated RBC's.

	Cell culture								
pH	GMK (primary)	GMK (secondary)	BSC-1	RK-13	Primary chick embryo	Primary duck embryo	Monkey testes	KB	Human embryo kidney
	Microscopic evaluation*								
4.6	++	+++	++	++	+	++	++	+	+++
5.4	++		++	++	++	++	+++	++	+++
5.8	+++	+++	++++	+++	++++	+++	+++	++	+++
6.9	++++	++++	++++	+++	—	—	—	—	—
7.5	++++	++++	++++	+++	—	—	—	—	—
8.0	++++	++++	++++	+++	—	—	—	—	—
8.6	++++	++++	++++	+++	—	—	—	—	—
9.3	++++	++++	++++	+++	—	—	—	—	—

* — = no hemadsorption; ++++ = apparent maximal adsorption.
Adsorption of chicken RBC's was for 30 min at 25°C in presence of 2 mM calcium.

cell cultures was confined to small areas.

Growth of cultures in Eagle's medium or Medium 199 and employment of rabbit, chicken, horse, human, or agamma calf serum did not alter hemadsorption responses.

Green monkey, guinea pig and sheep RBC's were investigated by the procedures described for chicken RBC's. These smaller mammalian RBC's adsorbed much less efficiently than did chicken RBC's, but positive results obtained were qualitatively similar.

Trypsinization of chicken RBC's or treatment with 0.01 M iodoacetamide, 0.1 M sodium nitrite or 0.1 M potassium sulfite did not preclude hemadsorption.

Treatment of chicken RBC's with 0.001 M sodium metaperiodate for one hour at room temperature was found to alter the hemadsorptive properties of chicken RBC's. Calcium ion was no longer required for adsorption. Nevertheless, as shown in Table V, cell cultures that adsorbed in the calcium-dependent system, adsorbed periodate-treated RBC's at pH 4.6 to 9.3. Primary chicken embryo cell cultures negative in the calcium-dependent system did not adsorb the periodate-treated RBC's at pH 6.9 to 9.3. Calcium was included in the suspension to maintain cell adhesion to the glass; however, similar results were obtained with omission of calcium.

Incubation of cell cultures with 0.2 to 0.5 mg purified neuraminidase or 0.02 to 0.05 trypsin or chymotrypsin per ml in Earle's solution for 60 minutes inhibited the calcium-dependent hemadsorption or adsorption of periodate-treated RBC's. The neuraminidase preparations, however, digested the casein at a rate equivalent to 1% to 2% of the same quantity of crystalline trypsin. Enzymic action was not found to destroy the reactivity of the RBC's.

Examination of the anomalous calcium-dependent hemadsorption with the electron microscope showed that extensive close direct contact existed between the RBC's and cultured BSC-1 cells (Fig. 2) and could be included under the descriptive term "cyto-hemadsorption" applied by Hotchin *et al* (14). In contrast, the sites of binding RBC's to MK-2 cells were through innumerable microvillae which characterized the surface of these cultured cells.

Discussion. The calcium-dependent hemadsorption and the adsorption of periodate-treated chicken RBC's appears to be characteristic chiefly of monkey kidney cell cultures, and to a minor extent to cultures of rabbit kidney cells. The inactivity of green monkey testicular cell cultures and localized areas of primary GMK indicates further the specificity of cell type involved. The histological identity of the positive reacting cells was not determined. The calcium ion is well known to have a role in cell-to-cell adhesiveness(15). It is not surprising that the ion can play some part in cultured cell-RBC surface relationships. The pH optimum was in a range in which divalent cationic calcium ion might be expected to form cross-bonds between anionic structures of the cellular surfaces. The reaction, however, was highly spe-

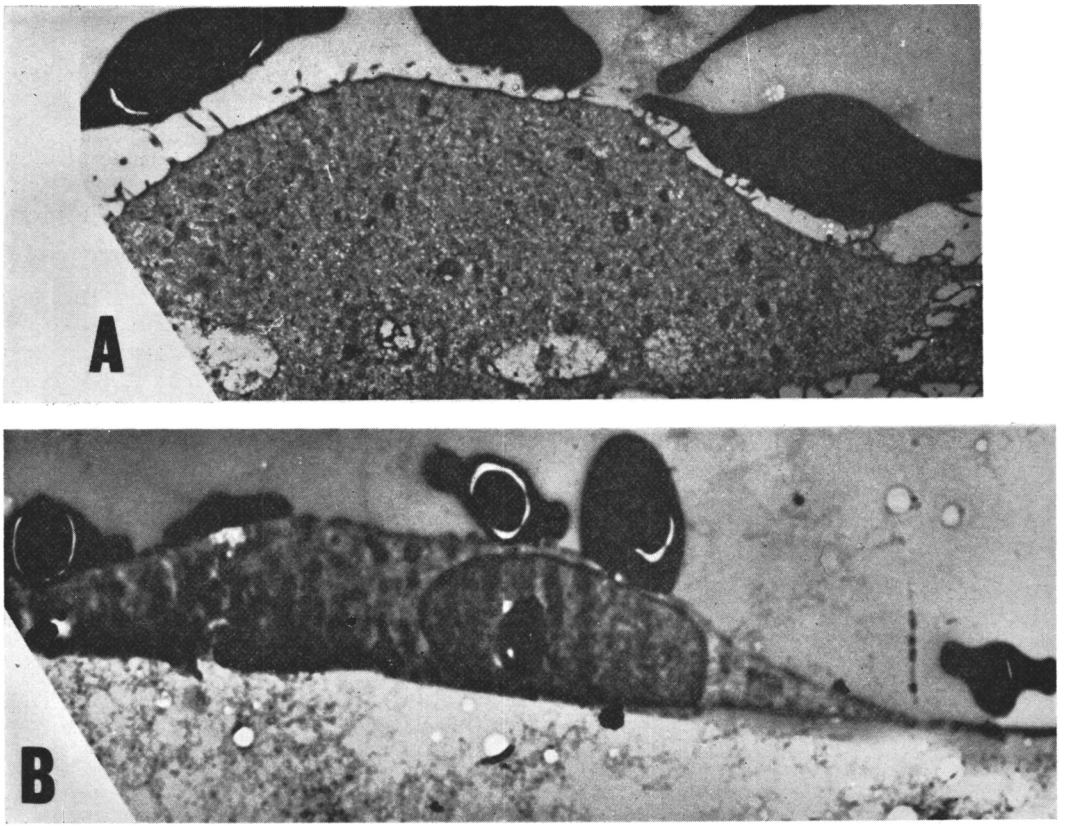

FIG. 2. Electron micrograph of absorbed chicken RBC's. 14,800×. A. Hemadsorption on MK-2 cell cultures showing attachment by microvillae. B. Hemadsorption on BSC-1 cell cultures showing direct "cyto-hemadsorption."

cific for calcium since barium and magnesium could not substitute for it.

The mode of action of periodate is obscure. The treated RBC's are changed so that calcium ions may be dispensed with; yet the cultured cells and only these responding to the calcium-dependent hemadsorption exhibit specific hemadsorption of the altered RBC's. Presumably, explanation for the anomalous hemadsorption characteristic in particular of monkey kidney cells may be found in special surface properties of these reactive cells. Inhibition of hemadsorption by treatment of cultured cells with proteolytic enzymes indicates involvement of protein in the cell membrane. Hotchin *et al* observed cyto-hemadsorption or direct and close binding of RBC's to cells in early influenza virus infection. At the stage of virus release, RBC attachment was indirect through filaments. In the present studies, the cultures were not known to be infected with virus although latent infection was posisble. However, the hemadsorption described here, unlike that induced by animal viruses, was dependent upon calcium ion or metaperiodate treatment. Furthermore, primary monkey kidney cell cultures from freshly isolated tissues consistently exhibited the anomalous hemadsorption. Apparently the anomalous hemadsorption was independent of viral infection. The cell strain determined the mode of attachment. BSC-1 cells, which displayed relatively few cytoplasmic projections, adsorbed RBC's by extensive contact. MK-2 cells adsorbed RBC's through multiple microvillae which characterized the cell surface.

Observation presented in the present work suggests the importance of thorough washing of cultures and omission of calcium from RBC suspension in viral hemadsorption studies.

Summary. Chicken RBC's in the presence of calcium ion or after treatment with metaperiodate were absorbed by cultured primary and stable line monkey kidney cells and to a lesser extent by rabbit kidney cells. An extensive variety of other primary or stable cell lines of human or animal origin did not adsorb the cells. Treatment of responsive cells with crystallized trypsin or chymotrypsin inhibited the hemadsorption. Apparently the anomalous hemadsorption was unrelated to viral infection. The morphology of attachment varied with cell strain. BSC-1 cells which displayed few cytoplasmic projections adsorbed RBC's through extensive contact in contrast to adsorption by MK-2 cells by microvillae which characterized the surface of these cells.

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Cytopathogenic Effects in a Cell Culture Infected with a Murine Leukemia Virus.* (30132)

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Quantitative studies of murine leukemia viruses have been hampered by lack of an established cell line in which they may be propagated. The lack of such a culture has likewise hindered the development of an *in vitro* assay based upon cytopathogenic effects (CPE) which might be elicited by these viruses. It is noteworthy, therefore, that Wright and Lasfargues have recently developed a mouse spleen and thymus tissue culture that supports the propagation of several murine leukemia viruses(1). Such infected cultures grown under the conditions described by Wright and Lasfargues (personal communication) have failed, however, to exhibit any obvious CPE. The present report describes conditions in which CPE have been observed in such a culture infected with the Rauscher murine leukemia virus.

Materials and methods. The modified NCTC 109 medium consisted of 2.0 g yeastolate, 0.5 g neopeptone, 6.8 g powdered NCTC 109 medium, 0.1 g each of penicillin and streptomycin, 0.5 g $NaHCO_3$, 250 ml inactivated human serum, 50 ml inactivated calf serum, and 700 ml triple-distilled H_2O. The medium was adjusted to pH 7.0 with NaOH, if necessary, and Seitz filtered. The 4XBME medium consisted of Eagle's basal medium(2) containing 4-fold concentrations of amino acids and vitamins, 0.1 g/l of peni-

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