A Simple Tissue Culture Maintenance Medium Utilizing Egg White. (25887)

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In a search for an efficient maintenance medium that would be simple to prepare and composed of readily available ingredients, it was decided to explore the practicability of a preparation containing albumin from avian egg. Antibacterial activity of egg white has been recognized for half a century, though the precise mechanism is not understood. It is also known(1,2) that antibodies may be transmitted from the female bird to the young through the egg, though their location in the latter is fortunately in the yolk(3). It remained to determine whether egg albumin could satisfactorily sustain developed tissue cultures in a healthy state for appreciable periods and whether it would exert any antiviral action against the enteroviral group of agents.

Material and methods. Egg white was separated from yolk of 12 fresh domestic hen's eggs under aseptic conditions and placed in sterile 250-ml centrifuge bottle containing a few glass beads. It was cooled and agitated in mechanical shaker for 10 minutes at 4°C, then frozen and thawed twice, inactivated at 56° for 30 minutes and finally centrifuged at 2000 rpm for 20 minutes. Three distinct layers were evident following centrifugation, *viz.*, a foamy upper, clear middle and dense, opaque lower layer. The clear middle layer, comprising about 80% of volume, was removed and used without further treatment; hereinafter it will be referred to as EA or albumin, although it is realized that egg white contains other chemically identifiable entities as well. The maintenance value of albumin was tested on cultures developed from freshly trypsinized monkey kidney and rabbit kidney, and on grown out cultures of 2 established cell lines, *viz.*, human amnion (FL) and our own human kidney line (HK/55). Egg albumin-saline (EAS) mixtures, containing 5%, 10% and 20% of albumin, were prepared and used as maintenance media, replacing the M150(4) and 10% calf serum (CS) after cell sheets had developed. Two variations were introduced as follows: (1) in half of primary tissue cultures and in half of established line subcultures, 10% EAS was substituted for usual growth medium (M150 plus 10% CS serum) when cultures were prepared, and (2) in half of all cultures the medium was renewed every 3 to 5 days; in the other half it remained unchanged. All cultures were observed for 2 to 3 weeks. Examination of our EA preparation for presence of antienteroviral factors was carried out in 2 ways. First, 38 cytopathogenic members of that group of agents, including the 3 poliovirus types, Coxsackie A-9 and B types 1-6 and Echo types 1-28 were exposed to 20% EAS for 90 minutes at room temperature (RT), with final virus concentration at approximately 100 $TCID_{50}$. Monkey kidney cultures were inoculated with virus mixtures, and 20% EAS was used as maintenance medium. This experiment was repeated with 4 separately prepared lots of albumin. Parallel virus control mixtures were also included, using M150 with and without 4% CS instead of 20% EAS. Secondly, monkey kidney cultures were inoculated with 3 enteroviruses, type I poliovirus, type B-1 Coxsackie and type I Echo virus, each diluted to 10^{-4}. When degeneration was complete, subcultures were made, diluting the fluid to 10^{-4}. In all, 3 successive subcultures were thus made, with EAS serving as maintenance medium in the original culture and 3 subcultures. Finally, the fluid from last serial culture was titrated for each virus in parallel using M150 (control) and 20% EAS as diluents. To test the practical value of EAS during viral isolation 25 known virus-bearing faecal or other clinical specimens were reexamined. Specimens represented the poliovirus (3 types), Coxsackie B (2 types) and Echo (one type) subgroups of viruses. Parallel monkey kidney cultures were inoculated, utilizing in one case

20% EAS and in the other (control) M150 as maintenance media. This experiment also provided additional evidence concerning possible presence of antiviral substances in albumin. Furthermore, we noted antibacterial activity of albumin. In 2 specimens bacterial contaminants that had apparently escaped preliminary treatment appeared in control cultures containing M150, obviously resistant to antibiotics (penicillin 100 i.u./ml and streptomycin 100 μg/ml) we normally employ in that medium. The 2 organisms were identified as *Alkaligenes fecalis* and *Aerobacter aerogenes*. Heavy broth cultures of each were prepared and each culture was titrated out to 10^{-6} in decimal dilutions in M150 (control) and M150 containing 5% EA. Adaptation of EA to use in plaque formation was attempted as follows: 89 ml of 1.7% NaCl solution (autoclaved and cooled), 10 ml EA and 1 ml of 0.2% neutral red solution (autoclaved and cooled) were mixed and pH reduced to 8 with *N* HCl. To aliquots of this mixture (at 40°C) were added equal quantities of 3% agar that had been autoclaved and cooled to 40°C. This was then poured over monkey kidney cultures already inoculated with the 3 types of poliovirus. The cultures were incubated at 35°C.

Results. The value of EAS as a maintenance medium proved quite satisfactory. With it the designated tissue cells survived in good condition for 7-10 days; renewal of fluid every 3-5 days insured survival for 2 weeks or longer. Use of EAS during isolation attempts also proved advantageous. Cytopathic changes appeared earlier in 13 instances with EAS than in M150 controls, on the same day in 10 cases, but never later with EAS.

During various experimental uses, 5% EAS was as effective as 20% EAS. In addition to serving as a maintenance medium, EAS supported growth of trypsinized second passage monkey and rabbit kidney cells, but not that of primary cultures of those tissues.

Although the 2 bacterial contaminants were resistant to penicillin and streptomycin, they did not appear in tissue cultures covered with M150 containing 5% albumin. The latter, while completely inhibiting *Alkaligenes fecalis,* was unable to suppress *Aerobacter aerogenes* in the lower 3 dilutions of the decimal titration series; it did so in the subsequent dilutions, however. Antibiotics in M150 controls were unable to inhibit either bacterial agent in any dilution.

Exposure of 38 enteroviruses to 20% albumin for 90 minutes at RT did not appear to have any more inhibiting effect than did the M150 without calf serum, as evidenced by time required to induce cytopathic effects. Antiviral activity of M150 with 4% CS, however, was observed in the case of each of 3 separate lots of CS represented. M150 with CS from Lot 1 inhibited for at least 7 days the production of cytopathic changes by Coxsackie B-5, Echo-1 and E-3; with CS from Lot 2 similar inhibition of E-12 and E-20 was noted, and with CS from Lot 3, cytopathogenic action by E-2 and E-23 was inhibited. Results in the titration experiment showed no significant difference in final titre between viruses that had been subcultured and titrated in 20% EAS and those subjected to M150 (controls).

Plaques formed by polioviruses using EAS-agar mixture were at least as large and as numerous as those observed when conventional and more elaborate salt-protein-agar mixtures were used.

EA appeared equally effective following lyophilization and reconstitution as it had been prior to this treatment.

Discussion. It is apparent from these results that albumin from domestic hen's egg, following simple preliminary treatment, can be utilized to serve several useful purposes in the application of current tissue culture technics to virology. When combined with physiological saline, not only is it effective as a maintenance medium during periods usually required either for viral isolation attempts or neutralization tests, but it also contains factors enabling second passage cells of monkey and rabbit kidney to establish themselves *in vitro* and grow. The albumin was unable to replace calf serum for outgrowth of primary cell cultures; unlike calf serum it was

not demonstrated to possess any antienteroviral substances.

Two beneficial characteristics of albumin were evident during its use in applied, or diagnostic, virology. First, the tendency toward earlier appearance of cytopathic changes observed when EAS was used, as compared with M150, may well be attributable to the relatively high pH (about 8.5) which characterizes EA and which the latter imparts to EAS. That acid reaction in tissue culture medium exerted an adverse effect on cytopathogenicity of certain viruses ("pH effect") and that this could be overcome by neutralization of the acid was pointed out by Barron and Karzon (5). Secondly, the antibacterial factor in EA is a particular advantage since bacterial contaminants occasionally escape preliminary centrifugation and antibiotics to which fecal specimens are subjected. It was obvious that the EAS medium was able at least to suppress multiplication of 2 bacterial agents in isolation cultures, as described, although 2 commonly used antibiotics were unable to do so. The active agent in albumin may be lysozyme (6).

Ability of EAS to serve as substitute for calf serum-balanced salt solution in agar overlay used for plaque formation was advantageous; so also was survival of its attributes following lyophilization and reconstitution.

Summary. Albumin from domestic hen's egg can be simply adapted for use as tissue culture medium for certain purposes. Combined with physiological saline it supports outgrowth of secondary cultures of monkey and rabbit kidney cells; it also sustains grown out cultures of fresh tissue and established cell lines for 2 weeks. Albumin possesses antibacterial properties, but no demonstrable antienteroviral activity. It can be lyophilized and reconstituted without loss of attributes.

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Clearance of Blood Coagulation Product I in Rabbits.* (25888)

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The hypothesis has been proposed that blood fluidity is preserved *in vivo* largely by cellular clearance of clotting intermediates (1). Data are reported which suggest that blood thromboplastin is removed from circulation by reticulo-endothelial cells(2). In the present study evidence is presented indicating that coagulation intermediate product I, a precursor of blood thromboplastin(3), disappears from blood of intact rabbits more rapidly than could be explained by action of circulating anticoagulants.

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Materials and methods. Experimental animals were male New Zealand rabbits weighing about 3 kg. Blood for preparation of product I was collected into uncoated glass tubes from central artery of ear. Plasma was obtained from blood collected into 1/10th volume of 3.8% sodium citrate, and aliquots were adsorbed with $Al(OH)_3$ as described by Pool and Robinson(4). Serum was prepared from blood incubated 1 hour after clotting at 37°C. Crude "cephalin" was made from human brain by method of Bell and Alton(5). Product I was prepared by mixing equal volumes of 0.025 M $CaCl_2$, undiluted serum, and