Neither the diethyl ethylidinemalonate (No. 2) nor the diethyl methylenemalonate (No. 4) is active; the unsaturated aceto acetic ester (No. 14) and itaconic ester (No. 15) are also inactive. The activity of the tetramethyl malonamide (No. 24) may be related to its greater lipid solubility. The ethoxymethylene grouping (No. 22) may impart polarity for a favorable partition coefficient.

The mode of action of these malonates might be explained by their interference with either of 2 biological pathways. Diethyl ethoxymethylenemalonate may be an inhibitor of succinic acid dehydrogenase, and consequently interfere with the tumor energy supply. Busch and Potter(6) found that succinate accumulated in tumor tissues of animals treated with sodium malonate. Another possible explanation for the activity may be considered relative to nucleic acid synthesis. It has been postulated, tentatively, that urea and oxaloacetic acid are involved in the synthesis of pyrimidines *via* orotic acid. It is conceivable that diethyl ethoxymethylenemalonate could either (a) block the condensation of urea and oxaloacetate, thus blocking orotic acid formation, or (b) condense with urea to give rise to an isomer of orotic acid, namely uracil-5-carboxylic acid. Either pathway for the utilization of diethyl ethoxymethylenemalonate might interfere with the build up of nucleic acids necessary for tumor growth.

Summary. Diethyl ethoxymethylenemalonate inhibited the growth of transplanted adenocarcinoma 755 in C57 black mice. The formyl and β-pyridylene malonic ethyl esters as well as two malonamides gave moderate inhibitory values. These compounds were inactive when tested against other transplanted tumors. Some possible modes of action are discussed.

1. Boyland, E., *Biochem. J.*, 1940, v34, 1196.
2. Gal, E. M., and Greenberg, D. M., *J. Am. Chem. Soc.*, 1951, v73, 502.
3. Gal, E. M., Fung-Haan Fung, and Greenberg, D. M., *Cancer Res.*, 1952, v12, 565.
4. Stock, C. C., Editor *Cancer Res.* Supp. Nos. 1 and 2, 1953, and 1955.
5. Petrakis, N. L., Bierman, H. R., and Shimkin, M. B., *Cancer Res.*, 1952, v12, 573.
6. Busch, H., and Potter, V. R., *ibid.*, 1952, v12, 660.

Received May 17, 1956. P.S.E.B.M., 1956, v92.

Preparation of Human Amnion Tissue Cultures.* (22535)

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Successful growth of human amnion in monolayer tissue culture was first reported by Zitcer *et al.*(1). A subsequent report from the same laboratory verified the usefulness of this tissue for culture of poliomyelitis virus (2). Because of the ready availability of placental tissue, attempts to culture human amnion were undertaken in this laboratory. Placentas from both vaginal and Caesarean section deliveries were used. These preliminary attempts were unsuccessful, however, and modifications of the method were undertaken, including a number suggested by Hok (3). The procedure here described evolved from these studies, and has proven to be reliable, simple, and economical. It requires little equipment that is not readily available in the average hospital laboratory. The tissue cultures are of uniformly good quality, and require a minimum of attention.

Method. 1) Human placentas from both Caesarean and vaginal deliveries have been collected by the obstetrical service of the King County Hospital System. Good growth

* This study was supported in part by grant from Office of Naval Research.

is obtained from either source; however, only placentas from uncomplicated vaginal deliveries are routinely used. The perineum is prepared with antiseptic soap, followed by a voluminous saline rinse. The placenta is delivered directly from the vagina into a dry, sterile basin, avoiding contact with the perineum, rubber gloves and antiseptic solutions. After examination, the placenta is placed in a dry, covered sterile beaker. Placentas collected from deliveries occurring after midnite are stored at room temperature until brought to the laboratory in the morning. 2) The placenta is placed in a sterile pan, and the amnion is separated from the chorion by blunt dissection with forceps. The amnionic tissue is placed intact in a large petri dish with 50 ml of Hank's balanced salt solution containing 100 units penicillin and 100 μg streptomycin per ml, and adhering blood vessels, mucus, and other debris are removed with forceps. Occasionally it is necessary to scrape the amnion with a spatula to remove accumulated debris. The relatively clean amnion is then put in 3 or 4 consecutive washes of Hank's balanced salt solution for 5-10 minutes each, with occasional gentle stirring. Manipulation and squeezing of the tissue is kept at a minimum. 3) The specimen is placed in a 250 ml Ehrlenmeyer flask containing approximately 100 ml of .25% trypsin (Difco 1:250) in phosphate buffer solution(4). The preparation is left at room temperature for approximately 1 hour, during which the residual debris becomes suspended in the solution, which is then discarded. An equal amount of fresh trypsin is added, and the preparation allowed to remain at room temperature for an additional 4½ to 6 hours without agitation. The trypsin solution remains relatively clear until the digestion process nears completion. With release of cells from the amnion, the solution becomes cloudy, and the surface of the amnion appears soft and fuzzy. A quick shake of the flask aids in releasing cells from the tissue. The process is arbitrarily terminated when the fluid appears almost opaque. Subsequent trypsinization has yielded few additional cells. 4) The cell suspension is decanted through 1 layer of sterile gauze into graduated centrifuge tubes and spun for 10 minutes at 1000 rpm. The supernatant trypsin solution is discarded and the cell pack suspended in 15-20 ml of medium 199. The tubes are spun again at 800 rpm for 5 minutes and the supernatant discarded. Approximately .5 to 1.5 cc of packed cells have been obtained from the average amnion treated in this manner. 5) The cells are suspended in sufficient culture medium to make a 1:50 dilution. The medium employed has been 20% inactivated, filtered beef serum in medium 199† containing 200 units of penicillin, 200 μg of streptomycin, and 25 units of nystatin per ml. Cell counts average 5 million cells per ml in the 1:50 dilution. 6) Approximately 50 million cells, ordinarily about 10 ml of the above suspension, are further diluted with culture medium to 30-40 ml and pipetted into 1 liter Roux bottles, then incubated at 37°C. Within 48 hours, the living cells attach themselves to the glass wall, and the medium is replaced with an equal volume of fresh medium to eliminate floating debris. Subsequently, the medium is changed every 5 to 7 days, depending primarily on pH changes. In 7-14 days, a solid sheet of large, clear cells completely covers the glass wall, and the cells are ready for harvesting. 7) The medium is decanted, and 4 ml of 2% trypsin in Hank's solution, 16 ml of medium 199, and 4 ml of 2.8% $NaHCO_3$ are placed in the bottle. After incubation for 30-60 minutes, the cell sheet can be seen floating in the solution and the suspension is pipetted in graduated 40 ml centrifuge tubes and spun at 800 rpm for 2 min. The supernatant is decanted and 5 ml of medium are added to the cell pack, which usually measures .2 to .4 ml. The cells are dispersed with a pipette and more culture medium is added to make a 1:75-1:100 suspension of the cells. One ml of this suspension is inoculated into screw top tubes which are incubated on slant trays. For further propagation in large bottles, 20 ml of the suspension are used per bottle. These

† Medium 199 was generously provided by Dr. Ralph Houlihan, Cutter Laboratories, Berkeley.

secondary cultures are frequently of a better quality than the primary cultures, and grow considerably faster. In the tubes, there is often a solid sheet of cells within 72 hours, and the cultures are ready for virus inoculation or other studies. In bottles, growth may be adequate for further harvesting in 4-7 days. 8) Cultures can also be started directly in tubes, and for this purpose 200,000-300,000 cells in 1 ml of medium are inoculated into screw top tubes and incubated on slant trays. Culture medium is changed as described in section 6, but the 48-hr. change is not necessary. Growth is slightly faster in the tubes, being adequate in 7-10 days for further studies, but the quality of the cultures is usually better when the cells are first grown in bottles and then transplanted to tubes. 9) Tubes with satisfactory cell sheets, not needed for immediate studies, can be stored in an incubator at 28°C. The medium is changed every 15-20 days, and the cultures can be maintained without apparent damage for periods exceeding 6 weeks. 10) For virus studies, the culture medium is discarded, the cell sheet is washed with Hank's balanced salt solution, and a serum-free synthetic medium consisting of medium 199 with antibiotics is introduced. Control cultures can be maintained in this medium for 15 or more days before significant degeneration occurs.

Results. Good growth has been obtained with approximately 80% of the amnions treated in the above fashion. Attempts to perpetuate a strain of cells from a single amnion have not been successful. Good growth of cells was obtained through 4 subcultures on one occasion, but subsequent attempts to reproduce these results have failed.

Zitcer(1) described a mixed population of large and small cells in amnion tissue cultures, but in our material this has not been observed. The cells are all large, clear, quite uniform in shape and appearance with little or no granularity, and have a very obscure, faintly visible nucleus. Cytopathogenic changes result from infection with virus. The changes attributable to poliovirus consist primarily of the rounding up of cells, increased granularity, and darkening and prominence of the nucleus. Ragged holes of various sizes appear in the sheet of cells. These changes are similar to those observed in monkey kidney cells, except that relatively few of the amnion cells fall away from the glass. Infection with various strains of APC virus results in characteristic cytopathogenic changes in which the prominent feature is the development of long, hyalinized, spindle shaped cells. In addition, there is usually rounding up of cells in the periphery of the sheets, but few cells fall away from the glass. Cytopathogenic changes resulting from infection with other viruses studied resemble the changes seen with poliovirus, but usually progress at a slower rate.

The susceptibility of amnion cells to various viruses has been studied (Table I). All strains of poliovirus tested produced infec-

TABLE I. Susceptibility of Human Amnion Tissue Cultures to Virus Infections as Reflected by Cytopathogenic Changes, Recorded on 5th or 6th Day.

Poliovirus	Type I		4+
	II		4+
	III		4+
*Theiler's mouse poliovirus		(TO-6)	0
†APC virus, Huebner-Hilleman			
	Type I	(Adenoid 71)	4+
	II	(Adenoid 6)	4+
	III	("GB")	4+
	IV	(RI 67)	4+
	V	(Adenoid 75)	4+
	VI	(Tonsil 99)	4+
	VII	(Gomen)	4+
	VIII	(Trimborn)	3+
	Simian I	(Bertha)	4+
	II	(Hull, WV)	4+
*Herpes simplex			4+
*Newcastle disease			0
*Western equine encephalitis		(8J-14)	2+
*Encephalomyocarditis virus		(MM)	0
‡Influenza	A	(GLNTC-1134)	0
*	A	(PR-8)	0
‡	B	(GLNTC-1760)	0
Coxsackie virus, Type		B-2	0
		B-3	4+
		B-4	0
		A-9	4+

0 = no change, 4+ = degeneration of almost all cells.

* Kindly supplied by Dr. Charles A. Evans, University of Washington School of Medicine, Seattle.

† Kindly supplied by Dr. Edwin H. Lennette, State of California, Department of Public Health, Berkeley.

‡ Kindly supplied by Naval Medical Research Unit No. 4, Great Lakes.

tion and cytopathogenic changes. Sensitivity equaled that of monkey kidney, both with stock cultures of virus and stool specimens from patients. Stool "toxicity" was no more troublesome than in monkey kidney. A marked difference between monkey kidney and amnion occurred in the Coxsackie B series. the susceptibility of amnion resembling that seen with HeLa cell cultures(5,6). Only type B-3 produced cytopathogenic effects in amnion, while 2, 3, and 4 did so in monkey kidney. Types B-1 and B-5, which are known to cause degeneration in monkey kidney(7) were not tested. Of the Coxsackie A group, only type A-9 has been studied, and this produced cytopathogenic changes both in monkey kidney and in human amnion. Strains of Influenza A and B, which caused cellular changes in monkey kidney, failed to produce similar changes in amnion. Hemagglutination studies of the supernatant were not performed. Herpes simplex and Western Equine Encephalitis viruses also produced cellular degeneration in both types of tissue. No "wild" viruses have appeared in amnion cultures.

Summary. 1) A simple, reliable, and reproducible method has been developed to grow monolayer cultures of human amnion. 2) Placentas from routine vaginal deliveries are used without modifying patient preparation or care. 3) Unminced, washed amnion is trypsinized at room temperature, and the resulting cell suspension is inoculated into bottles or tubes. The best results are obtained by first growing the cells in large bottles and transferring them to tubes. 4) Susceptibility of human amnion tissue cultures to a variety of viruses has been studied, and the results are reported.

1. Zitcer, E. M., Fogh, J., and Dunnebacke, T. H., *Science,* 1955, v122, 30.

2. Fogh, J., and Lund, R. O., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 80.

3. Hok, K. A., 1955, personal communication.

4. Dulbecco, R., and Vogt, M., *J. Exp. Med.,* 1954, v99, 183.

5. Crowell, R. L., and Syverton, J. T., *J. Immun.,* 1955, v74, 169.

6. Sickles, G. M., Mutterer, M., Feorino, P., and Plager, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 529.

7. Sickles, G. M., Feorino, P., and Plager, H., *ibid.,* 1955, v88, 22.

Received May 31, 1956. P.S.E.B.M., 1956, v92.

Plasma Amino Nitrogen and Creatine Values of Growing Chick and Laying Hen.* (22536)

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A search of the literature reveals few reliable standard values for amino nitrogen and creatine in chicken blood. Sturkie(1) does not present any amino nitrogen values in his summary of the chemical constituents of chicken blood, nor does Albritton(2) in his monumental reference on standard blood values. Edwards and Wilson(3) reported a higher blood amino nitrogen level in the non-fasted laying hen under conditions of hyperthermy. Hsu and Combs(4) noted that a vit. B_{12} deficiency of 4-week-old chicks resulted in significantly higher blood nitrogen compounds, including amino nitrogen. There is considerably more information in the literature on creatine(1,2) than on amino nitrogen values for chicken blood, but these determinations were made with alkaline picrate and without due correction for non-creatinine compounds that give a positive Jaffe reaction.

Methods. In this laboratory plasma cre-

* Paper of Journal Series, N. J. Agric. Exp. Station, Rutgers University, State University of N. J., Department of Poultry Husbandry, New Brunswick. Supported in part by a grant in aid from the Cooperative Grange League Federation Exchange, Ithaca, N. Y.