# TISSUE CULTURE MEDIA

## The Essential Non-Dialyzable Factors in Human Placental Cord Serum*

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Plates 4 to 6

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### INTRODUCTION

In studying the composition of tissue culture media one procedure is to separate complex media into dialyzable and non-dialyzable components. The investigation here reported was undertaken to determine what portions of the non-dialyzable fraction of human placental cord serum are needed to obtain active growth of fibroblasts in tissue culture.

At present, two methods of approach are being used to determine the metabolites necessary for growth in tissue culture. White (1, 2) and Morgan, Morton, and Parker (3) have eliminated all proteins from the medium by making up fully defined media containing the metabolites known to be essential for animals. They have been able to maintain cells in tissue culture for long periods but their cultures have shown little growth when compared with cultures fed the usual media containing serum and embryo extract. Fischer (4, 5) and his group, on the other hand, have fractionated plasma, serum, and embryo extract into dialyzable and non-dialyzable components. Little growth is obtained from either the dialyzable or the non-dialyzable constituents alone but growth is normal when they are combined. Fischer has been able to replace the dialyzable constituents by a relatively simple mixture of salts, amino acids, glutathione, glucose, and fructose diphosphate.

Although embryo extract has often been considered as the source of the growth-promoting activity of complex media, there are observations to indicate that embryo extract stimulates cell migration more than mass increase. Growth-promoting fractions have been obtained from adult tissue extracts and serum as well as embryonic tissues. Baker and Carrel (6) and Carrel and Ebeling (7) isolated a euglobulin fraction from chicken serum which had growth-promoting activity. Hoffman, Dingwall, and Andrus (8) obtained an active protein fraction from an extract of sheep heart and Davidson and Waymouth (9) obtained active protein fractions from brain, heart, and skeletal muscle of sheep embryos. Laser (10) found that tissue cultures fed only

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embryo extract showed a greater increase in area than cultures fed only serum; however, the increase in weight was greater in the cultures that were fed only serum.

### *Methods and Materials*

For this work we have used a strain of rat fibroblasts which was started in January, 1947, by Dr. H. Thompson in the Rockefeller Institute. It was derived from the pectoralis muscle of a 2 day old Wistar rat.

All cultures were set up in the culture flasks described by Porter, Claude, and Fullam (11). Each flask contained four implants in a clot of 7 drops chicken plasma and 7 drops 20 per cent chick embryo extract. We have as routine washed all cultures with four changes of 1 ml. of X-6.[1] One of these washes was an overnight wash. After the washes, the plasma clot was clear and colorless. The cultures were then fed and observed over one generation time (15 to 18 days). The basal medium, against which all our preparations were compared, consisted of 0.4 ml. X-6, 0.1 ml. human placental cord serum, and 0.1 ml. chick embryo extract. All cultures were fed twice a week.

The cultures were kept in a rotor run at 10 R.P.H. at 35–37°C.

The embryo extract is prepared by grinding 10 day old chick embryos in a glass homogenizer. The homogenate is diluted with four times its volume of X-6 and incubated for 4 to 6 hours at 37°C. The mixture is then centrifuged, the supernate quickly frozen in a mixture of dry ice and ethyl alcohol, and stored in the deep freeze. When thawed, a considerable amount of material precipitates. The embryo extract is centrifuged before use and only the supernate is taken.

All dialysis procedures were carried out in a cold room at 4°C. After dialysis, preparations were sterilized by filtration through Corning UF sintered glass filters.

Early in our work, we found that many of the globulin preparations from serum gave extensive clot lysis due to activation of plasminogen in these fractions by tissue kinase (12). Heating the fractions to 60°C. for 30 minutes inactivated the plasminogen without interfering with growth-promoting activity.

### RESULTS

(*a*) *Growth in Serum and Embryo Extract.*—We first compared cultures fed embryo extract or cord serum alone (in the same concentrations as in the complete medium) with cultures fed the complete medium. As shown in Figs. 1, 2, 3, and 7, the cultures fed the embryo extract alone had a larger area but the outgrowth was very thin, whereas those fed only cord serum were as dense as the controls. An estimate of the volume increase in 2 weeks indicated that the embryo extract cultures increased no more than two times in volume whereas the serum cultures increased their volume by a factor of 5 to 6 and those fed the complete medium increased by 6 to 8 times. Evidently the serum is more active in increasing the density of the cultures and the embryo extract is more active in stimulating migration of cells. The difference in density be-

[1] 8 gm. NaCl, 0.20 gm. KCl, 0.147 gm. $CaCl_2 \cdot 2H_2O$, 0.203 gm. $MgCl_2 \cdot 6H_2O$, 0.213 gm. $Na_2HPO_4$, 1.01 gm. $NaHCO_3$, and 1.00 gm. glucose per liter.

tween cultures fed only embryo extract and those fed embryo extract plus serum is easily made out by gross examination as shown in Fig. 7.

The growth-promoting effect of serum was visible in the gross when the medium contained only 0.025 ml. serum and was still present microscopically when the medium contained 0.0125 ml. of serum.

The increase in density of cultures thus provided us with a simple qualitative effect to determine the presence or absence of the serum growth-promoting factor(s), provided the responsible factor was present in a concentration greater than one-fourth the concentration in placental cord serum. In the experiments on the fractionation of serum, all fractions were tested for the serum growth-promoting effect in the presence of embryo extract. All cultures received 0.1 ml. embryo extract; those fed the various serum fractions plus embryo extract were compared with cultures fed cord serum plus embryo extract and some fed only embryo extract. The total volume of medium was always made to 0.6 ml. with X-6.

(*b*) *Dialysis of Cord Serum.*—Cord serum was dialyzed against an equal volume of X-6 for 24 hours to obtain the dialyzable constituents. It was then dialyzed against cold running tap water for 24 hours followed by three changes of X-6 over a period of 24 hours. The cultures fed the non-dialyzable fraction plus embryo extract were as dense as those fed the complete medium. The cultures fed the dialyzable fraction plus embryo extract were no better than those fed embryo extract alone. These results are shown in Figs 7 to 9. Thus the growth-promoting activity of the serum is associated with the non-dialyzable fraction.

(*c*) *Fractionation of Cord Serum.*—Cord serum was fractionated into the albumin and globulin fractions by precipitation of the globulins with half-saturated $(NH_4)_2SO_4$. The globulin fraction was dissolved in water, reprecipitated, and washed with half-saturated $(NH_4)_2SO_4$ three times and finally made up to the same volume as that of the serum with distilled water. Both fractions were dialyzed against cold running tap water for 17 hours (some of the globulins precipitated during this procedure) and then against two changes of isotonic saline (the precipitated globulins went back into solution) followed by two changes of X-6.

The globulin fraction had all the growth-promoting activity of the serum. However, the cultures fed the globulin fraction showed peripheral toxic changes after a few days (Fig. 4) whereas those fed the albumin fraction did not (Fig. 5). Furthermore the cells in the cultures given the albumin fraction plus embryo extract were in better condition, being less granular, than those given only embryo extract. Finally, in cultures fed the recombined globulin and albumin fractions (Fig. 6), the growth-promoting activity was present and no toxic changes appeared. Thus all the growth-promoting activity is in the globulin

fraction, but there is also a factor in the albumin fraction which is required to prevent the above mentioned toxicity.

(*d*) *Albumin.*—The above experiments were repeated in all details except that the albumin fraction was replaced by 0.05 to 0.1 ml. of a 5 per cent solution of crystalline bovine serum albumin (Armour) that had been dialyzed against three changes of X-6. The results were the same as those reported above. Thus the toxicity-preventing factor is the serum albumin.

This action of albumin is suggestive of similar findings with some bacteria. The growth of *M. tuberculosis* (13–15), *H. pertussis*, (16), and *L. bulgaricus* (17) is inhibited by traces of free oleic acid. Serum albumin prevents the growth inhibition by binding the oleic acid.

Experiments were therefore set up to test the toxicity of fatty acids and the effect of serum albumin on the toxicity of oleic acid. After the cultures were washed, the fatty acids were introduced by putting 0.01 ml. of a solution of the fatty acid in absolute alcohol on the side of the culture flask opposite the implants. The alcohol was immediately evaporated out of the flask by passing a stream of filtered air over it. The cultures were then fed 0.1 ml. chick embryo extract, 0.1 ml. of the globulin fraction of human placental cord serum, and 0.4 ml. of X-6. When albumin was added to the medium, it replaced some of the X-6. The albumin used was gotten by dilution of one stock solution of albumin. The stock solution was obtained by making a 6 per cent solution of crystalline bovine serum albumin in physiological saline. This was dialyzed against four changes of X-6 over a 24 hour period and then filtered. There was a slight increase in volume of the solution during dialysis and there is always a small loss of albumin in the filters. We estimate that our final solution had 5 to 6 per cent albumin. In the tables and the calculations in the text, it was assumed that the stock solution was a 5 per cent solution. This uncertainty is of little importance since our toxicity gradings are crude and calculations from them can only be considered "order of magnitude" calculations.

The cultures were examined 1 and 2 days after feeding and the toxic damage was graded from 0 to 4+ in terms of cell granularity.

Table I shows the toxic effect of stearic, oleic, and linoleic acids when no albumin is added to the medium.

The controls showed a toxicity of 1+; the controls to which 0.5 mg. of albumin had been added showed no toxicity. Stearic acid was considerably less toxic than oleic or linoleic acid, the damage in the two lowest concentrations being no more than in the controls.

Table II and Figs. 10 to 15 show the effect of albumin on the toxicity of oleic acid.

As the albumin is increased at a constant amount of oleic acid, the toxicity decreases. Within the error of the grading technique, there is an approximately linear relationship between the amount of albumin required to reduce the

toxicity to any predetermined level and the amount of oleic acid present. From the data in Table II, we calculate that 1 mole of albumin prevents the toxicity of 2 to 3 moles of oleic acid.

Extraction of fatty acids and titration with sodium ethylate indicated that in the albumin-free medium (0.1 ml. cord serum globulins, 0.1 ml. embryo extract, and 0.4 ml. X-6), there were about 30 gamma (0.106 micromole) of free fatty acid (expressed as oleic acid) and about 45 gamma of total fatty

TABLE I

*Toxicity of Fatty Acids*

| Micromoles per flask | Stearic | Oleic | Linoleic |
|---|---|---|---|
| 0.0197 | 1+ | 2+ | 2–3+ |
| 0.0395 | 1+ | 2–3+ | 3+ |
| 0.079 | 1–2+ | 3+ | 3–4+ |
| 0.158 | 1–2+ | 3–4+ | 4+ |

TABLE II

*The Prevention of the Toxic Effect of Oleic Acid by Albumin*

| Oleic acid | Albumin | | | | | | |
|---|---|---|---|---|---|---|---|
| Micromoles per flask | 10 mg. | 5 mg. | 2.5 mg. | 1.25 mg. | 0.63 mg. | 0.31 mg. | 0 mg. |
| 0.158 | 0 | ±–1+ | 2–3+ | 4+ | | | |
| 0.079 | | 0 | ± | 1–2+ | 3–4+ | 4+ | |
| 0.0395 | | | 0 | ±–1+ | 2–3+ | 4+ | |
| 0 | | | 0 | 0 | 0 | 0–± | ±–1+ |

acids (soaps plus free fatty acids). After twenty hours' incubation, the values were 40 and 55 gamma respectively. The values after 48 hours' incubation were the same as after 24 hours' incubation. The increase after 24 hours' incubation is about the error of the titration so that we are not justified in asserting there is an increase on incubation, although an increase would be expected since there are lipases in the serum and embryo extract which could release free fatty acids from phospholipids and fats. If we assume that the same ratio of oleic acid to albumin holds for the reversal of the toxicity in the controls (0.5 mg. albumin needed to prevent all toxicity in the absence of added oleic acid) as was calculated from Table II, it turns out that the toxicity in the controls is equivalent to the toxicity of 4 to 6 gamma of oleic acid. This amount could be present in the medium from the titration data on total fatty acids. Furthermore, if we plot the data in Table I and extrapolate to zero toxicity, we find that the toxicity in the controls is equivalent to the toxicity of 5 to 8 gamma of oleic acid.

(*e*) *Globulin Fraction.*—Further fractionation of the globulins with 33 and 40 per cent saturated $(NH_4)_2SO_4$, did not give a clear-cut separation of the growth-promoting activity, slight activity being present in all fractions. Fractionation by dialysis against distilled water did give a sharp separation. The euglobulins which precipitated on dialysis against distilled water had some activity, the remaining globulins had no or slight activity. However, the euglobulins had considerably less activity than the original serum or the total globulin fraction. Recombination of the euglobulins and the water-soluble globulins gave no more or slightly more activity than the euglobulins alone. Thus the loss in activity was probably not due to separation of the growth-promoting activity into two factors, one in each of the two fractions. Part of this loss was thought to be due to denaturation of the active material in the sintered glass filters but most of it was probably due to denaturation during the dialysis and precipitation of the euglobulins. The precipitate from dialysis against distilled water was not entirely soluble in X-6. There was always an insoluble residue. The use of a solution of bovine serum albumin in X-6 as solvent increased the solubility of the euglobulin precipitate but there was still some insoluble residue; this increased the recovered activity only slightly. Up to the present, we have not been able to prevent this loss of a large part of the serum growth-promoting activity. Thus, although we have been able to trace the activity to the euglobulin fraction, our recoveries have been poor.

(*f*) *Extraction of Cord Serum Lipids.*—Dried cord serum was obtained from Difco (No. B356, control 399572). One sample of this was extracted with benzene, chloroform, and ether and the other was kept as a control. Both were reconstituted to the original volume of the serum with distilled water and were then dialyzed against cold running tap water overnight followed by four changes of X-6 over a 24 hour period. The reconstituted serum from Difco had somewhat less growth-promoting activity than fresh cord serum but the lipid extraction did not lead to any further decrease in activity.

(*g*) *Pure Proteins.*—Fraction II (bovine gamma globulin, Armour, Lot No. C904) had no growth-promoting activity at concentrations equivalent to the gamma globulin concentration of serum.

Anterior pituitary growth hormone (Armour) and insulin (Lilly)[2] had no growth-promoting activity singly or in combination.

The above compounds were tested only for their ability to replace the serum growth-promoting factor but not in the presence of the growth factor to see whether they could further stimulate growth.

(*h*) *Relation of Growth Factor to Endocrine Functions.*—Sera from 30 to 35 day old Wistar rats have almost as much growth-promoting activity as hu-

[2] We wish to express our gratitude to Dr. Irby Bunding of Armour and Co. and Dr. W. R. Kirtley of Eli Lilly and Co. for the samples of growth hormone and insulin respectively.

man placental cord sera. To test the effect of endocrine deficiencies on the serum content of the growth factor, 30 to 35 day old rats (4 rats per group) were thyroid-parathyroidectomized, hypophysectomized, or adrenalectomized. The thyroid-parathyroidectomized rats were given 0.25 per cent $CaCl_2$ to drink for 4 days after operation, the hypophysectomized rats were given 5 per cent sucrose in their drinking water, and the adrenalectomized animals were given 0.85 per cent NaCl in their drinking water. Thirteen days after the operations, all animals were bled by heart puncture and autopsied to make sure of the complete removal of the endocrines. Serial sections of the regions involved were also made. All the adrenalectomies were considered complete. Only one of the hypophysectomized rats had the proper weight loss; at autopsy also, this was the only animal that was considered to have a complete hypophysectomy. Serial sections indicated that only one of the thyroid-parathyroidectomies was complete. The other animals had small thyroid rests further up in the laryngeal region. The sera from the rats that were considered to have had complete removal of the respective endocrines and sera from four controls were tested for growth-promoting activity. All showed the same activity.

## DISCUSSION

To date, we have concentrated on a study of the non-dialyzable factors in human placental cord serum which are necessary for active growth of fibroblasts in tissue culture. So far, it is evident that there is at least one growth promoting factor in the euglobulin fraction of cord serum. This factor would seem to correspond to the growth-promoting factor described by Baker and Carrel (6) and Carrel and Ebeling (7) in the euglobulin fraction of chicken serum and perhaps to the protein fraction isolated from sheep heart by Hoffman, Dingwall, and Andrus (8).

Besides the growth-promoting protein(s) of the globulin fraction, serum albumin is also needed in the tissue culture medium to prevent the appearance of a characteristic toxicity. We have been able to show that: (*a*) stearic, oleic, and linoleic acids are toxic to fibroblasts in tissue culture and that the unsaturated acids, oleic and linoleic, are more toxic than stearic acid, (*b*) this toxicity can be prevented by serum albumin, and, (*c*) there are sufficient free fatty acids in the tissue culture medium to account for the toxicity observed in the absence of albumin and added oleic acid. From these data, there is every reason to believe that albumin acts by binding the fatty acids, thus keeping the concentration of free fatty acids below the toxic level. The demonstration by Boyer *et al.* (18, 19) that serum albumin can bind considerable quantities of fatty acids lends support to this thesis.

These results are similar to those found in the cultivation of some bacteria. The unsaturated fatty acids have been shown to be toxic for many species of

bacteria, the toxic concentrations in many cases being of the same order of magnitude as found in our tissue cultures. In one instance, *H. pertussis* (16), oleic acid is released during growth and in the absence of a binder such as serum albumin, the concentration of oleic acid soon reaches a toxic level. The unsaturated fatty acids have also been shown to be growth factors for many microorganisms. Thus, for *Lactobacillus bulgaricus* (17), oleic acid stimulates growth at a level of 1 gamma per ml. and inhibits growth at 2 gamma per ml. Albumin prevents the growth inhibition. Oleic acid inhibits the growth of small inocula of *M. tuberculosis* (13–15) at a concentration of 1 gamma per ml., but stimulates growth at even higher concentrations provided serum albumin is present. In these cases, albumin acts as a buffer for oleic acid. It maintains the concentration of free oleic acid below the toxic level and at the same time acts as a reservoir to supply the organism with this essential metabolite. It is possible that albumin acts similarly in tissue cultures. However, because of the difficulty of obtaining growth-promoting media free of fatty acids, we have not been able to investigate this possibility.

## SUMMARY

1. Human placental cord serum contains a factor or factors necessary for active growth of fibroblasts in tissue culture and a factor which prevents the appearance of a characteristic toxicity.

2. The growth-promoting factor(s) is stable to heating to 60°C. for 30 minutes and is associated with the euglobulin fraction of the serum.

3. Extraction of dried cord serum with benzene, chloroform, and ether does not cause any decrease in growth-promoting activity.

4. Sera from hypophysectomized, adrenalectomized, and thyroid-parathyroidectomized rats show as much growth-promoting activity as normal rat sera.

5. Bovine gamma globulins, anterior pituitary growth hormone, and insulin do not replace the serum growth factor.

6. The toxicity-preventing factor is serum albumin.

7. Evidence is presented that the toxicity is due to free fatty acids in the medium and that serum albumin acts by binding the fatty acids.

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## EXPLANATION OF PLATES

### PLATE 4

Photomicrographs of 10 day old cultures. × 60.

FIG. 1. Fed embryo extract.

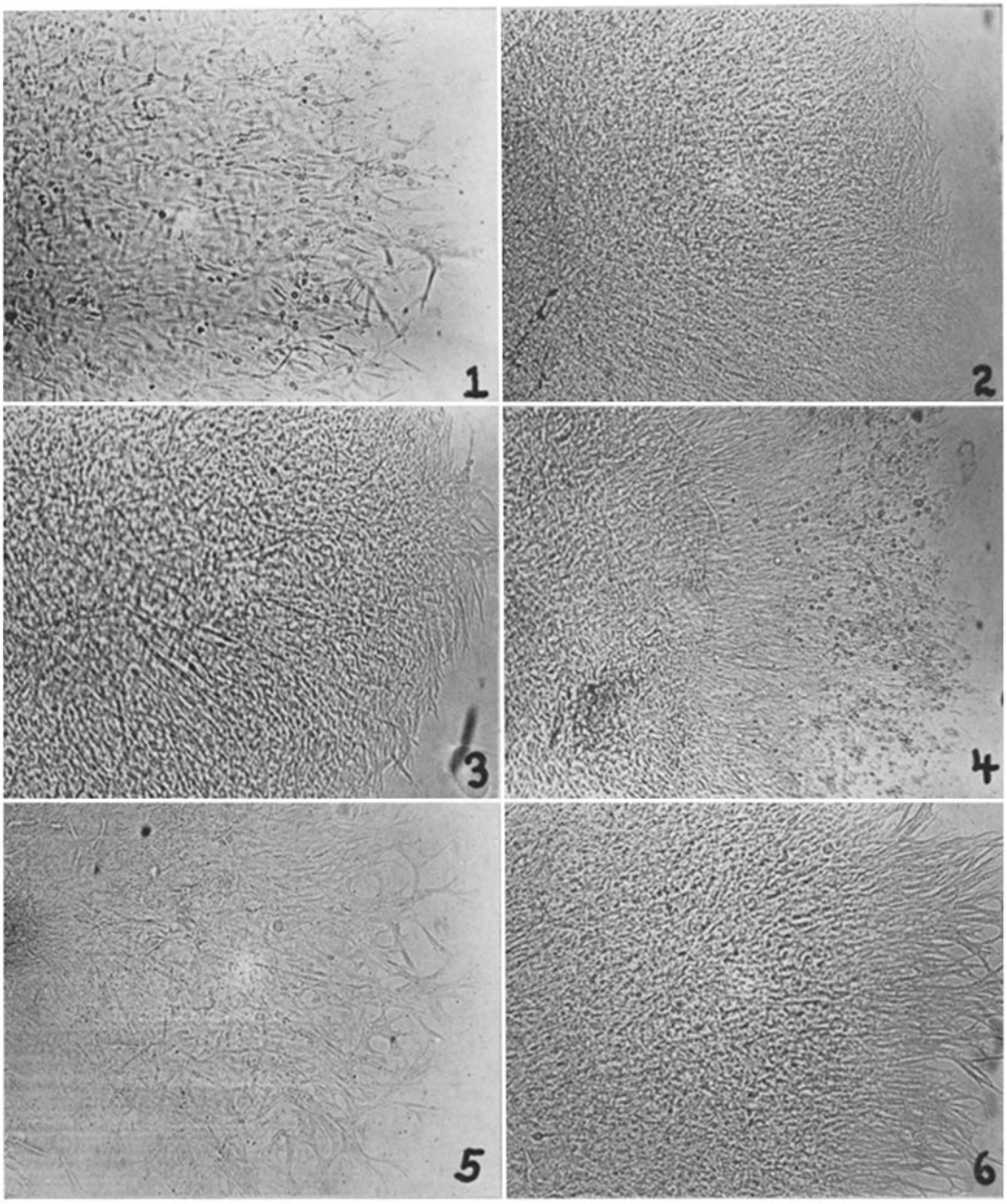
FIG. 2. Fed serum.

FIG. 3. Fed serum and embryo extract. Note the greater density of cultures that have serum in their medium.

FIG. 4. Culture fed globulin fraction of cord serum and embryo extract. Note density (serum effect) centrally and toxic damage peripherally.

FIG. 5. Culture fed albumin fraction of cord serum and embryo extract; no increase in density over cultures fed only embryo extract.

FIG. 6. Fed albumin and globulin fractions of cord serum plus embryo extract. Serum growth-promoting effect present and peripheral toxicity absent.




(Jacquez and Barry: Tissue culture media)

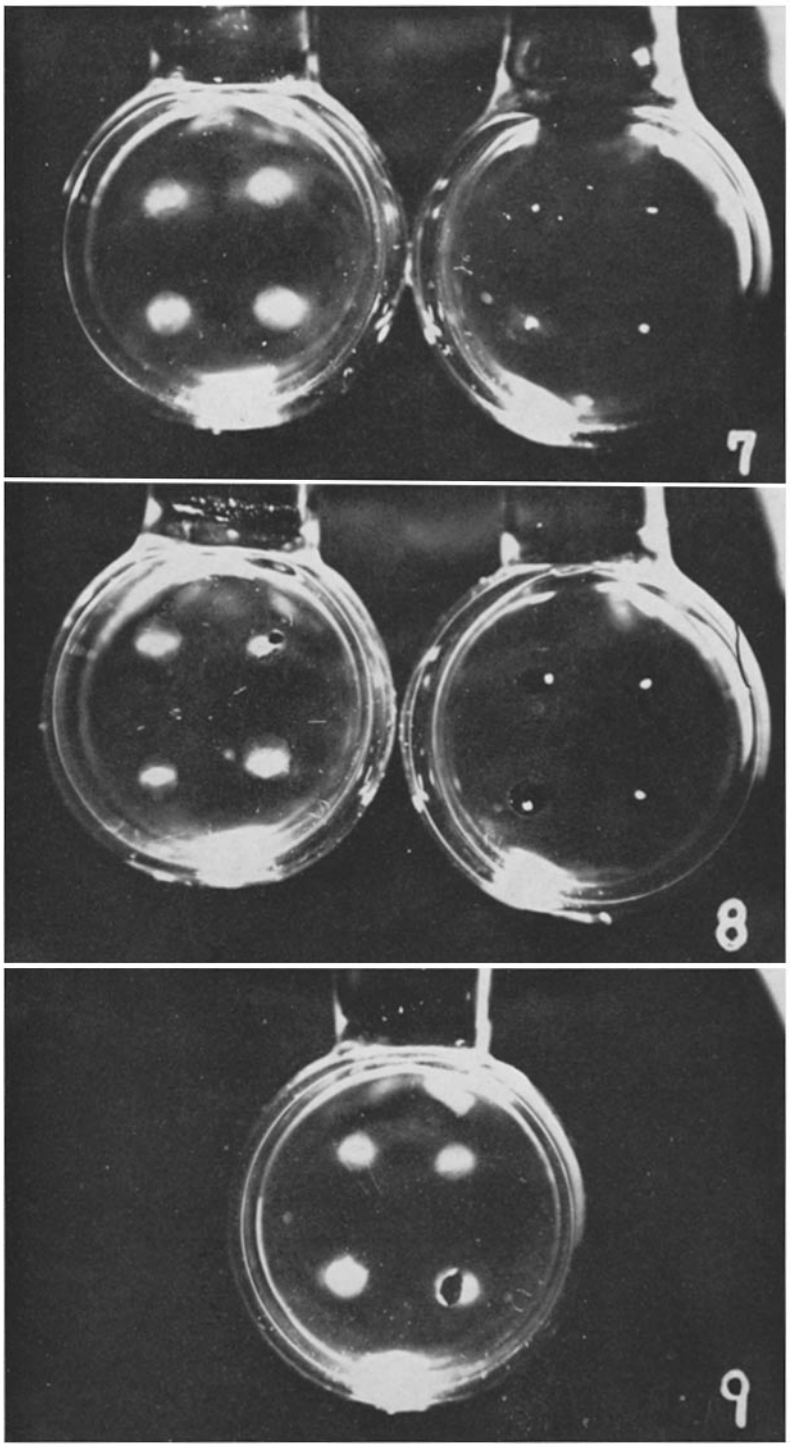
PLATE 5

15 day old cultures. The pictures were taken by illuminating the cultures from the side; the amount of light scattered thus gives some idea of the density of the cultures. × 1.5.

FIG. 7. The cultures in the flask at the left were fed the complete medium (cord serum plus embryo extract), those at the right received only embryo extract.

FIG. 8. Flask at left received embryo extract plus the non-dialyzable portion of cord serum, at the right, embryo extract plus the dialyzable portion of cord serum.

FIG. 9. Cultures fed reconstituted (dialyzable plus non-dialyzable) cord serum plus embryo extract.




(Jacquez and Barry: Tissue culture media)

## PLATE 6

Photomicrographs of 2 day old cultures. All cultures were fed 0.1 ml. embryo extract, 0.1 ml. of the globulin fraction of human placental cord serum, and X-6 or albumin in X-6 to make a total volume of medium of 0.6 ml. × 120.

FIG. 10. Control with no albumin in medium showing slight toxicity (dark granules in some peripheral cells).

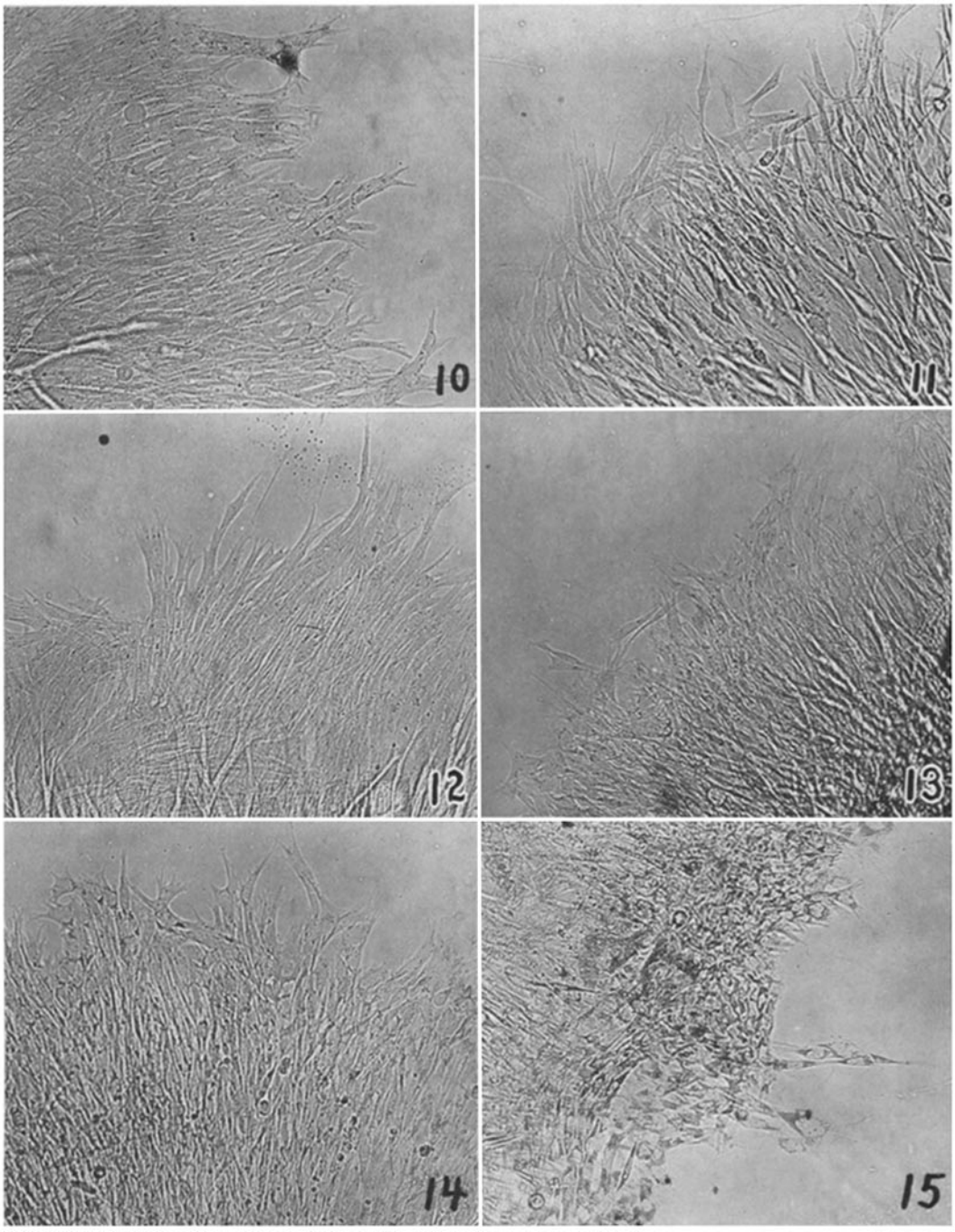
FIG. 11. Control with 0.5 mg. albumin in medium. No toxicity.

FIG. 12. 0.158 micromole oleic acid and 10 mg. albumin in medium.

FIG. 13. 0.158 micromole oleic acid and 5 mg. albumin in medium.

FIG. 14. 0.158 micromole oleic acid and 2.5 mg. albumin in medium.

FIG. 15. 0.158 micromole oleic acid and 1.25 mg. albumin in medium.




(Jacquez and Barry: Tissue culture media)