Heteromorphic Change of Adult Fibroblasts by Ribonucleoproteins*

By HELENA H. BENITEZ, MARGARET R. MURRAY, Ph.D.,
and ERWIN CHARGAFF, Dr. phil.

(From the Departments of Surgery, Anatomy, and Biochemistry, College of Physicians and Surgeons, New York)

PLATES 3 TO 6
(Received for publication, July 7, 1958)

ABSTRACT

Cultures of subcutaneous areolar fibroblasts from adult rats, when confronted in vitro with microsomes from rat liver or kidney, are changed heteromorphically so as to resemble cultures of nervous tissue. Similar effects follow exposure to the deoxycholate-insoluble fraction of microsomes, and to purified RNA from rat liver or from yeast. An equivalent ribonucleoside mixture has no heteromorphic effect.

The degree of heteromorphosis can be related quantitatively to the amount of RNA administered, up to a maximum of 150 μg per slide, above which toxicity intervenes. Ribonuclease destroys in considerable degree the effectiveness of the active agents.

Heteromorphosis cannot be induced in this adult tissue by a short exposure (1 to 3 hours) followed by removal to normal medium. A 24 hour exposure to microsome suspensions, however, is followed by partial change lasting for at least several days. Results are most clear cut when cultures of the explant type are maintained continuously in contact with the RNA-containing agents; nevertheless, cell suspensions exposed for 2 to 3 days to heteromorphic agents in suitable concentration appear to be permanently changed.

Interspecies experiments between rat and mouse indicate that rat fibroblasts are more labile than mouse, and/or rat microsomes are more potent as agents of heteromorphosis. Mouse liver microsomes have no morphogenetic effect on homologous fibroblasts, but exert a slight action on rat fibroblasts. Rat microsomes have a growth-stimulating effect, but no heteromorphic action, on mouse fibroblasts.

Purified protein from snake venom, which is highly active as a growth factor for avian nervous tissue, is growth-stimulating to rat fibroblasts but has no heteromorphic action on this material.

INTRODUCTION

The work reported here was undertaken in an attempt to investigate some of the chemical aspects of cytodifferentiation. The last few years have brought an increasing recognition of the specific nature of the highly polymerized cellular constituents; a specificity that characterizes not only the proteins, but also the nucleic acids, the polysaccharides, etc. As concerns the ribonucleic acids (RNA) and the ribonucleoproteins, their possible role in protein synthesis has been discussed very often, as has been the embryological evidence pointing to the accumulation of RNA in organs just before differentiation and to the general parallelism between RNA distribution and the morphogenetic gradients in the embryo. Pentose nucleoproteins are among the most effective of evocators. The concentration of microsomes in regions of cytoplasmic basophilia during development suggested to Brachet their possible identification with the agents of induction. The recognition of the chemical specificity of the nucleic acids led one of us a long time ago to propose "that just as the deoxypentosenucleic acids..."
of the nucleus are species-specific and concerned with the maintenance of the species, the pentose-nucleic acids of the cytoplasm are organ-specific and involved in the important task of differentiation” (4).

In this connection, recent, more direct evidence linking specific RNA to the synthesis of specific protein in enzyme induction should be mentioned (17, 19).

The writers set out to test the morphological response of cells isolated in vitro to exogenous nucleoproteins and related compounds. Adult mammalian cells were chosen as presenting the most refractory material from the standpoint of determination, and therefore the most meaningful in their morphogenetic response to differentiating agents; microsomes from homologous adult organs were selected for use as potential agents of cytodifferentiation.

Materials and Methods

For reasons of technical convenience we employed fibroblasts derived from subcutaneous areolar tissue of adult male rats. These were carried first as primary cultures in roller tubes for periods ranging from 3 to 95 days, then transferred as explants to Maximow slides for treatment and observation. This biological system has been in use in our laboratory for some 10 years as the material for studies on mitosis (1); its normal behavior and variation is therefore familiar. Degree and amount of morphological change were gauged subjectively, always by the same individual, who at the same time made photographs of the living cultures. Similarly, the degree of growth inhibition in irregular non-dividing outgrowth was of necessity an estimate. Photographs were checked by another author. Differences were not rated as significant unless they were clear cut and substantial. A 4+ scale was used in grading estimated results (see Table 1).

For the Maximow slides, cultures transferred from roller tubes were embedded in a plasma clot to which one drop of feeding solution was added. The experimental agent constituted 25 per cent of the feeding solution, the rest usually being composed of 25 per cent human placental serum, 25 per cent extract of 9 day chick embryo, and 25 per cent balanced isotonic salt solution as carrier for the antibiotics penicillin and mycinradin sulfate. However, the presence of serum or embryonic extract in the feeding solution carrying the sucrose suspension of microsomes is not necessary, balanced saline alone (with antibiotics) allows these cultures to survive in good condition for about 5 days, during which time maximal morphological change occurs. In feeding solution they can be kept on the coverslip without transferral for some 10 days, with re-feeding three times per week. Controls received balanced saline or sucrose solution in place of the experimental agents.

Microsomes from rat liver or kidney were prepared by the ultracentrifugation method of Hogeboom and Schneider (13) as modified by Palade and Siekewitz (26); whole microsomes were kept and used as a suspension in 0.25 or 0.88 molar sucrose. The lipide-free fraction was made by treatment of microsome pellets with 5 per cent Na-deoxycholate, followed by three consecutive sucrose washings and centrifugations. Ribonucleoside mixtures were composed of adenosine, guanosine, cytidine, and uridine in proportions equivalent to the molar phosphate ratios of cytoplasmic rat liver RNA (8).

The quantitative estimation of the RNA content of each preparation would have consumed too much material and was not practical; it was considered preferable to calculate the appropriate concentration of RNA in the various cellular preparations from the values for rat organs established in the literature. The microsome pellet when ready for use represents 1 gm. initial tissue pulp suspended in 1 ml. sucrose solution. For liver tissue the RNA value given by Leslie is 9.3 mg. and for kidney 6.5 mg. per gm. of pulp (24). Estimating 20 drops per ml. and ½ drop per slide, we administer amounts of the order of 115 γ liver RNA and 80 γ kidney RNA respectively, when standard microsome suspensions are employed. These values are of necessity approximate; an added variable is introduced by differences in ratio of stroma, fat, vessels, etc., to parenchyma that exist among organs from different individuals.

Purified RNA was prepared from rat liver and from yeast by hot saline extraction and precipitation, the method being a modification of several known procedures. Livers are removed from decapitated rats, weighed, and minced for 3 to 4 minutes in 2 volumes of 0.88 M sucrose in a teflon homogenizer at 0°C. After cold centrifugation in a Sorvall centrifuge at 20,000 g for 1 hour, 1 volume of 14 per cent trichloracetic acid (TCA) is added to the decanted supernatant and the mixture after being kept for 18 hours in the cold-room is centrifuged at 2500 g for 20 minutes. The residue containing the RNA is washed twice in the cold with 7 per cent TCA, twice with 95 per cent ethanol, once with ethanol-ether 1:3, once with methanol-chloroform 1:1, and twice again with ethanol-ether 1:3. A final wash with ether is carried out at room temperature. Traces of the solvent are removed by stirring occasionally at 40°C. for 15 minutes. The residue is then suspended in 10 volumes of 10 per cent NaCl and heated at 95-100°C. for 30 minutes. The residue is twice more extracted after cooling and centrifugation. To the combined supernatant 3 volumes of absolute ethanol are added, and the solution is kept at +4°C. overnight. The white flocculent precipitate obtained after centrif-
H. H. BENITEZ, M. R. MURRAY, AND E. CHARGAFF

The next morning is, in order to eliminate traces of NaCl, dissolved in a small amount of water and dialyzed in the cold for 4 hours against several changes of ice cold distilled water. The sodium salt of RNA so obtained is lyophilized. The authors are greatly indebted to Drs. J. J. Saukkonen and H. T. Shigeura of the Cell Chemistry Laboratory, Columbia University, for carrying out these procedures and making the purified RNA available.

Ribonuclease was obtained from Worthington Biochemical Company, Freehold, New Jersey, and from the Armour Company, Chicago.

Forty-eight experiments in all have been performed.

OBSERVATIONS

Whole Microsomes.—In optimal concentration, ca. 115 μg RNA per slide, suspensions of whole liver microsomes induce a marked change in the appearance and behavior of fibroblast cultures. This normally migrating cell, usually fan, flame, or spindle-shaped (see Figs. 5, 10, 20, controls) takes on a relatively sessile habit and emits long slender fibers of uniform width which frequently show pseudopodial growing tips (Figs. 1 to 4). Particularly within the first 4 days the treated cells migrate very little and divide little or not at all; at this concentration there is substantial inhibition of growth. At about the 4th day they become somewhat more active. When exposed to preparations of whole microsomes from which the lipide component has not been removed, the cells may gradually accumulate in cell body or processes small, rather uniform fat granules. Both nucleus and cytoplasm of the modified cells exhibit a marked basophilia when stained with cresyl violet, hematoxylin, or May-Grünwald-Giemsa (18); with the latter stain the coloring is blue or bluish purple. Smaller amounts of microsome suspension produce a proportional decrease in degree of modification and in number of affected cells: At 115 μg, 70 to 80 per cent of the outgrowth is modified; at 75 μg, 60 to 70 per cent; at lower concentrations the emerging cell population shows only limited spots of change from the normal fibroblastic form and growth habit. At concentrations of 150 μg and above, growth inhibition becomes extreme. Microsome suspensions in general are most active morphogenetically after being stored 10 to 20 days at 4°C. After 35 days at this temperature their effectiveness is considerably diminished; at 140 days all activity has been lost. The concentrations given above refer to preparations used during their period of maximum effectiveness; for older samples, concentrations must be increased in order to produce either morphological change or growth inhibition.

For kidney microsomes (10 to 20 days old), the most effective concentration is approximately 150 μg RNA per slide, with decrease in action proportional to decrease in concentration.

In one experiment a liver microsome suspension (116 μg concentration) was filtered through a Millipore filter (HA) with a calculated pore size of 0.45 micron. The filtrate, applied to fibroblast cultures, produced typical heteromorphosis, with exceptionally low fat accumulation. This is the finer of the two types of filters used by Grobstein (11) in his transfilter induction experiments.

Fractionated Microsomes.—Treatment of whole microsomes with sodium deoxycholate removes lipide and protein, and leaves a fraction consisting largely of dense particles of ribonucleoprotein. This material also, administered in graded amounts according to estimated concentration of RNA, produces the typical morphological changes described above (Figs. 6, 7), but at a somewhat higher effective concentration (150 to 175 μg RNA). Growth inhibition tends to be greater; this may be related to the difficulty of completely removing the detergent, even in three rinses. Reduced amounts (115, 85, 75 μg) of deoxycholate-insoluble fraction produce typical but less persistent changes. After about 4 days an overgrowth of more normal-appearance fibroblasts begins; the possibility exists that this ribonucleoprotein fraction deprived of its lipide complement is more labile than whole microsomes.

Ribonucleosides.—Because of the possibility that degradation products might be responsible for the morphological effects, lower molecular compounds were tested. Cultures were exposed to a ribonucleoside mixture composed of adenosine, cytidine, guanosine, and uridine in quantity and proportion equivalent to the liver RNA administered. This material produced no significant change in fibroblast morphology or behavior, though it was distinctly growth-inhibiting at concentrations equivalent to effective amounts of the preceding agents (Fig. 8).

Purified RNA.—Results being so far consistent with the hypothesis implicating ribonucleoprotein in cytodifferentiation, the next step was to apply the suspected agent in purified form. Saline-extracted RNA from rat liver (at 56, 115, and 150 μg) produces in fibroblast cultures morphological
changes identical with those produced by whole microsomes, with the exception that fat accumulation in the cells is greatly diminished. Purified RNA from yeast (at 115 and 150 °) is somewhat less effective, but nonetheless qualitatively similar in action (Figs. 11 to 13).

Ribonuclease.—Ribonuclease, in the amount of 10 γ per slide, was then applied simultaneously with effective doses of the agents which had been shown to induce morphological change in rat fibroblasts. In eleven experiments three batches were used, purchased from (a) Worthington, Freehold, New Jersey, 1953, (b) Armour, Chicago, 1955, and (c) Worthington, 1957. All had been kept in frozen state, and all were active when tested with RNA as the substrate.

The biological results were not uniform: reversal of RNA effects could be as high as 75 per cent or as low as 0 (Figs. 14, 15). In one experiment a large outgrowth of normal fibroblasts indicated the nearly complete inactivation of the transforming agent in presence of ribonuclease, especially within the first 48 to 72 hours. For unknown reasons a second experiment gave only negative results, although both enzyme samples were active when tested chemically with RNA as substrate. Irregular behavior of ribonuclease has been noted by other investigators (5) even at concentrations 10 to 40 times as strong as that used by us. In our experiments, yeast RNA was most susceptible and deoxycholate-insoluble fraction least susceptible to the enzyme, with whole kidney and liver microsomes being intermediate in response. Controls, to which ribonuclease alone was administered (10 γ per slide), sustained no morphological change, but some growth inhibition. It is probably significant therefore that the regularly observed growth inhibition exerted by RNA is somewhat reduced in the presence of RNase.

Time Relationships.—As a means of testing inductive aspects of the microsome effect upon fibroblasts, explants were incubated at 37° in the experimental medium containing liver microsomes for 50 minutes, 3 hours, and 24 hours, respectively, then planted in normal medium. Controls were incubated for corresponding periods in feeding solution to which balanced saline had been added instead of microsomes.

Exposures for 50 minutes and 3 hours were ineffective. Following 24 hour exposure to microsomes a partial heteromorphic effect occurred: such cultures were very similar in aspect to those which had been maintained in low microsome concentrations (58 or 23 γ). Appearance and behavior of control cultures were normal (Figs. 9, 10).

When cultures were planted in normal medium and the microsome component was added 3 hours later, after the clot was set, the morphogenetic effect was unchanged, and results were the same as if the cultures had been set out originally in the experimental medium. Cultures grown first for 3 days in normal medium can also be modified by the administration of microsomes.

If, after 2 to 3 days in the experimental medium, the agent is withdrawn from the feeding solution and replaced with saline or sucrose, the typical filamentous processes continue to be evolved at first; but later (after some 5 days) these are obscured by a welter of normal-appearing fibroblasts which then migrate out from the explant. When the explant of a modified culture is cut out after 7 to 9 days and transferred to normal medium the same sequence of events occurs. It is not clear therefore from the explant material how permanent is this morphogenetic effect; i.e., whether cells once modified remain so and are obscured by newly migrating cells from the interior which have not been exposed to the agent, or whether the effect has a span of several days only.

Cell suspensions prepared from primary fibroblast cultures (which are preferable to cell strains, in which spontaneous alteration is frequent) were at first found too fragile to withstand the experimental conditions. But by slightly modifying a trypsin-versene method used in the laboratory of Dr. Ian Leslie at Queen’s University, Belfast, and kindly communicated by him, we were able to maintain coverslip cultures of dissociated cells in which the experimental agents could be made equally available to all. In three experiments, dissociated cell cultures were exposed respectively to whole liver microsomes at 80 and 115 γ and to the (20 μ Millipore) filtrate of microsomes in 115 γ concentration. The agents were applied at the time of explantation and again after the first washing 2 days later; at all further washings only normal feeding mixture was used. The cells remained rounded and increased in size without dividing during the first 48 hours of this treatment, after which they began to elongate and take on a filamentous form similar to that seen at the edges of the explant cultures described above. These converted cells did not revert to fibroblastic
TABLE I

Action of Agents at Effective Concentrations

<table>
<thead>
<tr>
<th>Agent (amount per slide)</th>
<th>Rat fibroblasts</th>
<th>Brain tissue</th>
<th>Growth effect</th>
<th>Rat fibroblasts</th>
<th>Brain tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver microsomes 80 - 150 (\gamma)</td>
<td>++</td>
<td>23</td>
<td>±</td>
<td>6</td>
<td>++ (Inhib.)</td>
</tr>
<tr>
<td>“ 125(\gamma) + RNase 10(\gamma)</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td></td>
<td>++ (Inhib.)</td>
</tr>
<tr>
<td>Kidney microsomes 80 - 160 (\gamma)</td>
<td>++±</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>“ 160(\gamma) + RNase 10(\gamma)</td>
<td>+</td>
<td>1</td>
<td>±</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Fractionated microsomes 90 - 170 (\gamma)</td>
<td>++</td>
<td>6</td>
<td></td>
<td></td>
<td>+ + + (Inhib.)</td>
</tr>
<tr>
<td>“ 140(\gamma) + RNase 10(\gamma)</td>
<td>+±</td>
<td>1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonucleosides 120 (\gamma)</td>
<td>0</td>
<td>1</td>
<td>±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified liver RNA 115 - 150 (\gamma)</td>
<td>+++</td>
<td>4</td>
<td></td>
<td></td>
<td>++ (Inhib.)</td>
</tr>
<tr>
<td>“ 115(\gamma) + RNase 10(\gamma)</td>
<td>+</td>
<td>1</td>
<td>±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast RNA 115 - 150 (\gamma)</td>
<td>++</td>
<td>2</td>
<td></td>
<td></td>
<td>+ + (Inhib.)</td>
</tr>
<tr>
<td>“ 115(\gamma) + RNase 10(\gamma)</td>
<td>±</td>
<td>1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase 10 (\gamma)</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snake venom 1.25 - 0.8 (\gamma)</td>
<td>0</td>
<td>2</td>
<td>+ (Stimul.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

form during a subsequent culture period of 8 days in normal feeding mixture. The heteromorphosis therefore seems to have at least a relative permanence (Figs. 16, 17).

**Brain Tissues.**—These observations on the apparently neutralizing effect of microsomes upon fibroblasts led to experiments with nervous tissues. Two questions were asked of this material: (a) Will the experimental agent exert the same or a different action on a different tissue? (b) If the heteromorphic effect is primarily a toxic phenomenon, will it be expressed as extreme attenuation and ultimate breakage of the fibers and filamentous processes that are spontaneously produced by nervous tissues? In short will this material follow its commonly observed normal course of degeneration? Because of the large amounts of non-living materials (especially myelin) in adult brain and the difficulty of resolving them in vitro, newborn rat brain was used as material for explantation, and parallel cultures of subcutaneous fibroblasts from newborn animals also were run. The outgrowth from brain cultures, regardless of area, is glial in nature, with a few long neuritic processes from neurons which do not migrate but remain embedded in the explant. Little if any structural alteration was observed in the glial outgrowth following exposure to microsomes; the wandering proclivity of the cells was curbed somewhat, as was their multiplication; the length and conspicuousness of the cell processes appeared to be accentuated (Figs. 18, 19). Fibroblasts in these cultures, presumed to be of endothelial origin, appeared to be unaltered. Subcutaneous fibroblasts, however, from fetal rats sustained the same modification in form as those from adult tissue.

**Interspecies Experiments.**—So far, except for yeast RNA, homologous cultures and cellular preparations had been used. Tests as between two species, the mouse and the rat, were then undertaken; and it was found that the morphology of mouse subcutaneous fibroblasts is not altered significantly by either mouse or rat liver microsomes. Rat fibroblasts however are changed morphologically to a slight but perceptible degree by exposure to mouse microsomes (150 \(\gamma\)). When tissue from both species is explanted on the same slide, and exposed to rat microsomes (115 \(\gamma\)), the heteromorphic effect is exerted strongly on the rat.
30 HETERO MORPHIC CHANGE OF FIBROBLASTS

explant and not at all on the mouse. The mouse explant, however, receives a strong growth stimulus. In summary:

<table>
<thead>
<tr>
<th>Source</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>Heteromorphosis</td>
</tr>
<tr>
<td>Rat (115 γ) on Rat</td>
<td>++</td>
</tr>
<tr>
<td>Rat (115 γ) on Mouse</td>
<td>++</td>
</tr>
<tr>
<td>Mouse (150 γ) on Mouse</td>
<td>0</td>
</tr>
<tr>
<td>Mouse (150 γ) on Rat</td>
<td>++</td>
</tr>
</tbody>
</table>

**Snake Venom.**—Following reports by Cohen and Levi-Montalcini (7) on a nerve growth-stimulating factor isolated from snake venom, this material in purified active form was obtained by the courtesy of these authors and was tested in our system. Highest non-toxic concentrations of this purified protein (0.8 to 1.25 γ per culture) were growth-stimulating to control cultures of fibroblasts, but had no morphogenetic effect per se; nor did the purified protein, administered simultaneously with whole microsomes, modify the microsome effect perceptibly. Only traces, if any, of nucleic acids are present in this purified growth factor.

The observed action of the above experimental agents is summarized in Table I. Ratings are based on a scale in which 4+ = 100 per cent cells affected, read, and estimated always by the same investigator. Heteromorphosis ratings refer to percentage of outgrowth converted.

**DISCUSSION**

The observation has now been made consistently in a large number of experiments that adult rat fibroblasts when exposed to a variety of preparations containing homologous hepatic RNA undergo typical and reproducible morphological change, the degree of which is governed, within toxic limits, by the amount of RNA available. This change is heteromorphic in nature, resulting in cultures derived from subcutaneous areolar tissue taking on the appearance of cultures of nervous tissues. One is tempted on morphological grounds to call this transformation “neuralization,” if it be understood that the term includes glial derivatives as well as varied neuronal derivatives of neural crest. However, in this communication no information can be offered on the biochemical or functional activities of the changed cells: the term “neuralization” is used provisionally and for the sake of brevity. In the meantime much more work remains to be done before even authentic nervous tissues cultivated in vitro can be precisely or comprehensively characterized on morphological, biochemical, or functional grounds. In amphibians, skin and subcutaneous tissues also may stem from neural crest; it is not known whether this principle applies to mammals, and thus whether our fibroblastic material is of neural crest descent.

It is not appropriate here to attempt discussion or evaluation of the vast literature on embryonic induction. Little of this applies to mammals; in the realm of amphibian embryology, Toivonen (27, 28) performed pioneering experiments on fractionation of tissue extracts, using guinea pig liver and kidney and concluding that there exist two qualitatively different inducing substances, one thermostable and ether-soluble and the other not so. Kuusi (23) continued in this approach, implicating proteins rather than RNA and drawing attention to some conditions inconsistent with Brachet’s hypothesis that organizer and inductor substances are RNA. Brachet himself in 1955 (3) points out apparent inconsistencies. For a recent summary of the organizer literature, the reader is referred to Holtfreter and Hamburger (16).

When this investigation was undertaken, the expectation most in keeping with our own hypothesis of organ-specific RNA was that the cells exposed to liver microsomes might take on characteristics of this organ. At no time was such a change observed in these experiments; however, it is now reported by Niu (25) that organ-specific RNA can be obtained by phenol extraction, which was not the method used here. Since in embryonic development the most primitive inductive impulse is towards neuralization, our microsome and other active preparations may be operating in some such way as primary organizer substances.

Evocation in very early development may be achieved by a variety of agents physical and chemical which cannot be considered as specific but which probably perform a trigger-like function in an organism already mobilized for differentiation (10, 14, 15, 31). Although the possibility exists that we likewise are dealing with a non-specific agent, the failure of ribonucleosides and protein to effect morphological change, as well as the interspecies discrepancies between mouse and rat, suggest that our RNA preparations may be true differentiating...
agents, in crude form. Furthermore, the tissue on which they act is not embryonic, and since it comes from adult mammals, can be presumed to be fully determined as to tissue type. One would not expect it to be competent for evocation.

The large amount of RNA (75 to 150 \( \gamma \) per slide) found necessary to bring about heteromorphosis lends itself to two possible explanations:

1. Adult cells may require an overwhelming dose.

2. Such large, slowly diffusing particles or molecular aggregates as we are dealing with may make contact only with the cells in their immediate vicinity, hence the major part of the dosage, distributed through the medium, may be wasted.

Observation of living and stained cultures gives the strong impression that microsomal particles actually are incorporated within the cell bodies and processes: living cells contain many particles of the same color, refractivity and size as the suspension outside, and stained cells show a basophilia which greatly exceeds that commonly observed in filamentous processes, whether neuritic or fibroblastic. However, it cannot now be stated positively that the agent enters the cells; such a conclusion must await investigation by more precise methods.

It is apparent that in these experiments the agents which induce heteromorphosis are generally also inhibiting to growth and migration when applied in concentrations that are morphogenetically effective. As the concentration diminishes, both effects tend to diminish with it. However such a correlation is not found uniformly throughout any series of cultures. The ribonuclease experiments especially offer an indication that the two effects are separable, since some batches of enzyme diminish the growth inhibiting effects of microsomes but not the morphogenetic effects. In this regard, the refractoriness of mouse tissues to the heteromorphic action of microsomes is noteworthy, as is the growth-stimulating effect of rat microsomes on mouse fibroblasts. We do not know whether metabolic differences at the level of purine synthesis that exist between mouse and rat (12) may be relevant to any of our observations. There remains unexplained also the substantial effectiveness, on rat tissues, of purified RNA from yeast, as regards both heteromorphosis and growth inhibition.

Several investigators, e.g. Chèvremont and Firket (6), Kutsky (20), and Kutsky and Harris (21, 22) have reported growth-stimulating effects of RNA or homologous nucleoprotein fractions on fibroblasts or myoblasts. Though it is difficult to compare their systems quantitatively with ours, it appears that these authors were using substantially lower concentrations of RNA per cell than we. Kutsky’s biologically active factors, unlike ours, are streptomycin-precipitated.

Experienced workers in tissue culture are accustomed to associate shrunken cells and attenuated cell processes with poor nutrition, accumulated wastes, or certain other toxic conditions, and these possibilities were given primary consideration when the results described above began to appear. However, the whole aspect of the modified cells in form, behavior, refractivity, and capacity for survival seems to take them out of the class of unhealthy cultures and set them in a group to themselves. The authors also are well aware of the considerable modulation in form that characterizes healthy cells from various tissues when cultivated in vitro. Though the range of modulation for a given tissue type may be predicted, its actual occurrence is often quite capricious, and is governed by factors in the main unknown. The degree and quality of the morphological change observed in these experiments is entirely outside the authors’ experience in many years’ close observation of the changes in form and behavior adopted spontaneously by fibroblasts and other cells in vitro. It appears that the form of the outgrowth is not predicated upon the orientation of fibrin micelles, since heteromorphosis will take place if the agent is administered after the clot is set.

It has already been clearly shown by Fell and Mellanby (9) that a striking metaplasia can be elicited in relatively late and well differentiated embryonic tissue by a specific agent, vitamin A. According to Weiss and James (30) this effect can be induced in cell suspensions by a short exposure (of less than an hour), for embryonic epidermis. In our material a much longer time is required: 24 to 48 hours to produce a relatively stable heteromorphism. For a provocative discussion of tissue type stability the reader is referred to a recent paper by Trinkaus (29).

The nature of the change brought about in our adult rat fibroblasts by RNA may be elucidated by further experiments, which will be undertaken. Perhaps the chief and most permanent significance of these findings is the demonstration that a reproducible morphological change can be regu-
larly and quantitatively brought about in adult tissue by any agent.

BIBLIOGRAPHY
22. Kutsy, R. J., and Harris, M., Effects of nucleoprotein fractions from adult and juvenile tissues on growth of chick fibroblasts in plasma cultures, Growth, 1957, 21, 53.
EXPLANATION OF PLATES
PLATE 3

In this and following plates the original material consists of cultures of fibroblasts from subcutaneous areolar tissue of adult rats, with the exception of Figs. 20 and 21, which depict similar material from the mouse, and Figs. 18 and 19 which depict newborn rat brain.

Fig. 1. 5 days after treatment with rat liver microsomes. Living, phase contrast, × 150.

Fig. 2. 4 days after treatment with rat liver microsomes (115 γ RNA). Living, × 300.

Fig. 3. Culture kept in control medium 3 days, then treated with HA-filtered microsomes. Photographed 20 hours later, living, × 300.

Fig. 4. 24 hours after treatment with rat kidney microsomes (80 γ RNA). Living, phase contrast, × 600.

Fig. 5. 4 days in control medium. Living, × 300.
(Benitez et al.: Heteromorphic change of fibroblasts)
PLATE 4

Fig. 6. 28 hours after treatment with deoxycholate-insoluble fraction of rat liver microsomes (140 μg RNA). Living, × 280.

Fig. 7. 4 days after treatment as above.

Fig. 8. 5 days after treatment with nucleoside mixture. Fixed in Zenker's fluid, stained with phosphotungstic acid hematoxylin. × 185.

Fig. 9. Incubated 20 hours in microsome suspension then explanted in control medium. Partial heteromorphosis, photographed at 6 days, living, × 280.

Fig. 10. Sister culture incubated 20 hours in normal medium then explanted.
(Benitez et al.: Heteromorphic change of fibroblasts)
PLATE 5

Fig. 11. 3 days after exposure to purified RNA (150 γ) from yeast. Living, X 280.
Fig. 12. 4 days after exposure to purified RNA (115 γ) from yeast. Living, X 280.
Fig. 13. 4 days after treatment with purified RNA (115 γ) from rat liver. Living, X 140.
Fig. 14. 4 days after treatment with purified liver RNA and RNase. (Enzyme relatively ineffective biologically). Living, X 280.
Fig. 15. 5 days after treatment with yeast RNA and RNase (enzyme effective biologically). Living, X 140.
(Benitez et al.: Heteromorphic change of fibroblasts)
PLATE 6

FIG. 16. Suspension-culture 4 days after treatment with rat liver microsomes (80 γ RNA). Living, × 140.
FIG. 17. Control suspension culture at 4 days. Living, × 140.
FIG. 18. 5 day control culture from newborn rat brain. Living, × 280.
FIG. 19. Newborn rat brain, 9 days after exposure to rat liver microsomes. Living, × 280.
FIG. 20. Mouse subcutaneous fibroblasts, 54 hour control culture. Living, phase contrast, × 280.
FIG. 21. Mouse fibroblasts, 54 hours after exposure to rat liver microsomes (140 γ RNA). Living, phase contrast, × 280.
Plate 6
Vol. 5

(Benitez et al.: Heteromorphic change of fibroblasts)