Location of Absorbed Carcinogens within the Amphibian Cell

BY

C. H. WADDINGTON

(Institute of Animal Genetics, Edinburgh)

AND

C. B. GOODHART

(Department of Zoology, Cambridge)

INTRODUCTION

SOME of the polycyclic hydrocarbons derived from phenanthrene are biologically active in a number of different ways. Not only are they, as is well known, capable of causing the production of cancers and simulating the action of sex hormones, but they can also evocate neural tissue in the amphibian embryo (Waddington and D. M. Needham, 1935), and exert a rather feeble mutagenic activity (Demerec, 1947, 1948; Strong, 1947; Carr, 1947).

In none of these connexions have we a clear idea of the mechanism by which the effect is produced. This paper is, in the first instance, concerned to throw further light on the interaction between the carcinogenic substances and the embryonic amphibian cells on which they exhibit the activity of evocation. There are several questions which present themselves in this connexion. One is the relation between the carcinogen evocators and the substance, which may be called the 'natural evocator', which is presumably responsible for stimulating the ectoderm to differentiate into neural tissue during normal development. The demonstration of Waddington, Needham, and Brachet (1936) that evocation could be produced by treatment with methylene blue proved that evocation can be brought about by substances which are certainly not present in normal eggs. Waddington, Needham, and Brachet pointed out that such substances might act by killing a few of the neighbouring cells, thus releasing their stores of the natural evocator, which stimulates the surviving cells to neural differentiation. Holtfreter (1944b, 1945) and others following him at first suggested that all evocations by chemical implants were mediated by this mechanism. But recently Holtfreter (1948) appears to have convinced himself of the truth of the contention of Waddington, Needham, and Brachet that evocation is possible even when no signs of necrosis can be detected; and he seems ready to accept their suggestion that non-natural evocators may act, in some manner less drastic than killing, so as to release a store of natural evocator which is contained within

[Quarterly Journal Microscopical Science, Vol. 90, part 2, June 1949]
Waddington and Goodhârt—Location of the ectoderm cells and which then brings about an induction. It is very possible that the carcinogenic hydrocarbons owe their evocating power to an action of this kind. Alternatively, they might be related chemically to the natural evocator, about whose properties we are still completely in the dark. Waddington (1940) and Needham (1942) have suggested that the low dosage in which the evocating activity is manifested offers some support for this suggestion; but they admit that too much weight cannot be placed on the argument.

It was not to be expected that, from observations of the kind to be recorded in this paper, any direct evidence would be forthcoming on the general question of whether the carcinogens acted as substitutes for the natural evocator or in a secondary way as releasers of it. But it was anticipated that some light might be thrown on their relation to one of the other substances which have been claimed to be the natural evocator. Fischer, Wehmeier, and Jühling (1933) first showed that preparations of ribonucleic acid can act as evocators, but they did not exclude the possibility that this was a result of producing localized necrosis. Brachet (references and discussion in 1947) has confirmed the result, using purified preparations; and has also presented evidence to show that when dead tissue is digested with ribonuclease, it loses its evocating power. He argues that the synthesis of cell-protein is carried out mainly at the ultra-centrifugeable ribonucleoprotein granules in the cytoplasm, to which Claude (1941) had first directed attention; and he suggests that evocation is primarily a reaction which takes place at these granules. He appears to believe that the natural evocator substance is actually ribonucleic acid, which diffuses from the roof of the primitive gut into the overlying ectoderm and leads to an increase in concentration there of these cytoplasmic granules. And he further suggests that the activity of the carcinogens is due to their property of becoming attached to the granules.

In making the last suggestion, Brachet bases himself on the observations of Graffi (1939, 1940). This author studied the accumulation of various polycyclic hydrocarbons within the cells of normal and malignant tissues of the mouse. The technique used was to render the hydrocarbons water soluble by treatment with glycerine and blood-serum, to expose the cells to aqueous solutions containing up to about one part of hydrocarbon in 40,000, and to study the location of the chemical within the cell by observing the fluorescence of the cell constituents when illuminated with ultraviolet light. Graffi found that the hydrocarbons become attached to cytoplasmic granules. He speaks of these as 'lipochondria' and 'mitochondria', but it is notoriously difficult to be certain exactly what various authors mean by these terms; and it was perhaps not out of the question that Brachet might have been correct in suggesting that some of the granules which accumulate the substances may be identical with 'the ribonucleoprotein granules (which may be called 'microsomes') to which he attributes synthetic activity.

Although Brachet's theory provides an attractive way of envisaging the mode of action of hydrocarbons in both carcinogenesis and evocation, it is
clear that its observational basis is rather slender. We have therefore used methods essentially similar to Graffi's to investigate the accumulation of hydrocarbons in the cells of the amphibian embryo. This material has the advantage not only that it is the tissue on which the evocatory action is exerted, but that its microsomes have been described by Brachet, while its cytoplasmic lipoids have been fully studied by Holtfreter (1946a, b, c).

Apart from the somewhat involved questions relating to Brachet's theory of evocation, a much simpler problem remains to be solved and it was hoped that the present investigations would throw some light on it. It has been apparent for some years that the cell surface plays an extremely important part in many of the morphological changes occurring during development and in particular during the formation of the neural tube (Waddington, 1942; Holtfreter, 1943, 1944b). It might with some plausibility be suggested that the carcinogenic hydrocarbons owe their evocating power to their well-known surface activity (Moricard and Gothié, 1943a, b, 1944). If this were the case, one would expect to find them localized in the surface membrane of the cell.

**Technique**

Explants of the blastocele roof of early gastrulae of the newt *Triturus alpestris* were cultured overnight in Holtfreter's standard solution containing solubilized 3:4-benzpyrene. They were then examined microscopically by ultra-violet light which causes those structures in the cell which have accumulated any of the hydrocarbon from the culture medium to show a bright blue fluorescence. The light source was an Osram high-intensity lamp with a quartz lens and filter to exclude all visible light. At first a surface-silvered mirror and quartz condenser were used on the microscope, but it was later found that the standard glass mirror and condenser gave equally good results.

Weil-Malherbe (1946) has shown that various polycyclic hydrocarbons including 3:4-benzpyrene will form water-soluble addition-compounds with caffeine and other purines. In the present investigations this method was used for rendering the benzpyrene soluble, in preference to the more complicated way of using saturated solutions in hot glycerine diluted with serum, adopted by Graffi. A 1 per cent. solution of caffeine in Holtfreter's standard solution was stirred overnight with a slight excess of benzpyrene at 20°C and then filtered. The resulting solution, which shows distinct purplish-blue fluorescence in sunlight should, according to the figures given by Weil-Malherbe, contain about one part in 100,000 of dissolved benzpyrene. In one series of experiments this solution was diluted ten times again with Holtfreter's solution, which should give about one part in 10,000,000 of the benzpyrene. The cells of the explant cultured in this diluted solution were nearly as brightly fluorescent in ultra-violet light as were those in the stronger one and even in a solution diluted by a further factor of 10 the cells still showed a very faint bluish fluorescence. Amphibian gastrula cells cultured
in pure Holtfreter's solution or in a 1 per cent. solution of caffeine without any benzpyrene show practically no fluorescence, at the most only the very faintest greenish, not blue, tinge.

The cells of the explants in 1 per cent. caffeine in Holtfreter's solution, both with and without dissolved benzpyrene, tend to dissociate from one another and fall apart in the same way as when they are cultured in an alkaline medium. This happens also in a 1 per cent. caffeine solution buffered to pH 6-9. The dissociated cells appear otherwise to be quite healthy and remain alive for several days, although they are apparently not able to re-aggregate when transferred to a caffeine-free solution as happens when cells dissociated by alkali treatment are returned to a neutral medium (Holtfreter, 1947). Explants cultured in 0-1 per cent. caffeine with or without added benzpyrene show little or no tendency to fall apart. No cytological differences could be observed between the dissociated and undisassociated cells, and, as the former in the higher concentration of benzpyrene showed a rather brighter fluorescence in ultra-violet light, they were used for most of the observations.

A few observations were made with other types of cells, particularly to search for any evidence that fluorescent compounds were accumulated in the nucleolus (see p. 215).

**EXPERIMENTAL RESULTS**

After having been cultured for some time in a benzpyrene-caffeine solution, the cells of the explant will have fallen apart and become spherical with a diameter of about 60 μ, though there is considerable variation in size. Seen by ultra-violet light they show the characteristic blue fluorescence of benzpyrene in molecular solution, though otherwise it is hard to distinguish much structure in the spherical cell. The nucleus is seldom visible as it is obscured by the overlying fluorescent cytoplasm. When the cell is squashed slightly with a coverslip more of its structure becomes visible. The nucleus, as reported by Graffi, is entirely non-fluorescent and shows up as a dark patch in the middle of the cell. The fluorescent nucleolus described by Graffi as being occasionally visible in mouse cells was not observed in this amphibian material. Rarely a bright fluorescent liposome was seen outlined against the dark nucleus, which might have been mistaken for a nucleolus, but these were always outside the nuclear membrane.

Neither the cell membrane nor the nuclear membrane showed any fluorescence. This is true both of the intercellular membrane and of the 'surface coat' (Holtfreter, 1943, 1944a) which forms the boundary of the egg against the external medium. When a cell or group of cells is partly covered by surface coat containing pigment this material can be seen as a dark patch, itself not fluorescent, obscuring the light coming from the underlying cytoplasm. The cytoplasm showed a uniform bright blue fluorescence, though not infrequently the nucleus was outlined by a ring or crescent considerably brighter than the rest of the cytoplasm. This ring, which is usually rather homogeneous and does not contain yolk platelets, probably consists of the phospholipids
Absorbed Carcinogens within the Amphibian Cell

associated with the nucleus which, after fixation, constitute the so-called 'Golgi Apparatus'. It could sometimes be seen in quite fresh and unsquashed cells and was probably not an artifact. The rest of the cytoplasm in these early gastrula cells is closely packed with yolk platelets round which the lipochondria cluster in the way described and figured by Holtfreter (1946a). The lipochondria are brightly fluorescent and outline the considerably less bright yolk platelets. Scattered throughout the cytoplasm there are also a number of very much smaller brightly fluorescent granules or globules in Brownian movement as well as a few larger and very brightly fluorescent globules; both of these are considered to be liposomes. When the cells are squashed strongly so as to burst, or begin to dry up, and probably also simply as a result of prolonged exposure to ultra-violet light, they begin to show further changes. The lipochondria detach themselves from the yolk platelets and break up, their lipoid constituents running together to form the larger fat globules known as liposomes which are very brightly fluorescent. As the preparation deteriorates further the liposomes become larger and less numerous as they join up with one another. At the same time as the yolk platelets lose their attached lipochondria they also lose their never very bright fluorescence.

It is probable that in an entirely fresh state the gastrula cells would have most of their lipoid in lipochondria and that the liposomes appear as a result of the breakdown of the lipochondria under abnormal conditions. In cells of neurulae and later stages the liposomes do become conspicuous even in the freshest material, and they probably occur as such in the cells of the normal living embryo at those stages. In unsegmented eggs most of the lipoid is in the lipochondria associated with yolk platelets although liposomes and fat globules are also present. It is, however, impossible to examine the intracellular inclusions of an unsegmented egg in situ; the egg must be broken up for microscopic examination of its contents and the breaking-up process is very likely to damage some lipochondria and to release their lipide as liposomes. The contents of uncleaved eggs which had been cultured in benzpyrene-caffeine solutions were examined by ultra-violet light and their yolk platelets showed the same fluorescence picture as has been described for gastrula cells. Uncleaved eggs could not, however, be cultured whole in the benzpyrene-caffeine solution since, even when the vitelline membrane is left intact, the cell membrane and 'surface coat' disintegrate after 10 minutes or so.

Some of the explants that had been cultured in benzpyrene solution were then stained unfixed with Unna's methyl green-pyronine. In this the nucleus took up the methyl green while the cytoplasmic granules of ribonucleoprotein, or microsomes, stained red with the pyronine, as described by Brachet. By this means it is easy to distinguish between the microsomes, which are rather uniform in size just at the limit of microscopic resolution and so probably about 0.3 μ in diameter, and the unstained liposomes, the smallest of which were little larger than the microsomes, but which showed a graded series in size up to the largest of 1 μ or more. It is hard to distinguish between them
in unstained preparations and both are small enough to show Brownian movement. By the examination of a preparation cultured in benzpyrene solution and then stained with pyronine, first of all by visible light and then immediately afterwards changing to ultra-violet, it was possible to prove that the very small granules in Brownian movement that had taken up the benzpyrene and so fluoresced in ultra-violet light were small liposomes, while the pyronine-staining microsomes showed no fluorescence. It would therefore seem that Brachet was mistaken in assuming that the ribonucleoprotein microsomes of the amphibian gastrula are able to take up benzpyrene from the surrounding culture medium (Brachet, 1947, p. 479).

As well as the microsomes, which stain a darker red, the yolk platelets and especially their attached lipochondria stain pink with pyronine. After digestion with a solution of crude pancreatic ribonuclease the pyronine-staining properties of both lipochondria and microsomes are lost, although the microsomes still remain visible, not being dissolved away.

These observations of the location of the fluorescent material within the cell were supplemented by centrifuging experiments on single uncleaved eggs and on gastrulae ground up with Kieselguhr. In both cases, the material is sorted out (in an angle centrifuge giving about 3,500 g.) into four main zones; the lipoid accumulates at the centripetal pole, and is followed by a large, rather clear layer of cytoplasm, which is separated from the centrifugal layer of yolk granules by a thin zone of pigment and other granules. In material which had been treated before centrifugation with benzpyrene, the pigment layer did not fluoresce at all, while the fluorescence in the watery layer was also slight. The lipoid layer was extremely bright, and there was usually fairly strong fluorescence in the yolk layer, probably due mainly to lipochondria which had not been separated from the yolk granules. The main activity was certainly in the lipoid layer, as might be expected from what has been said above.

In such centrifugates, the nucleoprotein microsomes would be expected to come out at the bottom of the watery cytoplasmic layer, i.e. along with the pigment granules. No fluorescence could be found here in preparations made with a normal laboratory centrifuge. It might be argued that such an instrument would not suffice to sediment the microsomes, and that the slight luminosity of the cytoplasmic layer was due to benzpyrene absorbed on microsomes. Even if this were so, the microsomes would only account for a very small fraction of the total hydrocarbon absorbed by the cell. But the matter can be tested by further centrifugation. After a preliminary treatment in the angle centrifuge the watery layer was isolated and again centrifuged on a high-speed air-driven ultra-centrifuge of the Beams-King type. This succeeded in clearing the fluorescence; but the important point is that the granules to which the fluorescence was due moved centripetally, i.e. in the direction to be expected if they were very small fat or oil drops, but opposite to that expected if they were the microsomes discussed by Brachet.
Absorbed Carcinogens within the Amphibian Cell

DISCUSSION

It is clear from the above observations that the lipoid constituents of the cell—lipochondria, liposomes, and perhaps the phospholipids constituting the 'Golgi Apparatus'—have an affinity for benzpyrene and will take it up out of solution with caffeine. There is really no evidence that the non-lipoid constituents can accumulate it.

The cell membrane, in particular, seems to absorb less of the hydrocarbon than any other part of the cell; in fact by the present technique none whatever can be detected in it. This finding makes it appear very improbable that the evocatory power of the steroid hydrocarbons is due to any influence of their surface activity on the membrane, although it of course remains possible that an activity of this kind plays an important role in their reaction with some deeper-lying structure within the cell.

There is also no direct observational evidence of any accumulation of benzpyrene by nucleoprotein structures. It is certainly not directly absorbed by the nucleoprotein microsomes with the avidity with which it is taken up by the lipochondria. This conclusion is not in conflict with the published evidence of Graffi. That author did, however, claim that the nucleolus, which consists largely of ribonucleoprotein, occasionally shows fluorescence with benzpyrene. It does not seem to us possible to be certain that the structures described and figured by Graffi really were nucleoli. He only rarely saw them, and it is hard to distinguish them with certainty from liposomes lying just above or below the nucleus. In the amphibian material no fluorescent nucleoli were detected, though again it would be difficult to identify them with certainty.

In order to test this matter on more favourable material a series of observations was made on the oocytes of the pond snail Limnaea stagnalis, which have very conspicuous nucleoli and comparatively little lipoid (Raven, 1948). The ovotestis was dissected out and cultured overnight in amphibian Ringer solution diluted six times, containing 1 per cent. caffeine, and saturated either with benzpyrene or with another carcinogenic hydrocarbon, 20-methylcholanthrene. The oocytes were then examined either centrifuged or gently squashed under a coverslip. By visible light the nucleolus, which is about 15 μ in diameter, shows up very conspicuously; on switching over to ultra-violet light a certain amount of purplish fluorescence can be seen in the yolk cytoplasm but none at all in either the nucleolus or the nucleus. Fixed oocytes of Echinus kept for 24 hours in the hydrocarbon solutions also showed no trace of fluorescence in their conspicuous nucleoli. Some observations were also made on the salivary chromosomes of Drosophila cultured in benzpyrene-caffeine solution. If ribonucleoprotein absorbs the hydrocarbon it might have been expected that the heterochromatic regions would have been fluorescent in ultra-violet light, but no sign of this could be observed. Finally, a suspension of tobacco mosaic virus (a ribonucleoprotein), stood for some hours in solubilized 3:4-benpyrene and then centrifuged at high speed, showed no fluorescence in the centrifugate.
Weil-Malherbe (1946) detected a slight solubilizing effect on benzpyrene and other hydrocarbons by nucleotides and nucleosides in vitro. He suggested that this property, due presumably to their purine constituents, might be of some biological significance. It is conceivable that the ribonucleoprotein might have some slight affinity for benzpyrene but that the caffeine used as a solubilizer in this work holds on to it more strongly and so prevents its accumulation in the nucleoprotein structures. To check this point aqueous solutions of benzpyrene and methylcholanthrene were prepared according to the method described by Graffi (1939), glycerine and serum being used as the solubilizer. Limnaea oocytes cultured in these solutions and examined in ultra-violet light showed, however, no signs of fluorescence in the nucleolus. It must, therefore, be concluded that there is no evidence that structures containing ribonucleoproteins necessarily have any affinity for these carcinogenic hydrocarbons, whereas the evidence that the lipoid constituents readily take up the substance is direct and conclusive.

The evidence that benzpyrene is not accumulated to any significant extent by nucleoprotein structures makes it probable that the mutagenic activity of the carcinogens (Demerec, 1947, 1948; Strong, 1947; Carr, 1947) is indirect, the genetic mutations being produced as secondary consequences of a primary cytoplasmic effect. It is noteworthy that, as mutagens, these substances differ in several respects from other active chemical agents, which are thought to act directly on the chromosomes, such as mustard gas (Auerbach and Robson, 1947). Thus the increase in mutation-rate achieved with carcinogens in Drosophila is much less than with the mustards, and although exact dosages are known in neither case, the difference is probably real. Further it has been claimed by Strong and Carr that some carcinogens (methylcholanthrene) cause the mutation of specific genes: the effect is perhaps still open to some doubt, but nothing of the kind occurs with the mustards, and the phenomenon, if true, may prove to be characteristic of secondary mutagenic action (Hadorn, 1948).

We may now turn to consider the bearing of these observations on the way in which we envisage the mechanism of action of the hydrocarbon evocators. In the first place, the survival of amphibian embryonic cells for some considerable period in the rather strong solutions used in this work makes it unlikely that the evocation is to be attributed simply to the toxicity of the substances. Holtfreter (1945) appears to have advanced this suggestion on no better grounds than that it fitted in with his theoretical outlook. He produced no good observational evidence for it; whereas Shen (1942) noted that the explants which showed the best neuralization in solutions of 1:2:5:6—dibenzanthracene-o-β-endosuccinate were the ones which had the fewest damaged and cytolysing cells.

Sufficient attention has not always been paid to the delicacy of the balance which would have to be struck to operate the mechanism of evocation by the cytolysis of part of the exposed ectoderm. It is necessary that some cells should be actually killed, so that they release their evocator, while others
remain healthy enough to react to it by neural differentiation. It is comparatively easy to accept such an explanation in experiments where the stimulus is locally applied (e.g. by localized mechanical injury, or even by a localized implant of a relatively indiffusible chemical substance), but it is a rather less plausible explanation of the activity of an evocator which acts in solution. It then becomes necessary to suppose that some cells of the exposed ectoderm (e.g. those not protected by the 'surface coat') are much more readily accessible to the substance than the others, which are the survivors. This may in some cases be true; but the suggested mechanism has by this time become rather complicated, and there is usually no good reason to prefer it to the simpler supposition that some substances can act on the healthy ectoderm cells in such a way as to cause the release of their stores of previously inactivated evocator. If this release of the evocator occurs while the ectoderm still retains its competence then the cells would presumably respond by neuralization to the active evocatory stimulus now released within them. This is probably what happens when explants of competent ectoderm are exposed to artificial evicators in solution, where all the cells may respond by neuralization without any of them showing signs of necrosis (Shen, 1942). There is a certain similarity between an artificial evocator acting indirectly in this way and the normal evocator, since we know that when the latter diffuses from the mesoderm into the ectoderm it not only causes neuralization but also stimulates the ectoderm to produce more evocator substance, whose activity is exhibited in the phenomenon of ‘homoiogenetic induction’, i.e. induction of neural tissue by implants of neural tissue. We may speak of this as a physiological activation of the bound evocator, as opposed to the cytolytic activation which occurs when definitely lethal conditions, such as heat coagulation or mechanical disruption, are applied.

It is, then, very possible that the carcinogen evicators cause a physiological activation of the previously inactive evocator. If so, it would be most simple to suppose that the locus of this activation is at the lipocondria, where the hydrocarbon can be seen to accumulate. We have seen that after treatment with benzpyrene the lipocondria rather rapidly break down, their lipide constituents running together to form larger liposomes. Holtfreter (1946c) has shown that a similar process occurs in normal development and is noticeable first in the archenteron roof, that is, in the tissue in which the evocator first becomes active. It is tempting to suggest that this breakdown of the lipocondria is actually the process of liberation of free evocator from an inactive complex which was first adumbrated some dozen years ago (Waddington, Needham, and Brachet, 1936).

It should be noted that this suggestion does not entirely conflict with Brachet's hypothesis as to the importance of ribonucleoprotein granules in the induction process. Brachet (1943) has shown that such granules increase in number in the archenteron roof as it invaginates, that is, at the same time and place as the lipocondria break down. Now the lipocondria certainly consist not only of lipide but also contain protein material, probably arranged as an
envelope covering the lipide core (Holtfreter, 1946a). There is as yet no convincing evidence that this envelope is nucleoprotein in nature, but we have found that the lipochondria stain, albeit rather faintly, with pyronine and lose their affinity for that stain after digestion in crude ribonuclease. It is, therefore, by no means impossible that the appearance of Brachet’s granules is not merely correlated with, but is an actual consequence of the breakdown of the lipochondria, and it is possible to suppose that they are in fact the liberated evocator.

Holtfreter (1948) drew attention to the fact that during cytolysis, when the evocator is known to become liberated, the lipochondria can be seen to break down and their lipide constituents to become free. It is clear that he considered that the correlation between the two phenomena might be significant. In his discussion of the matter, however, he does not pursue it very far; instead he turns to a mention of the cytoplasmic basiphilic granules, which he derives, not from the protein part of the lipochondria, but by an ‘Entmischung’ from the originally clear cytoplasm. He suggests that both the mobilization of the lipides from the lipochondria and the precipitation of granules in the cytoplasm are secondary consequences of changes in the cell surface, these being of such a kind as to lead to increased permeability. It appears to us that the demonstration that the carcinogen evocators become accumulated directly at the lipochondria, and not at the cell surface, favours the simpler hypothesis advanced above, that the evocator-liberation takes place directly at the lipochondria.

Further work is required, however, before this can be accepted as more than one of a number of possibilities. It still remains possible that it is the lipoid component of the lipochondria which is the active evocator. And finally we cannot yet afford to overlook the possibility that the carcinogens are acting not as activators, either cytolytic or physiological, but as direct evocators which are chemically sufficiently allied to the natural substance to simulate its action on the ectoderm cell. What has been gained in this investigation is not a final resolution of these various alternatives, but a strong suggestion that the lipochondria are the site of evocator activation and possibly of evocator action.

SUMMARY

Cells from explants of newt gastrulae which had been cultured in Holtfreter’s standard solution containing the carcinogen 3:4-benzpyrene rendered soluble by caffeine were examined for fluorescence by ultra-violet light. The lipoid constituents of the cells, lipochondria and liposomes, showed the bright blue fluorescence of benzpyrene in molecular solution and there was also occasionally a structureless blue fluorescence round the nucleus corresponding perhaps to the so-called ‘Golgi Apparatus’. The yolk platelets were slightly fluorescent but this was probably due to the associated lipides and it largely disappeared in moribund preparations, where the lipochondria became detached from the platelets. The nucleus showed no fluorescence; nor was a fluorescent nucleolus observed. It could be shown that the pyronine-
staining cytoplasmic granules of ribonucleoprotein (microsomes) were not fluorescent and so probably do not have any affinity for the carcinogen. The very small fluorescent granules in Brownian movement that had previously been looked upon as ribonucleoprotein microsomes are in fact small liposomes and do not stain with pyronine.

It is suggested that the evocating power of the carcinogenic hydrocarbons is probably not due to cytolytic activation of the evocator, by the killing of some cells in the exposed ectoderm. It may be by physiological activation, operating through a specific action on the lipo-chondria, leading to their breakdown into liposomes and the release of active evocator substance. Some evidence is produced which suggests that the lipo-chondria contain ribonucleoprotein, and this fraction of them may constitute the active evocator; this would be congruent with the theories of Brachet (1947). It cannot yet be excluded, however, that the hydrocarbons act as direct evocators, simulating the effects of the naturally occurring substance.

The observation that benzpyrene is not accumulated by nucleoprotein makes it likely that the mutagenic activity of the carcinogens is indirect.

REFERENCES

— 1940. Ibid., 50, 196.
— 1944a. Ibid., 95, 171.
— 1944b. Ibid., 95, 307.
— 1946b. Ibid., 102, 51.
— 1946c. Ibid., 103, 81.