Factors Affecting Carcinogenesis

IV. The Effect of Tricaprylin Solutions of Cholesterol and Phospholipins

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In this series of publications (5, 6, 22) we have studied the incidence of local sarcomas resulting from the subcutaneous injection of a standard dose of 3,4-benzpyrene into mice. It was found (6) that there was a significantly higher tumor incidence when the solvent was a sample of mouse fat consisting almost entirely of neutral glycerides than with another sample of mouse fat (5) that was less pure and contained appreciable amounts of other lipids, notably cholesterol and phospholipins. It was of interest to study the effect of these two lipids independently in a synthetic medium (tricaprylin) at about the concentration in which they were present in the sample of mouse fat that inhibited carcinogenesis. We had previously found (22) a strong inhibitory effect of phospholipins, but as the concentrations then employed were very much higher than that in the mouse fat, these experiments did not prove that the inhibitory effect of mouse fat was due to its phospholipin content. Furthermore, in the light of more recent experience we feel that two objections may be raised against the experiments: (a) our mixtures of equal parts of lecithin or cephalin with tricaprylin were plastic pastes, the consistency of which differed greatly from that of the oily solvents with which they were compared. We now hold that, to exclude physical factors, the consistency of the solvents should be similar. (b) The implantation technic by which the lipid mixtures were applied in most cases does not give a sufficient guarantee against primary leakage of the soft material through the incision; and the slough that always forms on the wound may conceal a secondary leakage through ulceration. In view of these facts a reinvestigation of the phospholipin effect seemed indicated.

EXPERIMENTAL

PREPARATION OF PHOSPHOLIPINS

Two sheep's brains (280 gm.) were extracted 3 times at 0°C for 24 hours with acetone, 1 litre for each extraction. The dry acetone-insoluble residue (55 gm.) was shaken at room temperature under nitrogen in 4 changes of ethanol, 800 ml. The alcohol-insoluble residue was twice extracted with light petroleum at room temperature. The alcohol and petroleum solutions were evaporated (all distillations were done in nitrogen under reduced pressure), the combined residues were taken up in peroxide-free ethyl ether (300 ml.), and 1 litre of acetone was added. This acetone precipitation was repeated with the solution of the first precipitate in ether, and the resulting precipitate was dissolved in chloroform, washed with dilute sodium chloride solution, and the dried chloroform layer evaporated. The ether solution of the residue was cleared by centrifuging and then precipitated with acetone. This precipitation was repeated 5 times, until the material gave a perfectly clear ethereal solution. The final acetone precipitate was extracted with ethanol and the filtered solution evaporated, dissolved in ether, and precipitated with acetone. The acetone precipitate was dissolved in ethanol and the filtered solution evaporated, dissolved in ether, and precipitated with acetone. The acetone precipitate was dissolved in ether and left overnight at 0°C. It was then centrifuged and again precipitated with acetone. After solution in ethanol, the lecithin was finally re-precipitated from ether by acetone: the yield was about 1 gm. of dry substance.

The greater part of the material remained in the alcohol-insoluble fraction. This was dissolved in ether and the clear solution precipitated with acetone. The precipitated cephalin fraction was thoroughly washed with acetone and dried.
Preparation of Mixed Phospholipins for Injection Experiments

Portions of 1 gm. each of the cephalin and lecithin preparations were mixed, dissolved in ether, and the solution, cleared by centrifuging, was precipitated with acetone. The dried precipitate contained 3.96 per cent of phosphorus. Sufficient of the mixed phospholipin to give a 3 per cent solution was dissolved in a solution of 3,4-benzpyrene in tricaprylin containing 1 mgm./ml. of the hydrocarbon. As in all these experiments, each mouse received 0.3 ml. of the solution; i.e., 0.3 mgm. of benzpyrene.

The control solution contained the same quantity of benzpyrene in pure tricaprylin. The 3 per cent solution of cholesterol in tricaprylin used also had the same benzpyrene content.

Tumor Incidence

The results are summarized in Table I, which gives the numbers and percentage incidence of tumors after 20 and 30 weeks, and in Fig. 1. The incidence with tricaprylin as solvent is in good agreement with our other experiments in which we tested this solvent (22, 6), the actual figure being 46 per cent in the present series. The first sarcoma appeared after 11 weeks, and the median incidence was at 20 weeks. In 1 animal killed owing to the presence of an ulcer after 10 days, the bile and intestines showed brilliant fluorescence, presumably due to metabolism of the injected benzpyrene. No distant tumors were observed in any of the mice injected with these 3 solvents; all the neoplasms were local sarcomas.

In the cholesterol series the total incidence was high: 82 per cent. Two tumors appeared after 10 weeks, and the median time was 15 weeks. Animals that died 10 and 16 weeks after injection still had a local oil depot.

In the phosphatide series the main effect was a delay in tumor incidence, as compared with tricaprylin. The first tumor arose after 12 weeks, and the median incidence was at 24 weeks. Up to the 23rd week there were only 2 sarcomas in an effectual total of 17 mice, whereas at the same period in the tricaprylin series the total was 11 out of 26 mice. The difference from the cholesterol series was even more pronounced; the latter had at this period 22 sarcomas in 28 mice.

Statistical Analysis

The significance of the differences in tumor incidence according to the solvent used were analyzed by the $\chi^2$ test. The solutions containing cholesterol and diminution slowly until they were no longer palpable or until a local sarcoma began to appear, but in some mice no diminution could be felt. In the ulcerated mice (all of which were discarded) the lumps promptly vanished after the appearance of the ulcer. There was little difference between the tricaprylin and cholesterol series, but in the phosphatide series the lumps tended to be larger and more persistent; however, occasional large and persistent lumps were seen in the cholesterol series also.

Animal Experiments

The technic of injection has already been fully described (6). The only difference in the present series was the use of pure strain mice (males of the Glaxo FF strain). For each of the 3 solvents a group of 45 animals was injected; 30 were used for the observation of tumor incidence and the remainder for estimation of the rate of elimination of benzpyrene; again the details were as previously described (6).

Ulceration after the injection was negligible with the pure tricaprylin and the cholesterol solutions, but it was found on careful observation to be present at about the tenth day in one-third of the phospholipin series; all such mice were, of course, discarded. This unfortunately reduced the effectual total in the phospholipin series to about 20, and circumstances did not allow their replacement by similar mice. No late ulceration or sloughing occurred in any of these experiments.

The mice used for analysis were also closely observed for possible ulceration, and only those free of it were taken for benzpyrene estimations.

Observations During Latent Period

Soft subcutaneous lumps or cysts were palpable for periods varying from a few weeks to the whole duration of the experiment (30 weeks) in almost all the mice injected. The usual course was for the size to

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of mice</th>
<th>No. died before first tumor</th>
<th>No. with ulcers</th>
<th>Effectual total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatides (3%) in tricaprylin</td>
<td>30</td>
<td>2</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Tricaprylin</td>
<td>30</td>
<td>2</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>Cholesterol (3%) in tricaprylin</td>
<td>30</td>
<td>1</td>
<td>1</td>
<td>28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total local sarcomas</th>
<th>20 weeks</th>
<th>30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>23</td>
</tr>
</tbody>
</table>
phospholipins respectively were compared with the pure tricaprylin solvent as the standard of reference for two periods; i.e., after 20 and after 30 weeks. The results are shown in Table II. In the comparison between the phospholipin solution and tricaprylin after 20 weeks one of the "expected" values was below 5 (4.75); Yates' continuity adjustment was therefore applied in this case (9). A P-value of 0.13 was found.

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Without correction for continuity P=0.06. The true value of P lies between these two values, near 0.1. This is not a significant result in the statistical sense, but it suggests that more extensive data might satisfy the criterion of significance. After 30 weeks the percentage incidence in the phospholipin series had risen to that in the tricaprylin series. These two groups were therefore combined for the comparison with the cholesterol series. The higher tumor incidence in the latter is highly significant, whether compared with the tricaprylin group after 20 weeks or with the combined tricaprylin and phospholipin groups after 30 weeks.

We now have available the results of three experiments with tricaprylin as solvent. In addition to the present experiment the first was described in Part II (22), and the second in Part III (6) of this series of publications. A combination of these results, which are in reasonable agreement, provides sufficient material for a standard of reference by comparison with which it is possible to distinguish between cocarcinogenic and anticarcinogenic activity. Table II includes some data taken from our earlier papers, which are now compared statistically with the combined tricaprylin results. The original sample of mouse fat (mouse fat A, fresh), which we have so far regarded as anticarcinogenic, is shown not to differ significantly from tricaprylin, although, as in the case of the present phospholipin experiment, the fairly low value of P=0.16 suggests a tendency towards inhibition. On the other hand, the results of the sesame oil experiment and the combined results of the sesame and arachis oil experiments reveal a highly significant cocarcinogenic activity of these solvents. It has previously been shown that our sample of mouse fat B possessed similar properties (6).

**Table II: X² Test Applied to Figures of Tumor Incidence**

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>X² (N-1)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFTER 20 WEEKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13/14</td>
<td>3.641</td>
<td>0.06</td>
</tr>
<tr>
<td>15/14</td>
<td>2.435</td>
<td>0.13</td>
</tr>
<tr>
<td>15/14</td>
<td>7.361</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AFTER 30 WEEKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(13 + 14)/15</td>
<td>9.014</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3/TC</td>
<td>2.037</td>
<td>0.16</td>
</tr>
<tr>
<td>1/TC</td>
<td>13.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(1 + 2)/TC</td>
<td>10.67</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* 1 = sesame oil (5),
  2 = arachis oil (5),
  3 = mouse fat A, fresh (5),
  13 = tricaprylin containing 3% phospholipins,
  14 = tricaprylin,
  15 = tricaprylin containing 3% cholesterol.
  TC = combined tricaprylin experiments.
† Corrected by Yates' continuity adjustment.

**Elimination of 3,4-Benzpyrene**

Figs. 2 to 4 represent for each of the three series the results of benzpyrene analyses performed with mice selected at random and killed at regular intervals of 1 week. In the Figures, log S (= log of quantity of benzpyrene in micrograms remaining) is plotted against the number of days. The straight line is the linear regression curve. The numerical values of the linear regression coefficients and their standard errors are
Fig. 2—Rate of elimination of 3,4-benzpyrene after its subcutaneous injection in tricaprylin.

Fig. 3—Rate of elimination of 3,4-benzpyrene after its subcutaneous injection in tricaprylin containing phospholipins.
TABLE III: ELIMINATION OF BENZPYRENE WITH VARIOUS SOLVENTS

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Solvent</th>
<th>No. of observations</th>
<th>Linear regression coefficient, b</th>
<th>Standard error of b (S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Phospholipins (3%) in tricaprylin</td>
<td>11</td>
<td>-0.00619</td>
<td>0.00106</td>
</tr>
<tr>
<td>14</td>
<td>Tricaprylin</td>
<td>11</td>
<td>-0.0157</td>
<td>0.00116</td>
</tr>
<tr>
<td>15</td>
<td>Cholesterol (3%) in tricaprylin</td>
<td>12</td>
<td>-0.0274</td>
<td>0.00354</td>
</tr>
</tbody>
</table>

Table III shows the elimination of benzpyrene with various solvents. The table includes the serial number, solvent type, number of observations, linear regression coefficient, and standard error of the coefficient. The elimination is fastest in the cholesterol solvent experiment, intermediate in the tricaprylin experiment, and slowest in the phospholipin experiment. All the differences are highly significant.

FIG. 4.—Rate of elimination of 3,4-benzpyrene after its subcutaneous injection in tricaprylin containing cholesterol.

In considering cocarcinogenic or anticarcinogenic effects, it is important to have a stable and reproducible standard of reference, which should itself be absolutely neutral towards carcinogenesis. Tricaprylin, which we chose following the example of other authors (12, 18), seems to fulfill these requirements adequately. The results of the three independent experiments reported in this paper and in Parts II and III of the series (6, 22) are in reasonably close agreement, in spite of the fact that they were carried out on different mouse.

DISCUSSION

The linear regression coefficient, $b$, is the statistical mean value of $k$.
populations. The somewhat lower tumor incidence in the first experiment, 33 per cent (22), may have been due to the fact that old female mice of mixed stock were employed, for Leiter and Shear found the incidence of induced tumors to be 44 per cent higher in the males than in the females of strain A (12). The second and third experiments gave identical figures of percentage incidence (46 per cent), although the second was done on male mice of mixed stock and the third, reported in the present paper, on male mice of an inbred strain. Another important requirement that is satisfied in our experiments is that the carcinogenic dose should be so chosen that the tumor response in the experiment that is to serve as the standard amounts to about 50 per cent; in that case even weak cocarcinogenic or anticarcinogenic influences have an equal chance of shifting the result.

By comparison with tricaprylin the solvents so far tested in our experiments may be arranged as follows: (a) a group of cocarcinogenic solvents comprising sesame and arachis oil, mouse fat B, and tricaprylin containing 3 per cent cholesterol; (b) a group probably possessing anticarcinogenic properties; i.e., mouse fat A, and tricaprylin containing 3 per cent phospholipins; (c) cod liver oil, with a tumor incidence resembling that of tricaprylin itself. In the cocarcinogenic group we find a statistically highly significant increase of tumor incidence that seems to be associated with a shortening of the latent period. We also find, at any rate in the mouse fat B and the cholesterol-tricaprylin group, a significant increase of the elimination rate of benzpyrene. Statistical comparison between the linear regression coefficients for the elimination rate shows that the coefficient for mouse fat B, as determined in the preceding paper (6), is significantly higher than that found for pure tricaprylin, as reported in the present investigation (\( t_{0.025} = 3.126; P = 0.01 \)).

It is possible, though it cannot be proved at present, that the cocarcinogenic effects are due entirely to the sterol content of these solvents. This would of course presuppose that sitosterol, which occurs in oils of plant origin in considerable amounts, is in this respect equivalent to cholesterol. It is intended to test this point by experiment. The cholesterol content of mouse fat B (0.167 per cent) was low, but not negligible; but its cocarcinogenic effect was also lower than that of the other members of the group. An interesting cocarcinogenic effect of cholesterol has been described by Baumann, Rusch, Kline, and Jacobi (1, 17), who found that tumors induced by benzpyrene painting or by ultraviolet irradiation of mouse skin were stimulated by the application of cholesterol in an oily medium (cottonseed oil), but not by cholesterol in benzene.

In the group termed anticarcinogenic the effects are less striking. Though the percentage incidence of tumors with mouse fat A was lower than the combined results of the three tricaprylin experiments, the difference is not statistically significant. With phospholipins in tricaprylin the final tumor incidence reaches the level of that observed with tricaprylin, but there was a pronounced lengthening of the latent period. The impressive nature of the contrast between vegetable oil and mouse fat in our original experiment (5) was of course due to the fact that the comparison was unwittingly made with solvents that we have now reason to believe are strongly cocarcinogenic. Nevertheless, we still hold that there are solvents with anticarcinogenic properties, and that phospholipins are at least partly responsible for their action. Though our evidence may not yet be statistically convincing, it is reinforced by the following facts: (a) the association of accelerated elimination with shortened latent period observed in the cocarcinogenic group has its complete counterpart in the experiment with phospholipins in tricaprylin, where a lengthening of the latent period was accompanied by a much delayed excretion. (b) Though our older experiments, in which pellets of ox brain lipids (5) and of purified lecithin and cephalin (21) were implanted, are not up to the stringent standards we have now adopted, owing to the different physical state of the solvents and the possibility of unobserved leakage through the incision wound, they point at least in the same direction. (c) There can be little doubt that the crude extracts of “egg yolk fat” or “chicken fat” (15), of unpurified mouse fat (14, 16), and rat fat (20), by the use of which the inhibitory action of solvents was first observed, contained considerable amounts of phospholipins, in view of the fact that they were prepared from phospholipin-rich sources without purification. Some of them (14) were probably more powerful inhibitors than our preparation of mouse fat A, which had been partially purified by acetone precipitation. It seems also quite probable that the puzzling variability of different batches of lard as a solvent (12) resides in the very variable phospholipin content of this material (13).

The case of cod liver oil is rather complicated. Here we have a rapid elimination combined with a “neutral” tumor incidence. Cod liver oil contains from 0.5 to 2.0 per cent cholesterol (11), but it also contains an assortment of unusual lipids, sterols, steroids, hydrocarbons, and antioxidants, any one of which may exert an antagonistic effect on cholesterol. Furthermore, cod liver oil causes a peculiar tissue reaction (6). The possible play and counterplay of a variety...
of factors precludes a simple explanation. The same is true for lanolin, which, though a rich source of cholesteryl esters, prevents carcinogenesis in the skin after methylcholanthrene painting (19). In order to explore the mechanism of cocarcinogenic or anticarcinogenic solvent action, it seems to us preferable to use in future simple synthetic systems where the conditions are more transparent.

Our present experiments are a first step in this direction. The correlation here observed between rapid elimination of the carcinogen, shortening of the latent period, and increased tumor incidence on the one hand, and between delayed elimination and lengthening of the latent period on the other, is not invalidated by the lack of such a correlation in other more complicated solvents where disturbing factors of a chemical, physical, or biological nature may intervene.

We suggest that one of the main factors determining carcinogenesis is the rate of metabolism of the carcinogen. From the investigations of Chalmers and Peacock (4) and of Berenblum and Schoental (2) the metabolism of 3,4-benzpyrene seems to be predominantly an oxidative process, of which the first stable product is the 8-substituted phenol. At the present time the view prevails that this reaction is a detoxication, and Fieser (7) even postulates a competition for benzpyrene between this supposedly "detoxicating" mechanism and some other unknown reaction that is assumed to be that involved in the actual carcinogenesis. This view is based mainly upon the assumption that the physiological metabolites are noncarcinogenic. This, however, is a question upon which the present evidence is insufficient. There may be stages of oxidation preceding the formation of a phenol, as suggested by Weigert's recent work (21). As to the phenols themselves, those hitherto tested have been for the most part not those formed in vivo. Also, the unstable character and greater water-solubility of these compounds make it difficult to maintain a sufficient concentration in contact with the tissues for a sufficient length of time, whereas this is assured if the phenol arises from the hydrocarbon by a steady process of metabolism. From this point of view it is of particular interest that 8-methoxy-3,4-benzpyrene gave no less than 80 per cent of tumors, when injected into mice in a dose of 2 mgm. (3). This is an activity approaching that of the parent hydrocarbon.

If it is assumed, then, that oxidation is an essential condition for active carcinogenesis, our results become understandable. Thus we interpret the cocarcinogenic action of cholesterol as the result of an increased rate of oxidative metabolism of benzpyrene. On the other hand, the presence of phospholipins in the solvent diminishes considerably the rate of oxidative metabolism of benzpyrene, and thus diminishes its carcinogenic activity. Phospholipins are well-known antioxidants, and we have shown in unpublished in vitro experiments that the induced oxidation of benzpyrene is in fact strongly inhibited by cephalin. By the study of other antioxidants, acting alone or synergistically (10), it is hoped to obtain further evidence in support of our hypothesis.

Whereas the antioxidant properties of phospholipins suggest a rational explanation for their anticarcinogenic activity, if only in the form of a provisional working hypothesis, the mechanism of the cocarcinogenic effect of cholesterol is quite obscure. Some steroids, such as deoxycholic acid (8) and cholestenone sulphonic acid (23), are known to form molecular compounds with hydrocarbons, thereby increasing their solubility in water; perhaps a similar process is at the basis of the cholesterol effect.

If the interpretation of our results as outlined above proves to be correct, some conclusions as to the actual carcinogen involved are justified. In the phospholipin experiment a considerable quantity of benzpyrene must have lain in contact with the tissues for a long time before any tumor resulted; we assume that the phospholipin inhibited the oxidation of benzpyrene, which is a necessary condition for carcinogenesis. It would therefore appear probable that the hydrocarbon is not itself the true carcinogen, but the metabolite derived from it, presumably as a first oxidation product.

SUMMARY

Three groups of mice were injected subcutaneously with a single dose of 0.3 mgm. of 3,4-benzpyrene dissolved in: (a) tricaprylin, (b) tricaprylin containing 1.5 per cent lecithin and 1.5 per cent cephalin, (c) a 3 per cent solution of cholesterol in tricaprylin. Observations were made of tumor incidence and rate of elimination of benzpyrene. Tricaprylin was taken as the standard solvent for comparison with the others.

The tumor incidence at 20 and 30 weeks was: cholesterol series, 79 and 82 per cent; tricaprylin, 38 and 46 per cent; phospholipins, 12 and 47 per cent respectively. The increased incidence with cholesterol is highly significant ($P<0.01$), but the retardation with phosphatides is not statistically proved ($P=0.1$) and a larger number of observations would be required to establish it. From a consideration of all the evidence it appears probable, nevertheless, that the inhibitory action of phospholipins on carcinogenesis is genuine. The anticarcinogenic effect observed by several authors for various samples of animal fats might well be due to their content of phospholipins. The complex com-
position of most natural oils makes it preferable to use pure synthetic vehicles where possible, as otherwise the interpretation of results is hardly possible (e.g., with cod liver oil). It is shown that, compared with tricaprylin, the solvents (arachis and sesame oils) previously used by ourselves and others for comparison with mouse fat as solvent, are themselves cocarcinogenic. The differences in carcinogenic activity previously attributed to the anticarcinogenic activity of mouse fat are in part due to this fact.

The rate of elimination of benzpyrene is accelerated when the solvent contains cholesterol ($k=0.027$), and inhibited in the phospholipin solution ($k=0.0062$), compared with tricaprylin ($k=0.016$); the differences in these rates are highly significant ($P<0.01$). The fact that the more rapid elimination of benzpyrene is associated with higher carcinogenic activity, and slower elimination with lower activity, leads us to suggest that, contrary to frequently expressed opinion, the rapidity of elimination of the carcinogen is associated within certain limits with high carcinogenic activity. We suggest that the oxidative metabolism of the carcinogenic hydrocarbon may be a necessary condition for its carcinogenic activity. If this is so, it is probable that an oxidative metabolite of the hydrocarbon is the true carcinogen rather than the hydrocarbon itself.

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