SUMMARY

When films of plastics or metals are imbedded s.c. in rodents, the material becomes enveloped by a connective tissue pocket wherein tumors may eventually appear. Biochemical studies were made on this tissue at different intervals after imbedding. These included assays for lactate, malate, and glucose-6-phosphate dehydrogenase activities; determinations of DNA; and analyses for soluble and insoluble collagen.

It was found that the activities of the 3 enzymes were highest at one month after imbedding and then decreased markedly. On the other hand, the nucleic acid concentration either remained constant or decreased to a small extent. The soluble collagen was elevated at the early stages and decreased to a significant degree, while the insoluble collagen increased with time until a maximum was reached. Subsequently, the latter either remained constant or decreased slightly.

The possible relationship of these changes with the ultimate appearance of tumors is discussed.

INTRODUCTION

When tumors are induced in rodents by implanting films of plastics, they arise in the connective tissue pocket which envelops the implanted material (10, 11). Although the latent period is generally more than a year, it is not necessary for the implant to remain in situ for the entire period. With polystyrene, it was found that tumors develop even if the film is removed after 6 months (11); with cellophane, the minimal period is even less (14). It is therefore probable that the changes which lead to the ultimate appearance of the tumor take place within the connective tissue pocket during the first few months after the imbedding.

In order to learn more with regard to the processes involved in the carcinogenic action of the imbedded films, histologic and chemical studies were carried out on the connective tissue pocket during the precancerous period. Previous publications have dealt with histologic studies (11–13) and with the composition and amount of muco polysaccharides of the pocket tissue (4). The present paper is a report on some enzymes of this tissue, and the amount of DNA and collagen. Most of the investigations were made on pockets formed after imbedding cellophane and glass. These were chosen because cellophane yields the highest percentage of tumors and glass a comparatively low number. It was expected that a comparison of the two might indicate some of the phenomena which are related to the carcinogenic process.

MATERIALS AND METHODS

Glass cover slips and films of cellophane were imbedded subcutaneously in Wistar rats according to procedures described previously (9). At monthly intervals after insertion, the imbedded material, together with the connective tissue pocket, was removed from the different groups of animals. The pocket was separated from the film and analyzed for the three enzymes, total protein, and DNA. Generally, 10 pockets were taken at each interval. The pockets were minced and homogenized at 0°C with 0.9% NaCl for 2 min. The homogenates were centrifuged for 30 min at 20,000 × g, and the supernatant solution was assayed for protein, lactate dehydrogenase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase. DNA was determined on the remaining tissue.

The enzyme assays were conducted by procedures based on the absorbancy of the pyridine nucleotides at 340 nm (17). In each assay system, the concentrations were such that the activity was directly proportional to the amount of extract added. A unit of enzyme activity was defined as the amount of enzyme which catalyzed the conversion of one micromole of substrate per min. Protein determinations were carried out on aliquots of the extracts by the procedure of Lowry et al. (7).

DNA was determined on the individual pockets by the indol procedure (1, 2) after purification and fractionation of the nucleic acid components according to Schneider et al. (16).
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RESULTS

The enzyme activities in pockets around cellophane and glass from 1 to 7 months after imbedding of the film are shown in Table 1. All the three enzymes studied had the highest activity 1 month after imbedding and these subsequently dropped to lower values. Throughout the entire period malate dehydrogenase activity was higher than lactate dehydrogenase. Glucose-6-phosphate dehydrogenase was detectable only during the 1st two months and then fell to values below the limit of the assay method. The malate dehydrogenase activity in the pocket formed around glass was about the same as that around cellophane with only a small difference in the 5th- and 6th-month period.

The lactate dehydrogenase activity in the pocket around cellophane at 1 month was considerably higher than that around glass. With both materials the activity decreased with time so that at 3 months there was no difference between them. Unlike the pocket around glass, the one surrounding cellophane showed a rise in lactate dehydrogenase at 5-6 months.

The pockets formed around cellophane were significantly thicker than those around glass. This can be seen by the difference in weight of these pockets at different intervals after the imbedding (Table 2). Both types of pockets contained about 80% water so that the dry weight is 20% that of the wet weight. The pockets around cellophane were approximately 3 times the weight of those formed around glass. The amount of cellular component in the pockets is reflected in the results on DNA analyses (Table 2). There was a relatively high degree of variability in the amount of DNA in given pockets even at the same interval after imbedding. Several generalizations may, however, be made. The DNA concentration in the pockets around glass does not change significantly during the 7-month period. On the other hand, the DNA in the pockets around cellophane decreases to a significant extent while the weight of the pocket increases. This situation would suggest that, during the later months, there is a denser cell population in the pocket around glass that there is in the pocket around cellophane.

The results on the collagen for cellophane and glass are quite similar to each other. The NaCl-soluble collagen is highest at 1 month then decreases significantly until it reaches minimal values after 6 months. The tissue was homogenized and extracted first with 2 ml of 0.5 M NaCl for 1 day and then with 3 ml of 0.5 M citrate buffer, pH 3.6. Aliquots of each of the 2 extracts and the insoluble residue were hydrolyzed with 6 N HCl for 18 hr. Each fraction was then assayed for hydroxyproline by the method of Prockop and Udenfriend (15). Values for percent collagen were obtained by multiplying the figures for percent hydroxyproline by 7.46 according to Neuman and Logan (8).

Another set of imbeddings was used for collagen determinations. The materials imbedded for these studies were cellophane, glass, polystyrene, nylon, Teflon, silver, and stainless steel. The pockets were removed at monthly intervals after imbedding for a period of 6 months. The tissue was homogenized and extracted with 6 N HCl for 18 hr. Each fraction was then assayed for hydroxyproline by the method of Prockop and Udenfriend (15). Values for percent collagen were obtained by multiplying the figures for percent hydroxyproline by 7.46 according to Neuman and Logan (8).
at 4-6 months. The citrate-soluble collagen is maintained at a higher level for about 3 months. Insoluble collagen increases during the first few months and reaches a maximum at 3-4 months. Subsequently, it drops to somewhat lower levels although the results are highly variable in this respect (Table 3).

A similar overall picture was obtained with five other materials (Chart 1). There are some variations as to the specific time at which the maxima or minima occur, reflecting differences in the rate of formation of new collagen. This, in turn, is dependent on the reaction of each specific imbedded material with the adjacent tissue.

### DISCUSSION

The principal components of the connective tissue pocket formed around imbedded films are similar to those found in granulation tissue during wound healing (5, 6). There is an accumulation of fibroblasts (10, 11, 14), an increase in mucopolysaccharides (4, 12), and a deposition of collagen.

The relative amounts of collagen in the different fractions is an index of the degree of collagen formation. Collagen synthesis apparently involves the preliminary formation of soluble collagen protein which is subsequently modified to a less soluble form, and finally converted to the extracellular insoluble product (3). The collagen in the early stage of synthesis is soluble in dilute neutral salt solutions, the material in the intermediate stage requires acidic buffers for solubilization, and the final aged product is comparatively insoluble (3, 6). The amount of collagen in the sodium chloride-soluble fraction of the connective tissue pocket is highest during the initial months after imbedding, thus reflecting active collagen synthesis. Subsequently, this decreases to a minimal level while the amount in the acid citrate fraction is maintained at a higher level for a more extended period. The relative amount of insoluble collagen increases gradually and reaches a maximum at 3-4 months. At this time the pocket is completely formed and synthesis of new collagen becomes minimal. This is the general sequence of events for all the pockets which were studied although there are variations between different materials imbedded as to the actual concentrations at specific time intervals.

The cellular component of the pockets, as reflected by the values in the DNA determinations, is somewhat higher in those formed around cellophane as compared with those around glass. The DNA in the cellophane pocket decreases slowly during the first 4 months and is about half the original value at 7 months. The DNA in the pockets around glass is originally lower than those around cellophane and decreases only slightly with time.

These studies were made on pockets obtained between 1 and 7 months after imbedding of the films because it was found previously that tumors did not appear if the films were taken out earlier (11, 14). On the other hand, if the films were removed after 6 months a certain number of tumors would eventually appear. This suggested that the neoplastic transformation occurred approximately in the 3- to 6-month period. Thus although various changes may have taken place before the first month elapsed, they would probably not be related to tumorigenesis.

Investigations by Woessner and Boucek (19) on connective tissue development around subcutaneously imbedded polyvinyl sponge showed that lactate and malate dehydrogenase activities passed through a maximum and then declined before the DNA reached its maximum value. However, results with sponges cannot be compared with those of films since the tissue reaction elicited by these two forms are not the same. Furthermore, the results with sponges described changes taking place within the first 32 days whereas the present studies are concerned with changes which occur later.

It is interesting to compare the present results by chemical analyses with previous histologic findings (14). With cellophane, the early stages show a pocket of moderate thickness and a layer of active cells at the surface of the lumen next to the film. At 2 months, the generalized activity is still marked and the collagen fibers are increased. After 4 months the cellular activity decreases while the fibers increase. By 5-6 months the pocket wall becomes almost quiescent consisting of densely packed fibers with fibroblasts and their nuclei flattened between them.

Imbedding glass cover slips, on the other hand, results in a

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**TABLE 3**

Analyses on Collagen in Pockets Formed around Cellophane and Glass

<table>
<thead>
<tr>
<th>Collagen fraction</th>
<th>Months after imbedding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NaCl-soluble, %</td>
<td></td>
</tr>
<tr>
<td>Cellophane</td>
<td>1.53 ± 0.23</td>
</tr>
<tr>
<td>Glass</td>
<td>1.27 ± 0.18</td>
</tr>
<tr>
<td>Citrate-soluble, %</td>
<td></td>
</tr>
<tr>
<td>Cellophane</td>
<td>1.09 ± 0.28</td>
</tr>
<tr>
<td>Glass</td>
<td>1.33 ± 0.24</td>
</tr>
<tr>
<td>Insoluble, %</td>
<td></td>
</tr>
<tr>
<td>Cellophane</td>
<td>30.2 ± 3.8</td>
</tr>
<tr>
<td>Glass</td>
<td>33.4 ± 3.3</td>
</tr>
<tr>
<td>Number of samples</td>
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<tr>
<td>Cellophane</td>
<td>7</td>
</tr>
<tr>
<td>Glass</td>
<td>8</td>
</tr>
</tbody>
</table>

* Percentages are based on dry weight of the pockets.
The quantiative data on the DNA and the histologic observations both indicate a decrease in cellular or fibroblastic activity in the pocket tissue at 5–6 months. However, the decrease in activity of the enzymes which were studied was considerably more pronounced than the decrease in cell population. This indicates that the cells in the older pocket have a much lower metabolic activity than those in the earlier ones. It would thus appear from the present results that the environment in the connective tissue pocket tends to suppress metabolic activity. This suggestion has also been made by Vasilev et al. (18) who concluded that “collagenized tissue is an inadequate environment for cells.” It is conceivable that these adverse conditions lead to a selection process whereby only the more resistant cells survive and multiply, and finally become malignant.

The relative decrease in activity of the metabolic enzymes from one to 6 months tends to be greater with cellophane than with glass. This, in addition to the fact that imbedded cellophane induces a more collagenous pocket, may be the reason for its capacity to induce a higher number of tumors. Histologic studies have shown significant variations in pockets induced after imbedding different types of plastics and metals. The carcinogenic potency of different substances may be related to the variations in the types of pockets they induce. Furthermore, the inability of powdered materials to induce tumors could be related to the fact that they bring about a different kind of connective tissue response than that which occurs around imbedded films (13).

**REFERENCES**


Biochemical Changes in the Connective Tissue Pocket Surrounding Subcutaneously Imbedded Films


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