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THE PATHOLOGICAL EFFECTS OF SUBCUTANEOUS INJECTIONS OF ASBESTOS FIBRES IN MICE: MIGRATION OF FIBRES TO SUBMESOTHELIAL TISSUES AND INDUCTION OF MESOTHELIOMATA

by

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Groups of 20 female CBA mice were injected subcutaneously in two sites with suspensions of crocidolite, amosite or chrysotile asbestos. Each injection consisted of 10 mg fibre suspended in 0.4 ml saline and each animal received 3 injections into each flank at sites well distant from the thorax. For comparison, two groups were similarly treated with crocidolite and amosite from which natural and contaminant oils had been removed by solvent extraction. The most remarkable feature of the results were, first, the apparently specific transport of asbestos fibres of all three types to the submesothelial tissues of the thorax and abdomen, and secondly, the development of extensive inflammatory and proliferative changes in these regions culminating, in 10 instances, in the formation of mesotheliomata. Six animals developed sarcomas at the injection site but, in most mice, there was little cellular response to asbestos in the subcutaneous tissues in contrast to the vigorous reactions seen in serosal membranes. All three types of asbestos induced both injection-site tumours and distant mesothelial changes and the removal of oils from amosite and crocidolite did not abolish—though it may have reduced—these manifestations of carcinogenic activity.

An association between pleural mesothelioma and a history of exposure to asbestos dust was first observed in man by Wagner *et al.* (1960); in an addendum to this paper, the authors also referred to one case of peritoneal mesothelioma in a person exposed to asbestos. Since then, both peritoneal and pleural mesotheliomas have been reported in asbestos workers, their relatives, and in people living near asbestos mines and factories (for review, see Harrington, 1967). The carcinogenic effects of asbestos have also been studied in experimental animals. Schmahl (1958) reported the induction of local sarcomas in rats injected subcutaneously with asbestos but did not mention the type of fibre used. Subsequently, pleural mesotheliomas were induced in rats by intrapleural injections of chrysotile, crocidolite

or amosite (Wagner, 1962, 1966) and in hamsters by intrapleural injections of chrysotile or amosite (Smith *et al.*, 1965a, b).

The mechanism of carcinogenesis by asbestos is unknown, but three important pieces of information have recently come to light. First, certain forms of asbestos (notably crocidolite and amosite) normally contain appreciable quantities of mineral oils (Harrington, 1962, 1965). Secondly, asbestos is also readily contaminated with exogenous mineral oils such as oils from jute bags which are used to store and transport asbestos fibres (Harrington and Roe, 1965). Thirdly, natural and contaminating oils have been extracted from commercial amosite and crocidolite and shown to have tumour-promoting activity on mouse skin (Roe *et al.* 1966).

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Despite the demonstrable activity of these oils, their significance is disputed at the present time (see Wagner, 1966) and further investigations have now been carried out in an attempt to clarify their role. The experiments to be described below were devised to compare the carcinogenic effect on subcutaneous tissues of commercial asbestos with that of fibres from which the oils had been extracted. Three types of asbestos were used—crocidolite, amosite and chrysotile. In the event, the results were not conclusive but they proved to be of considerable interest in another respect. It was observed that asbestos injected subcutaneously was subsequently found in conspicuous amounts in the mesothelium of the pleural, pericardial and peritoneal spaces; striking reactions developed in these sites including the appearance of mesotheliomata.

MATERIAL AND METHODS

Mice. One hundred and twenty CBA female mice were used, aged 6 to 10 weeks and weighing 30-35 g. This strain has been maintained by brother-sister mating in the Chester Beatty Research Institute since 1951 when it was introduced from the University of Minnesota. The animals were fed a cubed diet (Diet 86, Messrs. Dixon Limited, Ware, Herts., England) and water *ad libitum* and they were kept in metal cages, 10 mice in each.

Asbestos. The three types of asbestos used—crocidolite, amosite and chrysotile—were derived from the same batch of finely-divided fibres used in earlier experiments by Wagner (1965), Harington (1965) and Harington and Roe (1965). Natural and contaminating oils were extracted from crocidolite and amosite under reflux with 8 solvents of increasing polarity; these were, successively, petroleum ether (60-80), cyclohexane, carbon tetrachloride, benzene, chloroform, ether, ethyl alcohol and methyl alcohol. Further details of the process are described by Harington (1965). Five preparations were thus available for study: crocidolite, extracted crocidolite, amosite, extracted amosite and chrysotile.

The mice were divided into 6 equal groups, 5 of which were treated with a preparation of asbestos. The fibres were suspended in sterile physiological saline and injected through wide-bore needles (serum 2, 19 gauge) into the subcutaneous tissues of the flank. Each animal was injected in both flanks with 10 mg fibre/

0.4 ml saline and this procedure was repeated twice at intervals of 5 weeks. The control group received saline only.

The animals were examined regularly for the development of tumours at the injection site or other lesions. Sick mice were killed and the survivors sacrificed approximately 2 years after the beginning of the experiment. Except where precluded by post-mortem changes, a careful necropsy was carried out on all animals. Tissues showing any macroscopic abnormality were fixed in Bouin's solution. Paraffin sections were prepared at 5 μ and stained with haematoxylin and eosin, haematoxylin and Van Gieson, Masson's trichrome, Weigert's method for fibrin, and the modified Foot technique for reticulin fibres.

RESULTS

Histological changes at injection sites

The incidence of tumours at the sites of injection is shown in Table 1. In all animals, the tumours were single unilateral lesions, despite the fact that asbestos was injected into both flanks. Six of the seven tumours were sarcomata; five of these were spindle cell lesions (Fig. 1) while the sixth was a pleomorphic tumour with numerous multinucleate giant cells. In one mouse, a mesothelioma developed from the peritoneum of the underlying body wall (Fig. 2). No clear difference in the incidence of injection-site tumours was established in the five experimental groups: extracted crocidolite and amosite appeared to be slightly less active than the corresponding untreated fibres, but the shorter survival time of mice which received extracted crocidolite and amosite may have been responsible for this difference.

The local changes in animals which did not develop tumours were consistent and showed little variation according to the type of asbestos injected (Fig. 3). Asbestos fibres accumulated in the dermis, lying free or within macrophages, just deep to the panniculus carnosus. The free-lying fibres were usually thin needle-like structures but, in some instances they appeared to be greatly thickened and resembled "asbestos bodies". Apart from the presence of macrophages, other signs of reaction were few: fibrosis was both slight and infrequent and inflammatory infiltrates were rare. Asbestos-laden macrophages showed some tendency to coalesce,

TABLE I
INCIDENCE OF INJECTION-SITE TUMOURS

Treatment	No. of mice examined post-mortem 40 or more weeks after first injection	Average survival time of mice examined post-mortem (weeks)	No. of mice with injection-site tumours	Time of appearance of tumours listed in previous column ¹ (weeks)
I. Crocidolite	17	75.3 (Range = 62 to 105)	2 Spindle cell sarcoma	65, 105
II. Extracted crocidolite	14	64.6 (Range = 41 to 104)	0	—
III. Amosite	13	96.4 (Range = 41 to 118)	3 Spindle cell sarcoma (2) Local mesothelioma close to injection site (1)	108, 118 62
IV. Extracted amosite	15	79.7 (Range = 41 to 109)	1 Spindle cell sarcoma	109
V. Chrysotile	12	75.5 (Range = 41 to 108)	1 Pleomorphic sarcoma	108
VI. Saline controls	15	104 (Range = 71 to 108)	0	—

¹ i.e. time when tumour first evident on macroscopic examination of living animal



FIGURE 1
An early injection site sarcoma, surrounded by asbestos fibres. Haematoxylin and eosin, $\times 160$.



FIGURE 2
Mesothelioma developing in abdominal wall beneath injection site. Haematoxylin and eosin, $\times 160$.



FIGURE 3
Injection site showing abundant asbestos, lying free and within macrophages. Note the paucity of the tissue response. Haematoxylin and eosin, $\times 160$.

forming multinucleate giant cells, and in animals surviving for long periods of time foci of necrosis and calcification appeared in the injection sites, these changes were more prominent in mice treated with chrysotile.

Distribution of asbestos at distant sites and the associated tissue reactions

The distribution of asbestos and the reactions evoked by this material are summarized below: the principal histological findings in the thorax and in the abdomen are shown in Table II. Although no clear-cut differences were established between the various types of asbestos which were studied, several important features emerged.

First, it is clear that asbestos fibres are not only disseminated widely from the sites of injection but are also distributed in a highly selective fashion. In the chest, the main sites in which asbestos accumulated were the visceral and parietal pleura and pericardium with involvement of the adjacent parts of the lungs, chest wall, diaphragm and heart. In the abdomen, asbestos was seen on the serosal surfaces of the stomach, intestine, liver and spleen, in the

TABLE II
HISTOLOGICAL REACTIONS IN THE THORAX AND ABDOMEN ASSOCIATED WITH ASBESTOS

Treatment	No. of mice examined post-mortem 40 or more weeks after first injection	Macroscopic deposits of asbestos on serosal surfaces		Inflammatory changes in serosal surfaces		Non-malignant mesothelial proliferation in serosal membranes and adhesions		Mesothelioma ¹		
		Thorax	Abdomen	Thorax	Abdomen	Thorax	Abdomen	Thorax	Abdomen	Thorax and Abdomen
I. Crocidolite	17	10	8	1	2	9	9	2 (71, 82)	1 (65)	1 (71)
II. Extracted crocidolite	14	14	12	2	1	8	11	1 (41)	0	0
III. Amosite	13	4	8	2	1	4	8	1 (104)	3 (59, 68, 109)	0
IV. Extracted amosite	15	7	8	1	2	7	7	0	0	0
V. Chrysotile	12	3	5	1	0	3	5	0	0	0 (73)
VI. Saline	15	0	0	0	0	0	0	0	0	0

¹ Figures in parentheses equal times of post-mortem of mice with mesothelioma.

mesentery, and also in retroperitoneal structures, particularly around the pancreas and kidneys

Secondly, in contrast to the relatively inconspicuous response which was described in the subcutaneous tissues, the serosal elements usually reacted vigorously to the presence of asbestos fibres. A wide range of histological changes was seen although, in some circumstances, it was noticeable that the tissue reactions developed at a small distance away from the deposited fibres (Fig. 4). The most common response was the formation of adhesions, together with thickening of pre-existing fibrous capsules (Fig. 5 to 7). Such changes were usually confined to the surfaces of the organs concerned and were frequently unevenly distributed, forming discrete fibrous nodules. Asbestos fibres, either lying free or within macrophages, were readily seen within them. In two sites—the heart and the liver—these fibrotic zones sometimes extended inwards producing considerable local destruction (Fig. 6, 7). Not infrequently, dilated lymphatics were observed within adhesions; some of them, particularly those in the hepatic capsule and in subpleural zones, contained aggregates of lymphoid cells but asbestos fibres were not identified within them with certainty. Asbestos fibres were, however, often seen in lymph nodes, particularly in mediastinal, mesenteric and pararenal glands. In some animals, the adhesions were infiltrated with granulation tissue and chronic inflammatory cells, but the latter were never seen in large numbers. More frequently, the adhesions contained cells which were regarded as proliferating mesothelial elements. These appeared somewhat swollen and were usually arranged as plaques on serous membranes (Fig. 8). The individual cells varied in shape, ranging from flattened to columnar forms; the nuclei were usually vesicular with an open chromatin arrangement and the cytoplasm was diffuse and faintly eosinophilic. In some animals, a blood-stained ascites was found at autopsy.

The most striking response to asbestos fibres in these sites was, however, the development of mesotheliomata. Ten examples were seen: 4 were confined to the chest, 4 to the abdomen, and 2 involved both body cavities. The tumours were usually multifocal: in the chest, for example, nodules were commonly scattered in the subpleural parts of the lung, the chest wall and the



FIGURE 4

Deposit of asbestos fibres in superficial part of the myocardium. Two "asbestos bodies" are seen but there is virtually no tissue response. (cf. Fig. 6.) Haematoxylin and eosin. $\times 200$.



FIGURE 5

Dense adhesions around the pancreas which are infiltrated with asbestos fibres and macrophages. Haematoxylin and eosin. $\times 160$.



FIGURE 6

Large deposit of asbestos in pericardium with fibrosis and inward extension involving the myocardium. Haematoxylin and eosin. $\times 5.5$.

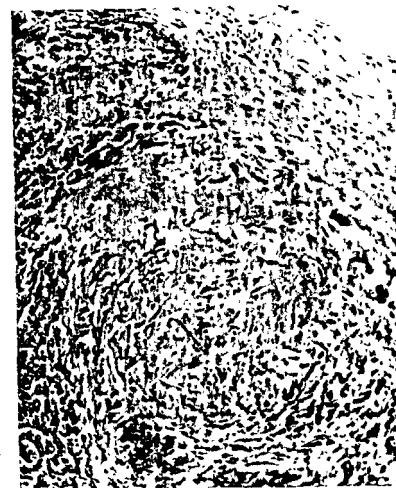


FIGURE 8

Subpleural zone showing an early mesothelioma which has developed on top of a layer containing granulation tissue and non-malignant mesothelial elements. Haematoxylin and eosin. $\times 160$.

diaphragm. No tumours were encountered in the pericardial cavity, but oesophageal involvement was observed in one mouse. In the abdomen, multiple tumours were commonly seen, particularly in the mesentery, in adhesions binding together the liver, stomach, spleen and intestine, and near the pancreas and kidneys (Fig. 9). These lesions were usually arranged either as solid plaques or in more complex configurations with pedunculated or papilliferous patterns. The cells were relatively homogeneous in appearance and, in most instances, their origin from mesothelial cells was readily apparent (Fig. 10, 11). Early neoplastic lesions presented considerable

FIGURE 7

Massive fibrosis of liver capsule extending into the parenchyma. There are numerous asbestos fibres. Haematoxylin and eosin. $\times 160$.

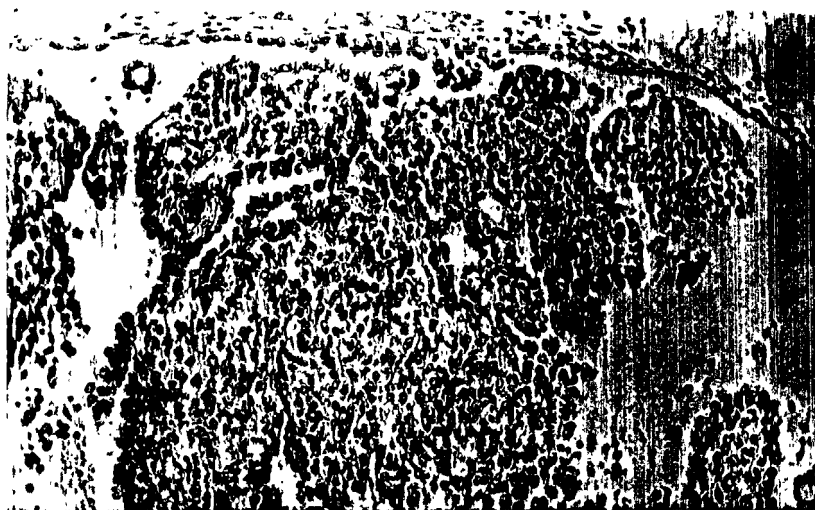


FIGURE 9

Mesothelioma arising from the mesentery, showing a characteristic papilliferous arrangement. Haematoxylin and eosin. $\times 200$.



FIGURE 10

Peritoneal mesothelioma which is infiltrated with lymphoid cells. Haematoxylin and eosin. $\times 200$.



FIGURE 11

Pleural mesothelioma which contains a granulomatous type of lesion at its centre. Haematoxylin and eosin $\times 160$.

TABLE III

INCIDENCE OF TUMOURS OTHER THAN INJECTION-SITE SARCOMAS AND MESOTHELIOMAS

Treatment	No. of mice examined post-mortem 40 or more weeks after first injection	Mammary tumours	Malignant lymphoma	Hepatoma	Pulmonary adenoma	Other tumours
I Crocidolite	17	0	2 (74, 76)	0	0	1 Granulosa-cell tumour of ovary (76)
II Extracted crocidolite	14	0	0	0	0	0
III Amosite	13	0	2 (41, 118)	0	0	0
IV. Extracted amosite	15	2 (65, 109)	2 (41, 109)	1 (109)	1 (109)	0
V. Chrysotile	12	1 (73)	1 (66)	0	1 (108)	0
VI. Saline	15	0	1 (108)	2 (108, 108)	2 (108, 108)	0

NOTE: Figures in parentheses refer to time, measured in weeks, at which the tumours were observed

problems in diagnosis, particularly when inflammatory and fibrotic reactions to asbestos were also well developed in the same region (Fig. 8). In such circumstances foci of mesothelial proliferation were regarded as neoplastic if the

cells concerned were present in reasonably large numbers, showed any tendency to form clumps or to invade underlying tissues, or exhibited the usual cytological features associated with malignant cells. Established tumours were not

infrequently infiltrated with chronic inflammatory cells (Fig. 10), lymphocytes and macrophages often accumulated in clusters within tumours and, in a few instances, ill-defined granulomatous zones were observed (Fig. 11). Asbestos fibres were rarely seen within tumours. Lymphatic channels adjacent to tumour-bearing areas were sometimes dilated and putative tumour cells were occasionally seen within them.

Incidence of tumours other than injection site sarcomas and mesotheliomas

The incidence of neoplasms at other sites, such as malignant lymphoma, hepatoma and pulmonary adenoma, is shown in Table III. A low incidence was observed in all groups and no significant differences were established.

DISCUSSION

Two main findings emerge from the present work. First, asbestos, injected subcutaneously into female CBA mice, is disseminated widely but selectively and accumulates in serosal membranes in the thorax and abdomen. Secondly, the presence of asbestos in these sites evokes a varied and vigorous cellular response, not infrequently culminating in the formation of mesotheliomata. These observations are equally applicable to crocidolite, amosite and chrysotile and although there is a suggestion that removal of mineral oils reduces the carcinogenic properties of the fibres, it has already been stressed that the difference in activity may be distorted by the shorter survival time of mice treated with extracted fibres. The reasons for the poorer survival of mice in these two experimental groups are obscure, but they effectively preclude any firm comparison between the effects of extracted and untreated asbestos.

The distant changes seen in serous membranes are all the more remarkable when contrasted with the relatively feeble response which the various types of asbestos evoke in the subcutaneous tissues. Although a few sarcomas were induced at the sites of injection, these appeared late and, in the majority of animals, there was negligible cellular response to asbestos even though this material persisted for long

periods of time. Conversely, mesotheliomas appeared earlier and in a higher incidence, despite the fact that the concentration of asbestos in serous membranes must be far less than at injection sites.

The histological reactions to asbestos in and around serous membranes of the chest and abdomen have already been described in detail, but certain general points may be considered. It must be stressed that the subcutaneous injections of asbestos were given with great care to ensure that no fibres were introduced into the peritoneal cavity. Routine histological examination of the injection sites indicated that the asbestos deposits were invariably localized to the body wall, just deep to the panniculus carnosus muscle; in no instance was there any indication of a needle-track leading into the peritoneal cavity. Accidental injection into the pleural or pericardial spaces is impossible since all injections were made low down in the flanks, at least 1 cm distant to the lowest part of the twelfth rib. There can be no doubt, therefore, that asbestos fibres are transported from the subcutaneous tissues to submesothelial regions; on histological grounds, this distribution appears to be specific. Certain questions inevitably arise—in particular, how are the fibres transported? Is this tropism to the mesothelium a peculiarity of asbestos or does it occur with other, less readily identified, materials? Such evidence as is available at the present time suggests that tropism to mesothelial tissues may indeed be a special attribute of asbestos fibres but the mode of transport is unknown. The pattern of distribution, their occurrence within lymph nodes, and the presence of dilated lymphatics in serosal membranes, however, favour the view that the fibres may be disseminated by lymphatic channels.

Finally, the mesotheliomas themselves present a number of noteworthy features. The tumours were relatively homogeneous in histological appearance, in contrast to the pleomorphism which has been described in mesotheliomas from other experimental animals (Wagner *et al.*, 1962; Smith *et al.*, 1965b) and also in man (McCaughy, 1958, 1965; Churg *et al.*, 1965). Secondly, many of them were infiltrated with chronic inflammatory cells. The significance of this finding is uncertain but the most likely explanation is that such cells are survivors of an earlier

inflammatory response. Thirdly, like other mesotheliomas reported in the literature, the tumours examined in the present work hardly ever contained recognizable asbestos fibres

Further studies based on the present findings, particularly with respect to the mode of transport of asbestos and its apparent predilection for the mesothelium, are now in progress

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EFFETS PATHOLOGIQUES DE L'INJECTION SOUSCUTANÉE DE FIBRES D'AMIANTE CHEZ LA SOURIS: MIGRATION DES FIBRES VERS LES TISSUS SUBMÉSOTHÉLIAUX ET INDUCTION DE MÉSOTHÉLIOMES

Des groupes de 20 souris CBA femelles ont reçu en deux points, par injection sous-cutanée, des suspensions d'amiante (crocidolite, amosite ou chrysotile). L'injection était faite avec 10 mg de fibre en suspension dans 0,4 ml d'eau physiologique; chaque animal a reçu 3 injections dans les deux flancs, en des points éloignés du thorax. Aux fins de comparaison, deux groupes ont été traités de la même manière avec de la crocidolite et de l'amosite dont on avait éliminé des huiles naturelles contaminantes par extraction au moyen de solvants. Les deux faits les plus remarquables que l'on ait observés sont, premièrement, la migration apparemment spécifique des fibres d'amiante des trois sortes vers les tissus submésothéliaux du thorax et de l'abdomen; deuxièmement, dans ces régions, des modifications étendues de caractère inflammatoire et proliférant, aboutissant dans 10 cas à la formation de mésothéliomes. Six animaux ont eu des sarcomes au point d'injection mais, chez la plupart des souris, on n'a observé qu'une faible réaction cellulaire à l'amiante dans les tissus sous-cutanés alors que la réaction était très vive dans les membranes séreuses. Les trois types d'amiante ont provoqué à la fois des tumeurs au point d'injection et des modifications mésothéliales à distance; l'élimination des huiles de l'amosite et de la crocidolite n'a pas supprimé, mais elle a peut-être diminué, les manifestations d'activité carcinogène.

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BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE IV. DEVELOPMENT OF RESISTANCE TO 5-AZACYTIDINE AND SIMULTANEOUS DEPRESSION OF PYRIMIDINE METABOLISM IN LEUKEMIC MICE

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The development of resistance of leukemic AKR mice to 5-azacytidine administered in small doses was investigated. The activity of uridine kinase decreased simultaneously with the development of resistance. The gradual shortening of the survival time of treated animals and the depression of enzyme activity had a continuous character. The application of 5-azacytidine along with hydrocortisone or actinomycin D did not appreciably affect the development of resistance. In leukemic cells resistant to 5-azacytidine the incorporation of uridine, cytidine and 5-azacytidine itself into ribonucleic acids was depressed in accordance with the impaired anabolic transformations of 5-azacytidine in vivo as well as with the soluble fraction in vitro.

5-Azacytidine which has been synthesized in these laboratories (Piskala and Šorm, 1964) has potent biological effects; its antileukemic properties in mouse leukemia are particularly pronounced (Šorm and Veselý, 1964; Čihák and Šorm, 1965; Veselý and Šorm, 1965). Preliminary clinical trials revealed that 5-azacytidine might be suitable for therapeutic application.

Like 5-fluorouracil (Chaudhuri *et al.*, 1958; Reichard *et al.*, 1961), 6-azauridine (Habermann and Šorm, 1958; Pasternak *et al.*, 1961), 1- β -D-arabinofuranosyl-cytosine (Chu and Fischer, 1965) and other pyrimidine analogues (Welch, 1963), 5-azacytidine is phosphorylated in animal tissues and incorporated into ribonucleic acids (Jurovčík *et al.*, 1965). The low stability of incorporated 5-azacytidine (Pithová *et al.*, 1965) is probably responsible for its striking biological activity. Furthermore, 5-azacytidine 5'-phosphate inhibits orotidylic acid decarboxylase (Veselý *et al.*, 1967).

MATERIAL AND METHODS

Reagents. 5-Azacytidine was synthesized by Dr. A. Piskala of this Institute. Sodium hydrocortisone succinate was supplied by Spofa (Prague, Czechoslovakia). Lyovac Cosmegen Dactinomycin was obtained from Merck, Sharp and Dohme (West Point, N.J., USA). Sodium adenosine 5'-triphosphate was obtained from Calbiochem (Lucerne, Switzerland). 5-Azacytidine-4-¹⁴C (4.1 μ C/ μ mol) was prepared by Dr. J. Morávek of this Institute. 6-Azauridine-4,5-¹⁴C (72 μ C/ μ mol), cytidine-U-¹⁴C (30 μ C/ μ mol) and