A TRACK-RADIOAUTOGRAPHY STUDY OF THE INTRACELLULAR LOCALIZATION OF COBALT IN NEOPLASTIC TISSUES

by

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In spite of the fact that cobalt does not belong to the group of elements which are essential to life, there is strong evidence that in trace concentration it plays an important role in cell metabolism, and cobalt deficiency is known to cause various plant and animal diseases. Like other mineral ions, such as zinc, it influences the activity of enzymes and vitamins; it is necessary to activate arginase and it is an essential part of the anti-pernicious anaemia Vitamin B_{12} (RICKS *et al.*[1]). Like other cations, cobalt unites directly with peptides (GILBERT *et al.*[2]), with amino acids (MICHAELIS[3], BURK *et al.*[4a]), and with purine bases (LIQUIER-MILWARD[5a]). Its great biological interest is suggested by its effect on the growth and respiration of various aerobic and anaerobic micro-organisms (CALVIN *et al.*[6], BURK *et al.*[4b,4c]), animal tissues and tumours (HEARON *et al.*[7]).

Little is known, however, on the metabolism of cobalt and on its mode of fixation in mammalian tissues. Early investigations with radioactive isotopes of cobalt were concerned only with the uptake and distribution of this element in the organs of polycythemic rats (BERLIN[8], COPP AND GREENBERG[9]) and with the selective fixation in brain tumours of cobalt-labelled dyes as a means of detection (WEYMOUTH *et al.*[10]). More recently, a study has been made of the uptake of ^{60}Co by normal and cancerous tissues (LIQUIER-MILWARD AND HEATH[5b]), and preliminary results on the gross distribution pattern in mice bearing an implanted leg sarcoma indicated a difference between the neoplasm and normal tissue.

In the present work, this research has been extended to mice bearing different kinds of tumours, two sarcomata and one adeno-carcinoma. The localization of cobalt within the tumour cell and its nucleus has been studied by the radioautographic method, in order to investigate the possibility of internal irradiation with ^{60}Co.

MATERIAL AND METHODS

Animal tissue

Three types of tumours have been used:

1. A transplanted sarcoma, in its 48th transplant, in the flank of a IF-strain mouse. This originally appeared to be a breast adeno-carcinoma, but during transplantation the histological character became of the spindle-cell-sarcoma type.
2. A leg sarcoma in Strong A-strain mice.
3. A carcinoma of the breast in IF-strain mice.

Animals bearing tumours of optimum size were selected and, in each of the various experiments, two mice were treated simultaneously. As the margin between normal requirement and toxicity is very narrow, a material of high specific activity was required; this was prepared in the uranium pile of the Atomic Research Establishment, Harwell, according to the nuclear reaction $^{59}Co(n,\gamma)^{60}Co$, by irradiation for six weeks of a thin wire of cobalt. A standard amount of cobalt, in solution as

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cobaltous chloride, was injected subcutaneously in the nape of the neck of the mice, the dose being 150 μg per 20 g body weight, corresponding to about 35,000 counts per minute, measured with a D.M.6 liquid counter.

The animals were killed 7 to 8 hours after injection and the tumours were immediately removed and fixed in a mixture of absolute alcohol and glacial acetic acid. The tissues were prepared for sectioning following the usual method of embedding in paraffin and sectioned at 4 μ. A Geiger-Müller counter was used to ascertain the presence of radioactivity in a number of serial sections from each tumour considered. The sections were not stained before taking the radioautographs, as staining at this stage was found to interfere with further processing.

Radioautographic technique

Different procedures have been applied, including those of EVANS[11] and DONIACH AND PELC[12], but the best results were obtained with the liquid emulsion method, coating the specimens with a thick layer of Ilford G5 nuclear emulsion. This technique allows an intimate and permanent contact between the sections and the gel, and it is especially suitable for the study of the soft beta radiation of ^{60}Co (0.3 Mev). With normal exposures, the random photographic density of the developed grains is greater than the one obtained with the thin film stripping technique, and a sharper radioautograph is produced: on the other hand, if the exposure has not been too long, one can localize individual beta-ray tracks and, as the spacings of the developed grains at the beginning of the tracks is not too great, it is possible to trace back the path of the particle to its origin.

On account of the great sensitivity of the nuclear gel employed, the background at ground level is of importance and special precautions were taken, the exposures being carried out at a depth of 600 metres, in a coal mine, with heavy shielding of lead. This reduced the number of electron tracks from 250 to 2.6 per mm^2 per day in a 200 μ thick emulsion layer (FREMLIN AND WALTERS[13]) and proved a great help as it made possible a precise localization of the radioactive element within the cells themselves, allowing investigations on isolated nuclei.

Prior to coating, the paraffin wax was removed in xylol and the sections were taken through the usual succession of alcohol concentrations and thoroughly washed in water. It was found that a certain degree of moisture was helpful in ensuring good adherence between the photographic emulsion and the specimens. Fresh Ilford G5 gel, melted at 55° C, was poured on the slides, which were dried at room temperature under vacuum. Alternatively, if it is desired to reduce the period between coating and sealing in a lead container, a blower can be used, great care being taken to eliminate dust particles which, especially in the vicinity of a "hot laboratory", often contain radioactive matter. Photographs of the sections were taken before coating and only alternate sections in a sequence were coated.

After exposures varying from 22 to 41 days, the plates were developed by the normal technique employed for thick emulsions (DILWORTH[14] and HERZ[15]), soaking the emulsion in an amidol developer for about one hour at low temperature (5° C) and gradually warming up the plates to 25° C. To allow recognition of the section structure under the emulsion, the processed slides were stained with haematoxylin.

RESULTS

All the tumour sections examined gave well-defined radioautographs, as seen in Fig. 1 and Fig. 2; sections from sarcomata (1) and (2), respectively, are shown at low magnification, together with the radioautograph obtained with the adjacent section.

Fig. 3 shows, at high magnification, (a) the background at a distance from the sections, (b) the background near the edge of a section, and (c) the contrasting density of the beta-ray tracks which corresponds to areas of high radioactivity in the specimens. Photomicrographs (a) and (c) were taken before staining.

Examination of the stained sections with the radioautographic image superimposed showed that the areas of most intense activity correspond to the parts of the tumour where malignant cells are most dense. Although the emulsion layer is very thick (up to 250–300 μ) and takes up the stain, photomicrographs were recorded. The two micrographs in Fig. 4 show an area of sarcoma (2) which has a dense distribution of malignant cells, with the camera focussed in turn on the tissue level and on a layer in the emulsion some distance away. Heavy radioactivity is present. Tumours with more abundant connective tissue showed a consistently lower uptake.

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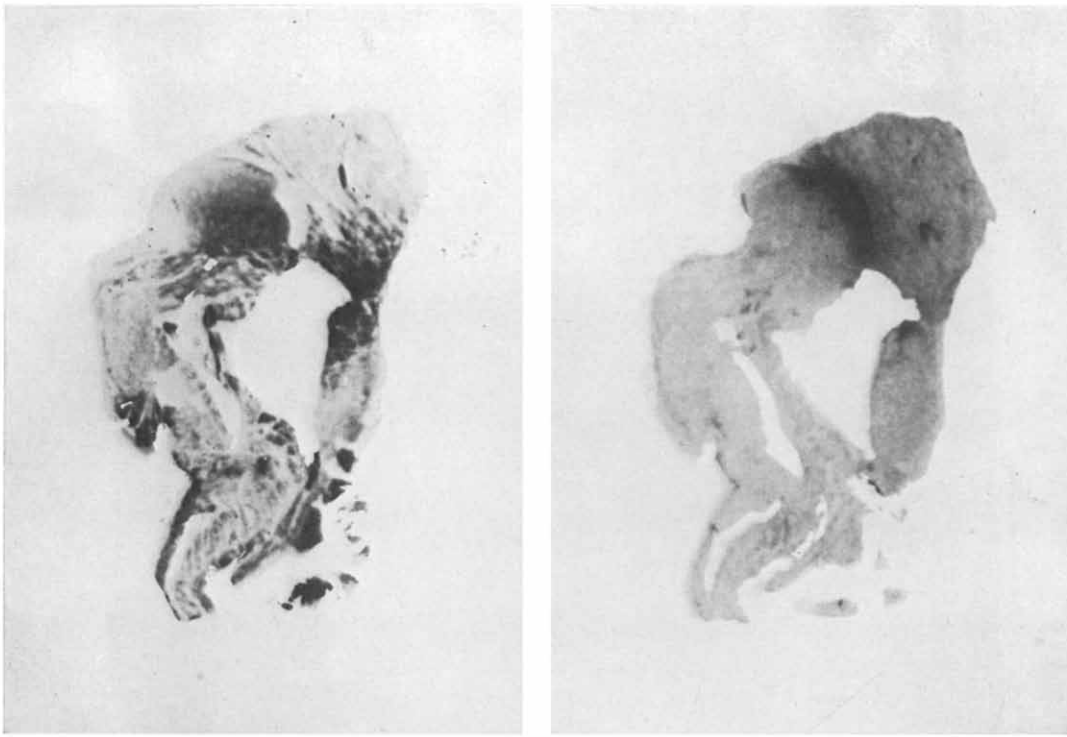


Fig. 1 a. Section from sarcoma (1), low magnification, 5 ×; b. Radioautograph, adjacent section, 5 ×.

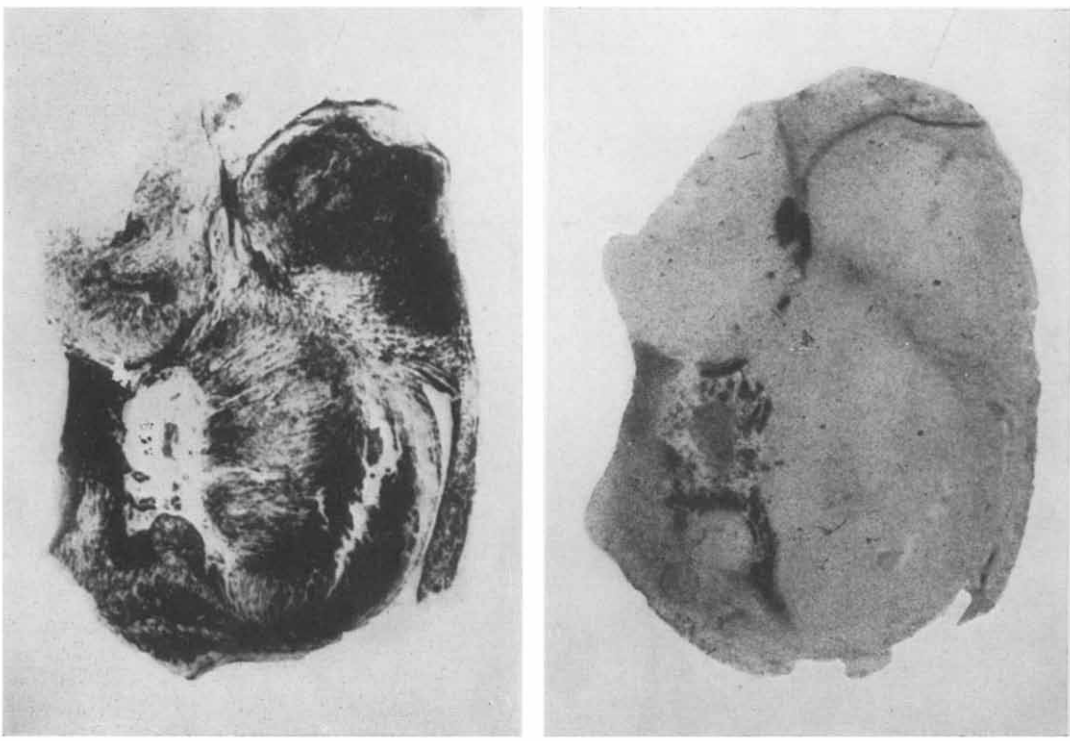
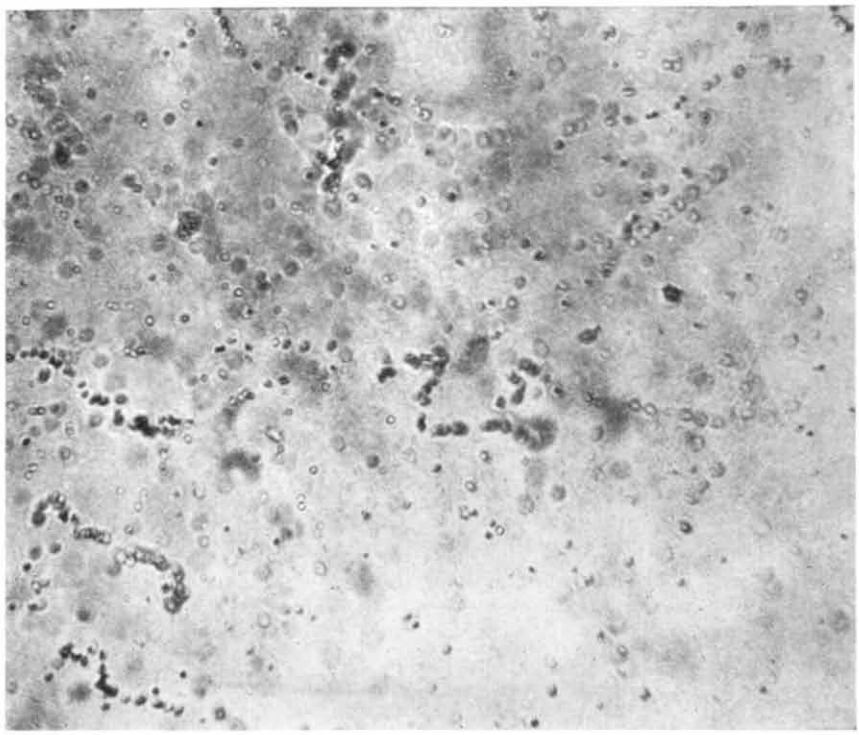
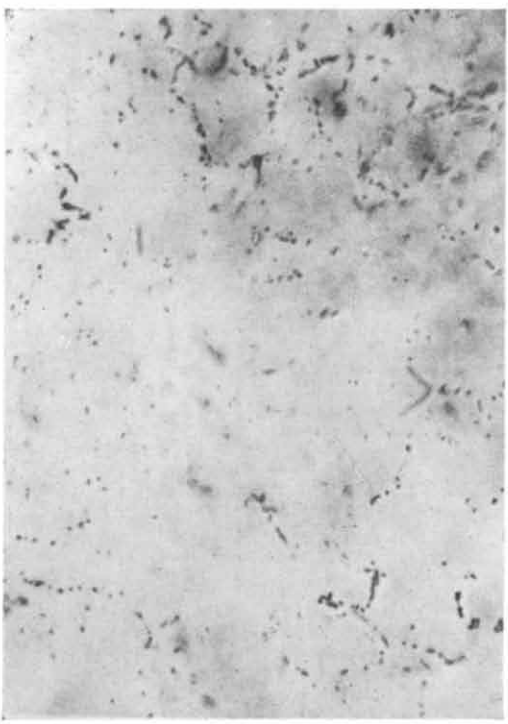


Fig. 2 a. Section from sarcoma (2), low magnification, 5 ×; b. Radioautograph, adjacent section, 5 ×.





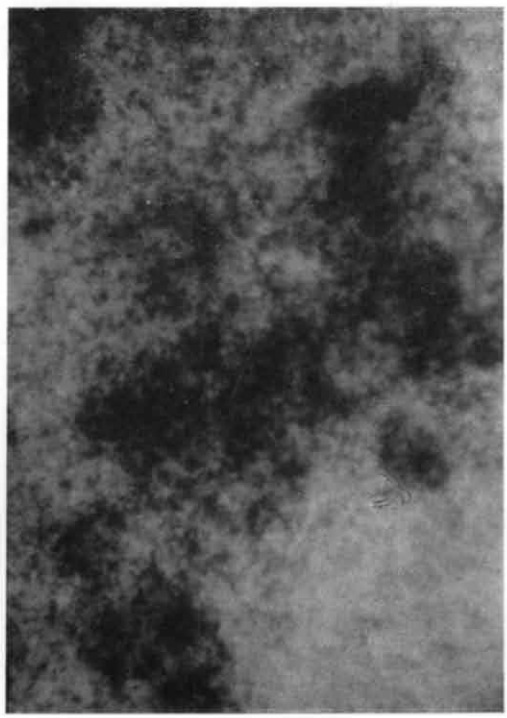


Fig. 3a. Background at a distance from sections, 800 ×; b. Background near edge of radioactive section, 800 ×; c. Density of tracks corresponding to darker areas in Figs. 1b and 2b, 800 ×.

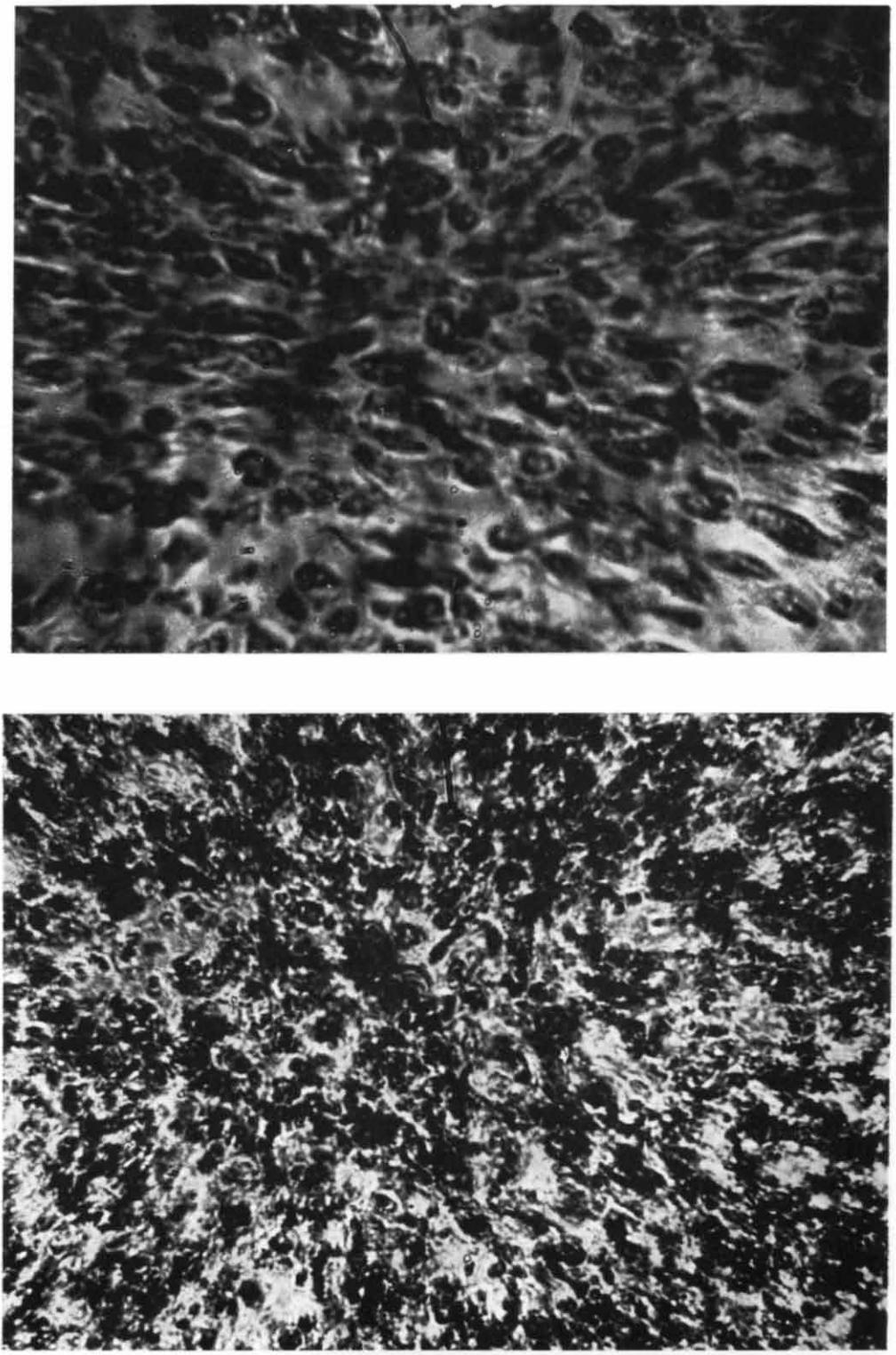
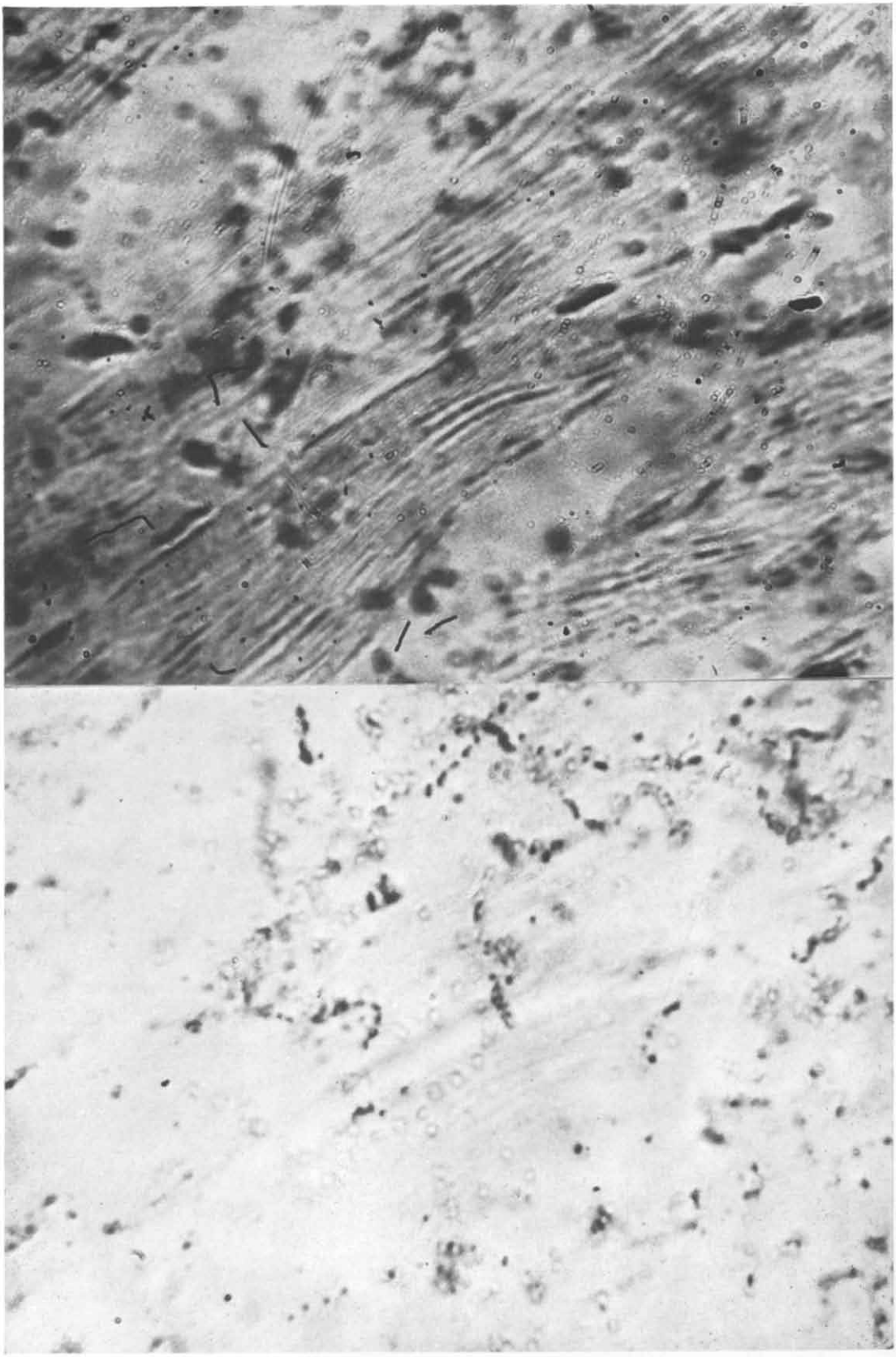


Fig. 4. Stained section from sarcoma (2), with dense distribution of malignant cells: (a) Camera focussed on cell level, and (b) Camera focussed on emulsion layer above tissue, 800 ×.



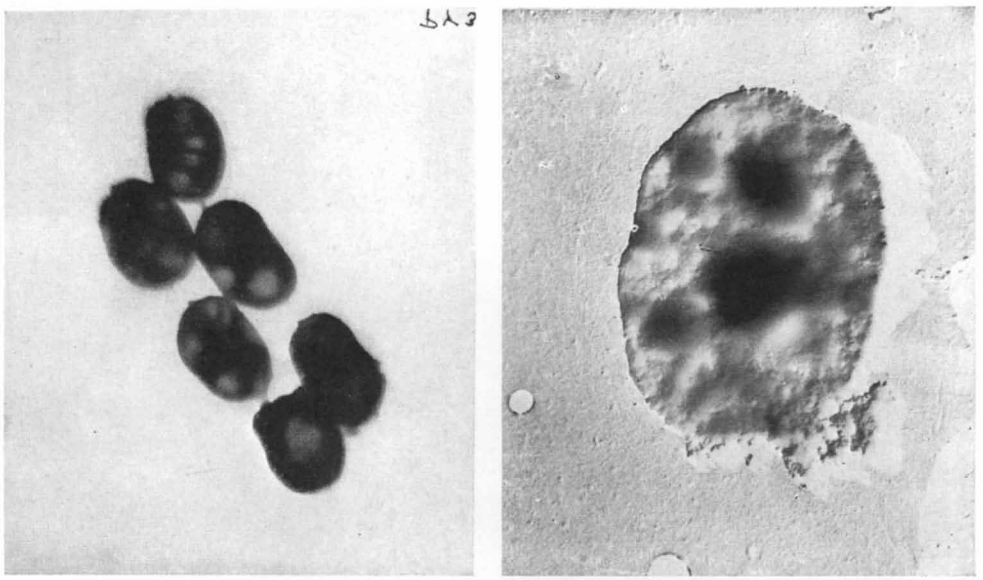


Fig. 6a. Nuclei extracted from sarcoma (1) at the end of separation process in citric acid, 3000 ×; b. Nucleus extracted from adeno-carcinoma (3), 7000 ×.

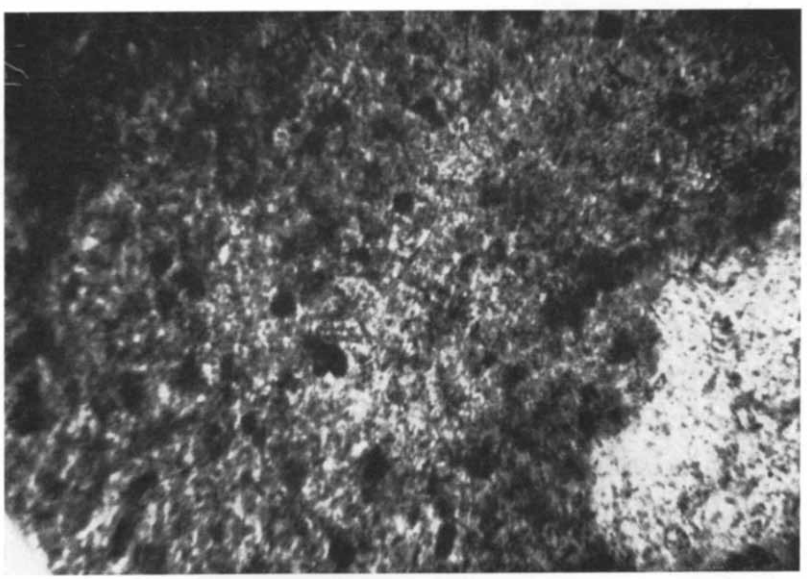


Fig. 7. Radioautograph, smear of nuclei emulsion corresponding to Fig. 6a, 400 ×.

← Fig. 5a. Region of stained section from sarcoma (2), camera focussed on tissue level, 1500 ×; b. Corresponding radioautograph, camera focussed immediately above level of fibres. Only a few beta-ray tracks are present, 1500 ×.

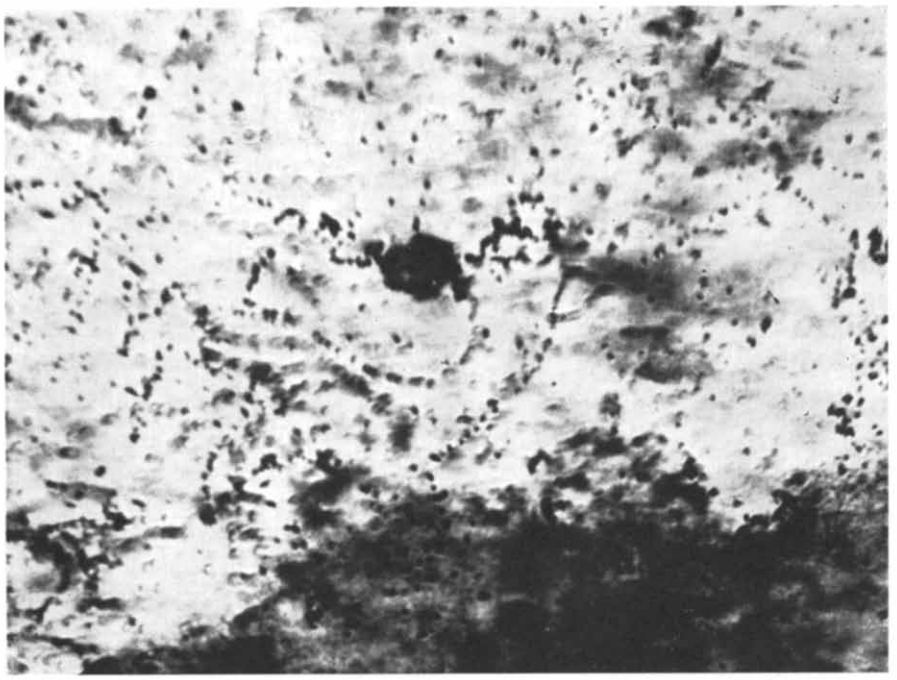


Fig. 8. Individual nucleus from sarcoma (2), at high magnification, showing electron tracks with origin against the surface of the nucleus, 1000 ×.

The regions of the section which contain muscle fibres and reticular tissue, and where the cancerous cells are few, gave rise to only a small number of tracks, as illustrated in Fig. 5 (a and b). A few sharply defined dense spots were found to correspond to the sections of blood vessels and what is left of their content (Fig. 10).

Nuclei

Cell nuclei were separated from neoplastic tissues, pooled from 10 mice in each experiment, by the method of DOUNCE[16], and washed repeatedly with 0.5% aqueous citric acid solution. Observations with the electron microscope were made at each step of the separation to check the degree of cleanliness of the nuclei (Fig. 6, a and b). The emulsions of nuclei were assayed for radioactivity in a Type D.M.6 liquid counter (20th Century Electronics) between washings, and a progressive loss of activity to the successive citric supernatants was observed; cobalt is either leached out or, if it is assumed that the metal is fixed on to the nucleoprotein molecule, this corroborates previous evidence for the loss of protein from the nucleus during the process of isolation from aqueous media, already reported by POLLISTER AND LEUCHTENBERGER[17] and by DOUNCE *et al.*[18]. However, after five or six washings and centrifugations, further treatment removed no significant amount of cobalt. The nuclei were dried and weighed and numerical results were as follows:

Nuclei extracted from sarcoma (1) gave a count of 12 cts/min per 200 mg, corresponding to a specific radioactivity of 60 cts/min/g of dry weight.

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Fig. 9. Individual nucleus (center of plate) sarcoma (2), camera focussed, as in Fig. 8, on a plane between surface of glass and emulsion layer, 1000 ×.

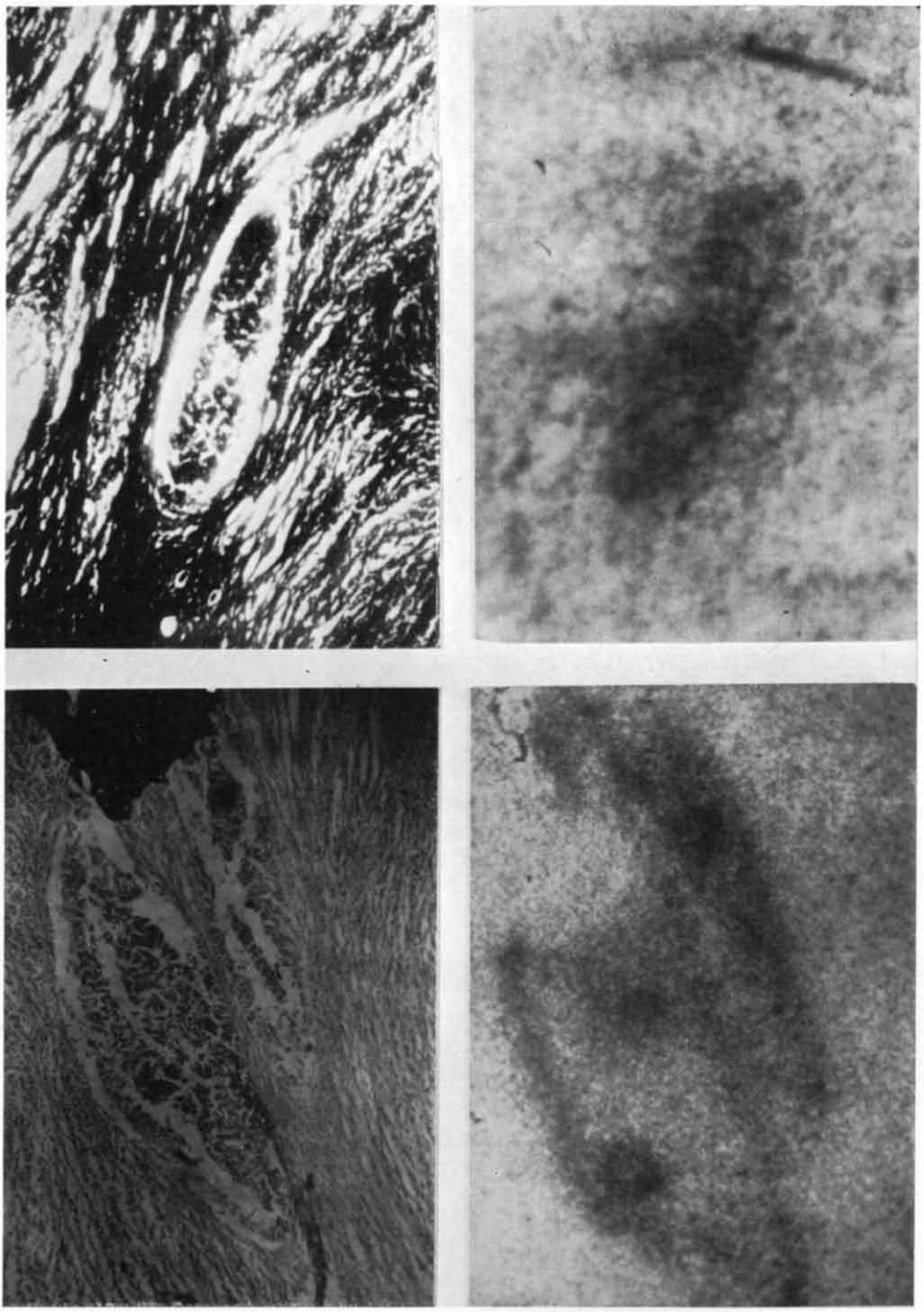

Fig. 10a. Blood vessel, and b. corresponding dense spot in radioautograph, sarcoma (1), 50 ×; c. Blood vessels visible in Fig. 2a, and d. corresponding tracks in radioautograph, 60 ×.

Nuclei extracted from adeno-carcinoma (3) showed a specific radioactivity of 113 cts/min/g of dry weight.

Radioautographs of smears of the nuclei emulsions were obtained (Fig. 7) and if the radioautograph exposure has not been too long, the smallness of the background makes it possible to follow the beta-ray tracks easily, to measure their length which corresponds to the range expected for the soft radiation of ^{60}Co, and to trace their origin to the surface of the nuclei. Photomicrographs, focussed on a level in the emulsion as near as possible to the nuclei, record (Figs. 8 and 9) a few beta-particle tracks emanating from the nucleus.

CONCLUSION

It can thus be concluded from this radioautographic study that, following subcutaneous injection of ^{60}Co, the malignant cells and their nuclei show a high uptake of radioactivity compared with the normal tissue elements.

It is intended to extend these observations to longer periods of irradiation, giving to the animals small successive doses of cobalt, within the limit of tolerance, in order to study the effect of internal irradiation on the malignant cells.

To ascertain if the fixation of the trace metal by the cancer tissue is connected with the mere process of mitosis and formation of new nucleoprotein, or if it is related to malignancy proper, a similar investigation is in progress on the uptake of cobalt by growing roots of *Allium cepa* and by various organisms growing in a medium containing radioactive cobalt.

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SUMMARY

The localization of radioactive ^{60}Co within tumour cells has been investigated by the track-radioautographic method. Sections from three kinds of tumours, two sarcomata and one adeno-carcinoma, have all given well defined radioautographs. The regions of higher density of cancerous cells show the higher uptake of cobalt, and the muscle fibres give rise only to a small number of beta-ray tracks. The presence of radioactivity within the cell nuclei themselves has been demonstrated.

RÉSUMÉ

L'absorption et la localisation du ^{60}Co dans les cellules de tissus néoplastiques (sarcoma et adéno-carcinoma) ont été étudiées par radioautographie, en faisant usage d'une épaisse couche d'une émulsion sensible aux électrons. Les régions des tumeurs où les cellules cancéreuses sont nombreuses donnent naissance à un grand nombre de traces, les fibres musculaires montrant au contraire une faible radioactivité. Les noyaux cellulaires ont été isolés et la présence du Cobalt 60 dans ces noyaux a été démontrée.

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ZUSAMMENFASSUNG

Die Lokalisierung des radio-aktiven ^{60}Co innerhalb von Tumorzellen ist mittels der Bahnradioautographischer Methode untersucht worden. Schnitte von drei Arten von Tumoren, zwei Sarcomata und ein Adeno-Karzinom lieferten alle wohlausgebildete Radioautographien. Die Gebiete wo die Krebszellen am häufigsten sind, zeigen höhere Kobaltaufnahme; die Muskelfasern erzeugen nur eine kleine Anzahl von Strahlenbahnen. Die Gegenwart von radioaktivem Kobalt 60 in den Zellkernen selbst wurde bewiesen.

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