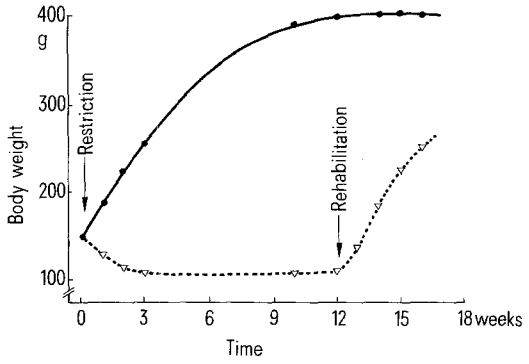

Fig. 1. Body weight of control rats (solid line) and of protein-energy restricted and rehabilitated rats (dotted line).

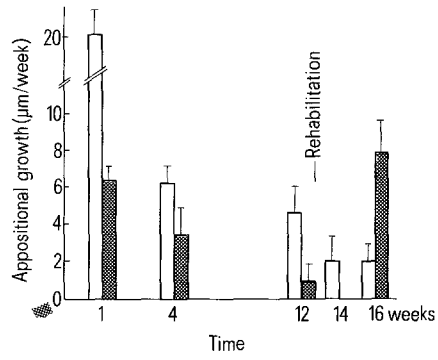

Fig. 2. Appositional bone growth of control rats (white bars) and of protein-energy restricted and rehabilitated rats (cross-hatched bars). Standard deviations are shown.

Studies from this laboratory[5] have demonstrated that, under the same experimental conditions as those described here, longitudinal bone growth slows during the 1st week of malnutrition and then ceases. During rehabilitation longitudinal bone growth resumes (although somewhat later than the resumption of weight gain) and, if rehabilitation continues for a sufficient length of time, bone length approaches that of the controls. The present study demonstrates that a similar pattern is characteristic of appositional bone growth, although the rate of growth suppression is slower. Periosteal growth slows during the 1st week of restriction and finally, by the 14th week, no further growth is observed. The later cessation of appositional growth, relative to longitudinal growth, would account for the changes in proportions of the long bones, as observed by Outhouse and Mendel[6].

Appositional bone growth does not resume immediately upon transfer to the control diet, but is delayed. However, if catch-up growth is defined as 'a growth velocity above the statistical limits of normality for age ... during a defined period of time'[10], then catch-up growth occurs during the early stages of rehabilitation (14–16 weeks). Whether this is temporally correlated with an accelerated rate of longitudinal bone growth remains to be established.

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Lymphatic metastasis of tumour; persistent transport of cells[1]

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Summary. A model of lymphatic metastasis established by injecting Walker rat carcinoma cells into the rat footpad was used to study the output of tumour cells from the footpad. The lymphatic efferent from the footpad was cannulated in a group of rats with advanced neoplasm; it was shown that the output of tumour cells was continuous over periods up to 90 min and ranged from 10^2–10^5 cells/min.

The common human cancers metastasize early by the lymphatics, unlike most experimental animal neoplasms. Experimental models which mimic human lymphatic metastasis are therefore of especial interest[3,4]. A useful model is to inject tumour cells into the footpad and follow their metastasis to the draining popliteal lymph node[5,6]. Among the tumours which successfully metastasize in this manner is the Walker rat carcinoma, but many tumours do not[7]. Detailed studies have not till now been carried out on the lymph directly draining experimental tumours which metastasize by lymphatics. Accordingly, it has not been clear whether tumour cells pass from primary to secondary, singly or in clusters, and discontinuously or continuously. Furthermore, while it has been possible to investigate the entry of tumour cells into the lymphatic vessels in the primary, it has not been possible to pinpoint the time at which this happened by detecting the cells within the lymphatic trunk. This report describes the cannulation of lymphatics efferent from an implanted ('primary') tumour, and the detection and enumeration of tumour cells therein.

A metastasizing model was established by injecting Walker rat carcinoma cells into the left footpad of out bred albino rats. The tumour cells were prepared as described elsewhere[5]. The presence of metastasis was established by histological examination of step paraffin sections of the ipsilateral popliteal lymph node. In newborn male rats the injection of 5 million tumour cells of better than 90% viability adjudged by trypan blue exclusion produced better than 95% successful metastasis in a series of 24 animals. In adult male rats the similar injection of 20 million tumour cells

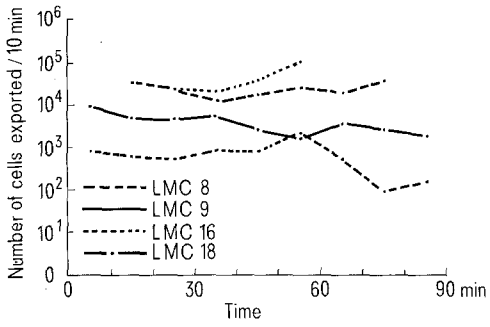

Fig. 1. Output of Walker Rat carcinoma cells in one lymphatic efferent from the rat footpad. Serial counts of the number of tumour cells passing out of the pre-popliteal afferent lymphatic per 10 min, in 4 rats bearing 8-day implants of 20×10^6 Walker rat carcinoma cells in the footpad. Cells were counted/0.0628 mm^3 at 10-min intervals. The mean between 2 times was taken as the flow rate of cells and calculated from the volume of fluid collected over the same period. LMC 8, 9, 16, 18 = individual animals.

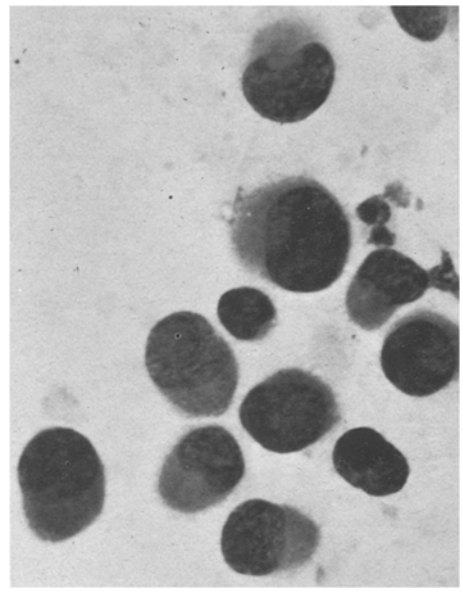

Fig. 2. Light micrograph of cells in lymph from lymphatic vessel efferent from footpad of rat with Walker rat carcinoma implanted into the footpad 8 days previously. The large cells with hyperchromatic nuclei are all tumour cells. H and E staining. × 720.

produced better than 90% successful metastasis. Because of physical size lymphatic cannulation could be attempted only in adult animals. Animals which showed regression were excluded from the series.

Lymphatic cannulation was carried out by inserting a 30-g disposable needle shaft carrying a PE10 polythene cannula into the lymphatic 1 cm proximal to the ankle joint in animals anaesthetized with nembutal and ether. Passive dorsiflexion plantar flexion movement of the ankle was carried out by holding the tips of the toes and the malleoli. Evans blue (20 µl of 30 mg/ml in 0.45% saline solution) was previously injected into the footpad to visualize the lymphatic vessels. Lymph was collected into a polythene microtube for electron microscopy and the volume was measured serially. The collection was continued for up to 90 min and the number of effluent cells counted by cutting off 1 cm of the polythene tube every 10 min. The lymph was expelled onto a glass slide, fixed in 10% formalin and stained H and E. Since the volume of 1 cm of the tube was known (0.0628 mm^3) an absolute count of the number of tumour cells was obtained. In every case successful metastasis in the popliteal node was established by histological examination. The presence of tumour cells in the cannulated lymphatic was verified at the end of 2 experiments by serial histological examination of the afferent lymphatic after ligation of the top and bottom ends of the lymphatic to retain the tumour cells within it. A further lymphatic from a non-cannulated rat was similarly histologically examined. The tumour cell counts are illustrated in figure 1.

12 tumour-bearing animals were cannulated (12/12) successfully, 7–9 days after transplantation. In all animals, tumour cells were seen in the lymph in all samples (figure 2). No such cells were seen in 12 rats similarly cannulated which did not bear tumour. The cells in the lymph of 6 8-day animals were counted for periods up to 1.5 h. Duplicate counts were carried out on adjacent 1-cm pieces of tubing in some cases, and a variation of ± 12% was seen in these. Cells did not appear to sediment in the tubing as low counts followed high counts successively. Lymph flow rate always fell off progressively with time, although total cell output varied less. The maximum volume collected was 0.52 ml in 70 min. There was a consistent fall in cells exported in the first 30 min following cannulation, followed by a later rise to higher levels. In animals with smaller footpads there was again a fall in cells exported at 40–50 min and again at 80–90 min, suggesting that the passive movement was causing synchronisation of cells passing into the lymph in waves. This time interval is similar to the time taken for polymorphonuclear cells to migrate through blood vessel walls in acute inflammation[8].

Broadly, the number of tumour cells leaving the footpad by a single lymphatic was directly related to the size of the primary tumour. Those showing gross oedema and extension of the tumour to the heel exported more cells than those where the heel appeared normal, and there was no obvious oedema with splaying of the toes. In all cases there were at least 2 lymphatic trunks visibly draining the area. In the footpads which were most grossly enlarged, injection of Evans blue into the usual site between the 3rd and 4th toes did not demonstrate the lymphatic trunks. In these animals the dye penetrated the lymphatics when injected into the heelpad, above the level of oedema. This suggests that the more distal lymphatics may have been totally or partially obstructed by the tumour. In these animals it was noticed that the adjacent vein showed immediate darkening on 1st injection of Evans blue, suggesting open endothelial junctions and/or a very rich capillary network.

Total numbers of cells exported ranged from about 1000 cells/10 min to just over 1,000,000 cells/10 min. In the group of animals with the larger footpads the number of cells leaving via 1 lymphatic was consistently in the range of about 100,000 or more cells/10 min.

Apart from 2 specimens from 1 animal, with counts of 1300 and 4300 cells/cm cannula it was unusual to see tumour cells in groups. About 2% of tumour cells were multinucleate or dividing. Tumour cells were readily identified by their large size and gross nuclear hyperchromasia and pleomorphism. The numerous lymphocytes and macrophages present in the specimens were readily distinguished, and were not counted.

All animals had histologically proven metastasis in the popliteal lymph node. Tumour cells were found in the medullary sinusoids and major efferent lymphatic vessel of the node. This is explicable on the basis that some tumour cells are not trapped and migrate through. Alternatively they may be trapped, divide within the node and their progeny migrate on. In all 5 animals where concurrent blood samples were taken from veins draining the primary, tumour cells were identified. Lung metastasis was not seen

histologically on random sections, although single tumour cells could be identified in the pulmonary capillaries.
It is possible that the flow of tumour cells was somewhat increased by the prior increase in tissue pressure induced by the introduction of 20 µl of Evans blue. However 1 serially sectioned lymphatic from the animal which did not receive Evans blue did not contain an evidently different number of cells from the similarly sectioned lymphatics from animals given Evans blue. It is unlikely that this is an important factor.
These findings establish for the first time the approximate number of tumour cells in lymph efferent from a transplanted tumour and show that lymphatic metastasis under the conditions studied is a continuous phenomenon.

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Copper-induced heart malformations in hamsters[1]

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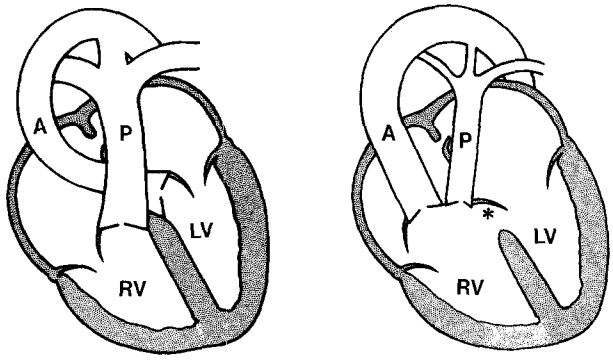
Summary. The injection of copper citrate into pregnant golden hamsters induces a specific pattern of cardiovascular malformations in their embroys. The syndrome consists of double-outlet right ventricle, pulmonary hypoplasia and a ventricular spetal defect.

Although the release of copper from an IUD during the early stages of pregnancy inhibits implantation of the blastocyst[2,3], the release of copper from wires placed in the uteri of several experimental animals was not found to have toxic effects on their post-implantation embryos[4]. On the other hand, mouse blastocysts were killed rapidly when cultured in association with copper[5] and there is evidence that the administration of copper to hamsters induces congenital malformations in their offspring. The embryos of pregnant hamsters injected i.v. with copper salts showed, amongst other abnormalities, thoracic wall defects with or without an associated case of ectopia cordis[6]. It is this latter finding that led to this study on the effects of copper on cardiac development.
Materials and methods. Pregnant golden hamsters were i.p. injected on the morning of the 8th day of gestation with a final treatment concentration of 2.7 mg copper citrate solution per kg b.wt. The day following the evening of mating is considered the 1st day of gestation. The mothers were individually caged, fed ad libitum and sacrificed at gestational days 12 and 13. A group of control hamsters were treated in the same manner except that the solution injected contained solely deionized water. Embryos were removed from gestation sacs, placed in 0.9% saline solution, checked for viability and live embryos were examined for gross pathologic and gross teratogenic effects. Embryos showing gross malformations (e.g. mostly tail and limb defects) or edema were embedded in paraffin, sectioned with a microtome through the thoracic cavity and examined for cardiac anomalies with a light microscope. Whenever a cardiac malformation was found the littermates of this embryo were then sectioned in a similar manner and examined for defects. In addition, several litters of copper-treated, normally-appearing embryos were also processed for light microscopy. Embryos from control group litters were selected at random and, in several cases, the entire litter was processed for light microscopic observation.



Normal day-12 or day-13 hamster embryo heart (left) and a hamster embryo heart of the same age affected by copper citrate (right). Note the narrowing of the pulmonary trunk (P), the malpositioning of the aorta (A) and the ventricular septal defect (*) between the left and right ventricles (LV and RV, respectively).

Results and discussion. Maternal survival was unaffected by the administration of copper. The effects of maternal copper treatment on the embryos, as well as the control data, are summarized in the table. Embryos collected from the water-injected mothers exhibited normal survival and were free of gross pathologic and teratogenic effects. Light microscopic sections of these embryos revealed normal cardiac development. In the copper-injected group, only embryos with edema had heart malformations but not all edematous embryos had cardiac defects. Nevertheless, the presence of edema served as a reliable clue for the detection of these copper-induced anomalies.
The unusual observation is the specificity of the heart malformations found in the day-12 and day-13 embryos of copper-treated hamsters. 4 of the 17 copper-treated mothers bore 1 or more embryos with a specific array of cardiac lesions. As shown in the figure, the pulmonary trunk of the normal day-12 and day-13 embryonic hamster heart has a greater diameter than the aorta. The cephalad portion of the aorta lies ventral to the pulmonary trunk. As these