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THE EFFECT OF TRANSPLANTED MAMMARY TUMOURS ON THE CALCIUM BALANCE OF THE RAT

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Abstract—A method is described for the preparation of rat femur and its use in *in-vitro* incubation experiments. Soluble calcium and acid and alkaline phosphatase were estimated in the medium. Blood serum from rats with mammary tumours caused a depression of calcium release in the test system.

When bone from rats bearing mammary tumours was used, there was a decrease in the release of calcium but incubation of normal bone with tumour slices caused an increase in both soluble calcium and acid phosphatase. The addition of cortisone (but not cortisol) to the reaction mixture caused increased calcium liberation from normal bone as did previous sensitization of the rat with dihydrotachysterol. The significance of these findings is discussed.

SINCE Virchow enunciated his idea of 'metastatic calcification', it has been assumed that systemic calcification develops in patients with destructive bone tumours because the organism is flooded with calcium and phosphate. This process plays an important part whenever the skeleton is attacked by metastatic carcinomas, multiple myeloma, leukemia, etc. but it has also become evident that hypercalcaemia sometimes conducive to calcinosis can develop in patients with malignant tumours which do not form metastases.[1–4]

In some patients, removal of the primary tumour was followed by reduction of the blood calcium to normal and it was assumed that malignant tumours can cause hypercalcaemia through the production of substances having some of the properties of parathyroid hormone[5, 6] or Vitamin D.

Transplantation to the rabbit of the carcinoma XV-2 caused hypercalcaemia, hypophosphataemia and nephrocalcinosis.[7] Bone resorption by giant cells took place and the general picture was similar to that of hyperparathyroidism. Some tumours may also produce parathyrotrophic substances which raise the blood calcium through direct stimulation of hormone secretion.[8] Hypercalcaemia in cancer can lead to fatal results through renal failure.

The present report deals with some *in-vitro* experiments on the effects of tumour derivatives on bone. The problem is one of providing evidence to support one or more of the three hypotheses although other factors may have to be considered in *in-vivo* experiments.

MATERIALS AND METHODS

Schartum and Nichols[9] have used mouse calvarium for their *in-vitro* experiments on bone metabolism but in the present experiments rat femur has been used exclusively since the femur is a common metastatic site.

Rats weighing approx. 250 g were used throughout. The animals were killed with chloroform, a sample of the blood was taken, and both femurs were removed. The bones were freed of periosteum and fragmented into 25 ml of Krebs Improved Ringer I, which has the concentration of electrolytes and organic acids similar to mammalian serum and contains intrinsic substrate. This medium was free from calcium, was not sterilized and was gassed alternately with oxygen and carbon dioxide until the pH was between 7·2 and 7·4. The fragmented bone was crushed into particles in the medium to free the marrow in the form of a suspension. The marrow was rinsed completely out of the bone and the rinsings made up to a standard volume with medium. The bone was dried with filter paper and equal amounts were weighed into 50-ml stoppered conical flasks. Ten ml of the marrow suspension was added to each flask.

Five flasks prepared in this way constituted the control group in each of the experiments to be described. Incubation was carried out for 18 hr at 37° with shaking vigorous enough to keep the bone in motion (approximately 140 oscillations/min). At the end of this time the calcium content of the medium was estimated by a complexometric method using disodium ethylene diamine tetra-acetic acid. The amount of calcium released from 100 mg of bone under these conditions was 260 ($\pm$ 40 μg S.E.M.).

Estimation of calcium

After the incubation of the bone *in vitro*, the contents of each of the ten flasks (five controls and five tests) were centrifuged and 5 ml used for the determination of soluble calcium. The rest was used for the determination of acid and alkaline phosphatase activities.

The medium was deproteinised with trichloroacetic acid, centrifuged, and brought to pH 9 with ammonium hydroxide. Calcium was precipitated as the oxalate which was dissolved in ethylenediamine tetra-acetic acid and the excess EDTA titrated with 0·001 M zinc sulphate. This method is based on that of Pilz.[14]

This method was most suitable on account of its high reproducibility (error $\pm 3{\cdot}2\,\mu$ per cent) but is not suitable for the determination of quantities of calcium less than 20 μg per test.

Estimation of acid and alkaline phosphatase activities

The activity of the two phosphatases was estimated by the method of Andersch and Szczypinski[10] with *p*-nitrophenol orthophosphate as substrate. One unit of enzyme activity/ml of sample was taken as the amount capable of liberating 70 μg of *p*-nitrophenol and optical density values were converted to units of enzyme activity from a calibration curve of *p*-nitrophenol concentration against optical density. The unit of phosphatase activity as defined by Andersh and Szczypinski is that quantity of enzyme present in 1 l. of serum which liberates 1 mM (140 mg) of *p*-nitrophenol in 1 hr at 37°.

RESULTS

Effect of aging on bone matrix

Femurs were removed from a female rat, crushed and the marrow made up into a suspension in the medium. Each of the five control flasks contained 240 mg of bone in medium with marrow. The test series contained bone matrix (240 mg), which had been taken from a rat of the same size and sex three days before and aged in physiological saline, and 10 ml of the fresh marrow suspension. A reduction of 15% in the amount of calcium released into solution followed aging of the mineralized part of bone. No enzymatic activities were measured.

Effect of aging on bone matrix and marrow

Bone (120 mg) from a male rat was fragmented and crushed in the medium containing marrow. The control series of five flasks each contained 10 ml of the complete enriched medium, but the suspension in the test series differed in that no acid substrates glucose or oxygen were added. A reduction of 50% in the amount of calcium released into solution in the medium followed deprivation of bone and marrow of acid substrates, glucose and oxygen. Acid phosphatase activity was depressed. The experimental figures are given in Table 1.

Effect of oestrone on bone

Bone (150 mg) was incubated in medium, without marrow, to which about 0·5 ml of calf serum in ten had been added. The test series of five flasks had 100 μg of oestrone in each, as the free steroid. A very small increase in the mean value for the calcium content of the test flasks over the controls was not regarded as a positive result.

Effect of serum from tumour-bearing rats on normal bone

Serum was collected from rats with implanted mammary gland tumours which had grown to a weight of between 40 and 50 g. Serum was also collected from normal rats of the same size. Bone from normal animals was fragmented and freed from marrow which was made up into a suspension in the enriched medium. The same amount of bone (about 120 mg) was placed in each of the five test and five control flasks, and 10 ml of the marrow suspension added to each. To each of the control flasks was added 0·5 ml of normal rat serum. The same amount of serum from tumour-bearing rats was added to the test series in the same way. The flasks were incubated and the results obtained are presented in Table 1. The amount of soluble calcium released into the medium of the test series was, on the average, about 10% less than in the control series, with a small depression in the activities of both acid and alkaline phosphatases.

Comparison of normal and diseased bone

Normal rat femur (360 mg) was fragmented and incubated in enriched Krebs–Ringer medium (10 ml) with the marrow in suspension. Femur from rats with a large implanted mammary tumour was also incubated under the same conditions, concurrently, as test series, and the soluble calcium estimated. The results, set out in Table 1, indicate that the bone from the animals with tumours had diminished capacity to release calcium into the medium. Alkaline and acid phosphatase activities were slightly depressed.

TABLE 1. THE EFFECT OF TREATMENT ON *in-vitro* CALCIUM RELEASE FROM BONE

Each figure is the mean value of five with deviation from mean in brackets.
Enzymatic activities are expressed in enzyme units as defined in the text.

Description of Experiment	Control Series			Test Series			Difference between test and control means—(calcium μg/ml)	Weight of bone matrix in medium (mg/ml)	Weight of tumour in animal or in medium (g)
	Calcium (μg/ml)	Acid phosphatase (E.U./ml)	Alkaline phosphatase (E.U./ml)	Calcium (μg/ml)	Acid phosphatase (E.U./ml)	Alkaline phosphatase (E.U./ml)			
Effect of aging on normal rat bone matrix	50 (±4)			43 (±6)			− 7	14	
Effect of aging on normal rat bone matrix and marrow	32 (±4)	4·20	5·20	16 (±4)	0·98	5·07	−16	12	
Effect of boiling for 10 min on normal rat bone matrix	53 (±2)			28 (±1)			−25	10	
Effect of serum from tumour-bearing animals on normal rat bone and marrow	28 (±2)			12 (±3)			−16	12	50
	35 (±4)	2·13	5·75	32 (±3)	1·83	5·33	− 3	12	25
	41 (±2)	1·75	1·25	38 (±2)	1·75	1·25	− 3	12	40
Rat bone without marrow	46 (±2)	2·18	1·55	42 (±2)	2·13	1·25	− 4	11	40
Comparison of bone and marrow from a rat with tumour and a normal rat	50 (±4)	3·68	4·55	23 (±4)	2·75	6·75	−27	35	40
	53 (±10)	2·90	5·65	35 (±3)	2·75	4·93	−18	36	45
Effect of incubating normal rat bone and marrow with mammary tumour	35 (±4)			73 (±4)			+38	25	500 mg/10 ml
	44 (±3)	3·05	6·05	82 (±4)	7·50	6·05	+38	25	500 mg/10 ml
Effect of cortisone acetate on normal rat bone and marrow	38 (±4)	3·50	5·33	46 (±3)	1·63	5·45	+ 8	12	
Effect of dihydrotachysterol on bone and marrow (DHT-sensitized rat)	36 (±1)	1·00	3·18	51 (±1)	1·05	4·43	+15	13	

Effect of incubating tumour and bone together

Normal rat femur was fragmented and incubated in 10 ml of enriched Krebs–Ringer medium with dispersed marrow and 500 mg of fresh sliced mammary tumour. This represented the control series. In the test series of five flasks the tumour had been frozen at $-70°$ and thawed five times before it was added to the medium with bone and marrow. The results indicated that destruction of the cellular structure of the tumour by freeze-thawing resulted in the liberation of some substance which could promote release of soluble calcium from bone. There was a continuous increase in the acid phosphatase activity of the test samples.

The possibility that the substance which caused the increased liberation of calcium in the test series of flasks might be dialysable was investigated in the following way. Sliced mammary tumour (5 g) was incubated in 50 ml of enriched medium for 6 hr at 37° and pH 7·4. The medium was centrifuged and dialysed overnight against 50 ml of fresh medium. Normal rat femur (200 mg) was incubated for 18 hr in the dialysate and the medium from the dialysis bag. The average figure for calcium among four flasks was 523 (range ± 63) μg in 10 ml of medium at the end of the experiment and for the four test flasks with additional non-diffusible solutes, 541 (± 20) μg.

This result demonstrated that diffusible solutes were responsible for the greater amounts of soluble calcium in the test series of the foregoing experiment. Sliced liver did not have the same effect as sliced mammary tumour in raising the final concentration of soluble calcium in the incubation medium under the same conditions. When the concentration of dialysable phosphate (NaH_2PO_3) in the control medium was raised to the same level as that in the test, as indicated by the molybdenum blue reaction, the amount of calcium released into the medium reached approximately the same final figure. Values for enzymatic activities of the phosphatases of both test and control series coincided closely, according to samples taken periodically.

Effect of cortisone and hydrocortisone on bone

Femur (110 mg) from a male rat was incubated in 10 ml of enriched medium under the usual conditions (control) and with 100 μg of hydrocortisone as the free steroid (test). The average value for soluble calcium in the controls (five flasks) was 267 (range ± 19) μg and in the test series 279 (± 23) μg. This result showed that hydrocortisone has no significant effect under our experimental conditions on the release of soluble calcium from bone *in vitro*. No enzymatic activities were measured

A similar experiment was carried out with 100 μg of cortisone acetate, in the test flasks. There was a 22% increase in the soluble calcium of the test series with a lowering of acid phosphatase activity (Table 1).

Effect of previous sensitization of a rat with dihydrotachysterol on the calcium exchange between femur and medium

One female rat (test) received 2 mg of dihydrotachysterol *per os* in 0·5 ml of vegetable oil 2 days before the experiment, according to the method of Selye and Padmanabhan.[15] An identical rat was treated with oil as a control. On the day of the experiment, the test rat weighed only 153 g. Femur (130 mg) was taken from each animal and incubated in enriched medium under the usual experimental conditions with dispersed marrow. The results of this experiment (Table 1) show that previous sensitization of

the animal brought about an alteration in its bone so that more soluble calcium than normal was released into the medium during incubation (41% increase with elevation of the alkaline phosphatase activity). It is generally accepted that Vitamin D and its analogues act directly on bone matrix to cause accumulation of citric acid and calcium release. None of the non-dialysable fractions of mammary tumours which were tested had the property of causing *in-vitro* osteolysis with raised alkaline phosphatase.

The addition of cortisone acetate to bone *in vitro* from a sensitized rat did not further raise the amount of soluble calcium during incubation under normal conditions.

DISCUSSION

Malignant tumours may act on bone directly, as in the case of a growth attached to bone or a metastasis from a primary tumour. Possible routes of indirect action by a tumour on bone include the blood circulation, trophic action on one or more of the endocrine glands, in particular the parathyroid, or by lowering the resistance of the body to stress, e.g. through the production of anaemia.

The purpose behind the experiments described here was the study of the influence of the substance(s) liberated into the blood by chemically-induced mammary tumour[11] on the calcium exchange of bone. In order to isolate bone from the influence of various hormones and Vitamin D, it was necessary to carry out experiments *in vitro* and then to add one or more factors at a time, in the hope of reproducing the situation as it might exist *in vivo*. By proceeding in this way it was possible to show that the medullary soft tissue or marrow, the bone matrix and the blood all play important parts in relation to the action of tumour on bone.

Bone with marrow from rats with the tumour released less calcium into the medium than normal bone with marrow, during incubation *in vitro* under the same conditions. Bone without marrow from rats with the tumour released the same amount of calcium into the medium (containing calf serum) as bone without marrow from normal animals. It was concluded from these results that a tumour of long duration of growth (about 6 weeks) progressively impairs the action of the calcium-mobilising elements in the bone marrow, finally reducing their effectiveness to about one-half of the normal level.

Normal bone matrix requires either marrow or serum in the medium before calcium is released into solution. Selective aging of the mineralized part of bone and of the marrow reduced the release of soluble calcium by 15% and 35% respectively. These experiments provided evidence that both the matrix and the marrow contained cellular elements necessary for the exchange of calcium between bone and the surrounding medium. Recent work by G. S. Gordan,[12] who kindly allowed us to see his manuscript before publication, has revealed that the release of soluble calcium from heat-killed bone matrix can reflect the level of parathyroid hormone in incubation media to which serum has been added. We have confirmed this finding, although the amount of calcium released from bone which had been boiled for 10 min was only half of the amount available from fresh bone. The action of serum on bone matrix appears to be due to parathyroid hormone attached to one of the serum proteins.

The question arises of whether the tumour itself secretes a substance with some of the properties of parathyroid hormone or Vitamin D, or whether it has a parathyrotrophic effect. If the former hypothesis is true, then incubation medium in which sliced

tumour has been incubated should have some effect in facilitating release of calcium from bone and the active factor, attached to serum proteins, should be retained by the dialysis membrane. In practice, it was found that the tumour did not appear to be capable of manufacturing any substance, apart from phosphate, which had any direct effect on calcium exchange of bone *in vitro*, and since the serum of rats with such tumours affected bone, it is probable that the tumour provokes a reaction elsewhere in the body which can affect bone. The action of the parathyroid gland may be altered by secretions from the tumour, but this simple explanation is rendered doubtful by the fact that patients with hypercalcaemia due to breast cancer do not respond to treatment with cortisone and that renal re-absorption of phosphate is normal, unlike the situation in hyperparathyroidism.

When bone is incubated *in vitro* in media containing inorganic phosphate, there is enhanced calcium release with raised acid phosphatase. A local metastasis from a primary tumour, lodged in bone and secreting dialysable phosphate could have this effect and may, to some extent, account for the bone erosion which has been reported clinically.

Cortisone acetate promoted the release of calcium from bone, *in vitro* with depression of acid phosphatases and this agrees with the findings of Clark *et al.*,[13] but the action of parathyroid hormone on bone *in vitro*, under our conditions, has not yet been investigated. It might be expected that the serum of a rat with a large tumour would contain less soluble calcium than that of a normal animal. In fact, of five animals which were tested, two had a raised serum calcium and three had normal.

One conclusion which can be drawn from these experiments is that tumour remote from bone does not exert a direct action on the exchange of calcium, for instance, by the secretion of a parathyroid hormone analogue or Vitamin D. If malignant tumours were to liberate such substances into the circulation, then the result would be a continuing osteolysis. Quoting Baker,[4] ". . . most tumours show a combination of osteolytic and osteoblastic elements, but all variations exist". Perhaps the simplest explanation of clinical and experimental findings is that malignant tumours have a trophic effect on one or more of the endocrine glands which affect the exchange of calcium, causing the release of calcium from the skeleton to vary according to the nature of the tumour and its state of development. Higher or lower levels of hormones than normal in the serum would determine whether the overall effect would be uptake or release of calcium by bone matrix.

Further experiments are in progress with human serum and fresh rat bone matrix in order to find whether some correlation can be made between the situation during *in-vitro* incubations, as already described, and the clinical state of patients.

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