The effect of zinc on alkaline phosphatase activity in rheumatoid synovial tissue

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SUMMARY   To examine the reported beneficial effect of zinc in rheumatoid arthritis, rheumatoid synovial tissue has been maintained *in vitro* in non-proliferative culture with or without zinc sulphate in the culture medium. Alkaline phosphatase activity was measured by microdensitometry of the cytochemical reaction in cryostat sections; the activity in blood vessels was measured separately from that in the supporting tissue below the synovial surface. Zinc enhanced this activity optimally at concentrations of between 10^{-5} and 10^{-4} mol/l.

In a double-blind trial, Simkin (1976) has shown beneficial effects of oral zinc in 24 patients with chronic refractory rheumatoid arthritis. He pointed out that one of the little known effects of penicillamine is to promote the gastrointestinal absorption of zinc (McCall *et al.*, 1967) and that the serum levels of this element in rheumatoid patients have been shown to be very significantly depressed (Niedermeier and Griggs, 1971). Although no one would suggest that zinc mimics all the effects of penicillamine (Lyle, 1976) and although the correlation between the clinical improvement and the increased concentration of zinc in the plasma in individual patients was not absolute, the effects were of sufficient interest to warrant further investigation. The use of non-proliferative adult organ maintenance culture of human synovial tissue has been shown to be useful in assessing the effect of anti-inflammatory drugs, giving similar effects to those obtained *in vivo* (Chayen and Bitensky, 1973; Bitensky *et al.*, 1974; 1977). Consequently it was decided to test the influence of zinc on human rheumatoid synovial tissue *in vitro* to try to determine whether this element had a marked direct biochemical effect on this tissue. The most striking response was seen in the alkaline phosphatase activity, which is the basis of this communication.

Material

Synovial tissue was obtained from 3 patients, all of

whom had definite or classical rheumatoid arthritis according to the criteria of the American Rheumatism Association (Ropes *et al.*, 1959). Specimen 2039 was obtained at operation for carpal tunnel decompression on a male patient, aged 59 (treatment: indomethacin and distalgesic); No. 2050 was from a synovectomy of the knee of a male patient, aged 72; No. 2056 from a synovectomy of the wrist in a female patient, aged 66. The last 2 patients were currently on prednisolone therapy (more than 5 mg/day). The specimens were taken in a bloodless field within about 10 min of the application of the tourniquet, and were placed immediately on a sterile gauze, soaked in Trowell's T8 medium, in a sterile tube.

Methods

Small pieces (about $5 \times 5 \times 3$ mm) were maintained at 37°C *in vitro* in Trowell's (1959) adult organ maintenance culture for 20 hours at pH 7·4 (Poulter *et al.*, 1970). The medium was the synthetic Trowell T8 medium (Trowell, 1959); for some specimens zinc sulphate (Zn SO₄·7H₂O, British Drug Houses Analar reagent) was included in the medium at various concentrations (from 10^{-3} to 10^{-6} mol/l).

At the end of the period of maintenance culture the pieces of tissue were chilled to $-70°C$ for up to 1 min in n-hexane (free from aromatic hydrocarbons, boiling range 67 to 70°C, obtained from BDH). For comparison, some biopsy specimens, which were not maintained *in vitro*, were chilled immediately they were received. The chilled specimens were transferred with cold forceps to dry glass tubes

at −70°C and stored at this temperature until required; storage did not exceed 5 days. They were then sectioned at 10 μm in a Bright's cryostat with the cabinet temperature of about −30°C and with the knife cooled to about −70°C by having its haft packed with solid carbon dioxide (Chayen *et al.*, 1973).

Sections were reacted for glucose 6-phosphate dehydrogenase, for lysosomal naphthylamidase, and for alkaline phosphatase activity. The methods used for the first two activities were the same as those described by Bitensky *et al.* (1974; 1977). Alkaline phosphatase activity was demonstrated by the Gomori calcium method as described by Chayen *et al.* (1973), the time of reaction being 30 min at 37°C. The specificity of the reaction for alkaline phosphatase activity was tested by including l-p-bromo-tetramisole oxalate (Aldrich Chemical Co.) at 10^{-4} mol/l concentration; the dextro-form of this substance (d-p-bromotetramisole oxalate), which lacks inhibitory power (Borgers and Thoné, 1975), was used as a further control of the specificity of this response.

The reaction-product was brown-black. Because of its optical properties and distribution it was measured by means of a Joyce Loebl double-beam recording microdensitometer equipped with an integrating system. The section was placed on the stage of this instrument and the image of the specimen, projected through a ×20 objective, was observed on the recording screen. The section was then positioned so that the image of the region just below the synovial lining cells impinged on the slits in the recording screen. These slits were set to a horizontal size of 0·3 mm and a vertical size of 1 mm corresponding to a size of $7·5 \times 25$ μm at the level of the section. Thus when the table was set in motion it scanned a region of these dimensions in the section, approximately parallel to and about 200 μm from the surface of the synovium (as indicated in Fig. 1).

In this instrument, one beam of light traverses the specimen while the second passes through a wedge of known neutral density (having a range of optical density of 0·88 to 1·5). Automatic mechanical movement of the wedge balances the optical densities
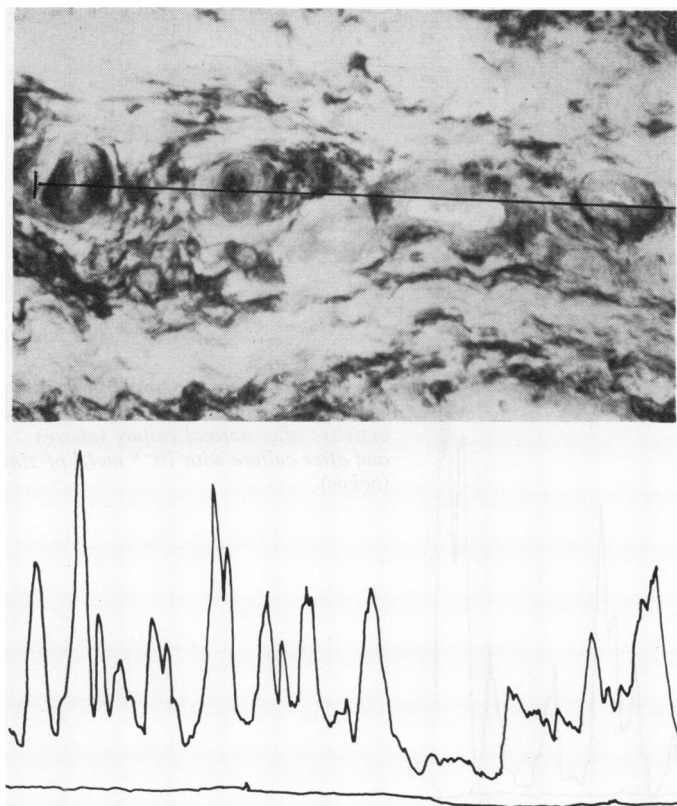

Fig. 1 *A densitometric trace through a region of a section reacted for alkaline phosphatase activity. The region traced is shown by the line in the photograph of the section (magnification ×240). The synovial surface is at the bottom of this photograph. The magnification of the trace has been adjusted so that the major peaks can be related to the edges of the blood vessels in the photomicrograph.*

of the two beams, and this movement is recorded on a chart which is synchronised with the slide-bearing table. A ratio arm of 100:1 increases the magnification of the record. A very similar instrument is used in clinical biochemistry for scanning electrophoretic strips and recording the amount of a serum protein or of activity of a particular isoenzyme.

A defined length of scan was recorded for each specimen (2·5 mm of the section), with the integrating unit switched on during the scan. This recorded a certain amount of integrated optical density. A second scan was then made in the blank field outside the specimen. The integrated optical density recorded for unit length of this scan represented the amount of light lost from the optical system by non-specific absorption through the glass of the slide and coverslip, and through the mountant. The amount of true absorption in the specimen, per unit length of the scan, was calculated by subtracting this figure from that measured within the specimen.

To evaluate changes in activity in the walls of blood vessels the height of the major peaks, usually 10 (as in Fig. 1), was measured in each trace. The differences were evaluated by the Student's *t* test.

Results

QUALITATIVE RESULTS

Exposure to zinc caused no great change in either the naphthylamidase or the glucose 6-phosphate dehydrogenase activities.

Alkaline phosphatase activity in the biopsy specimens was considerable; the activity diminished after the tissues had been maintained *in vitro*. The activity was strongest in blood vessels and was also present in small linearly orientated structures suggestive of small capillaries or lymphatics. There was moderate activity at the edges of the synovial lining cells and in and around infiltrating cells. When zinc was included in the culture medium the activity of all the stromal elements, but particularly that of the blood vessels, was markedly increased (Fig. 2).

QUANTITATIVE RESULTS

Integration of the amount of optical density for a scan of 2·5 mm in each specimen gave a quantitative measure of the qualitative observations. In both specimens in which a biopsy specimen (not subjected to maintenance *in vitro*) was measured, the activity after 20 hours in normal culture was about half that of the biopsy material. Including zinc in
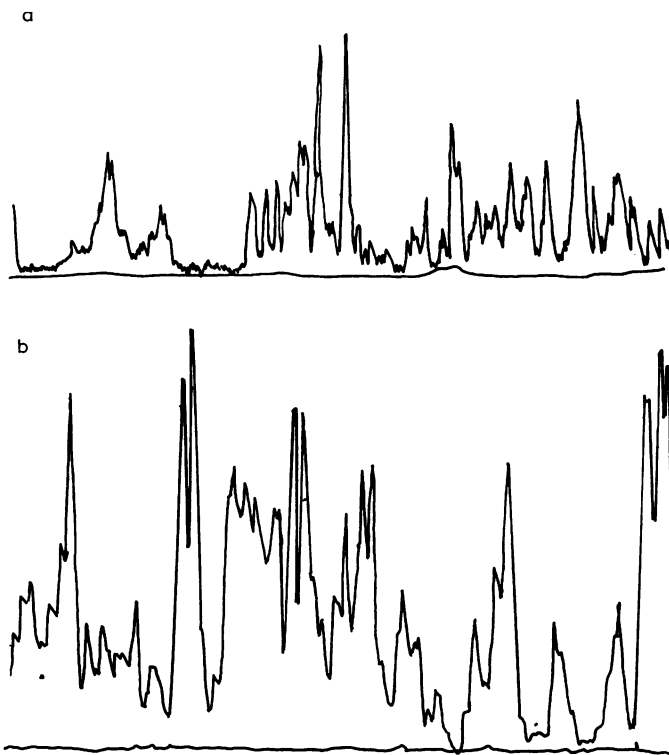
Fig. 2   *Densitometric traces through sections reacted for alkaline phosphatase activity: after normal culture (above) and after culture with 10^{-4} mol/l of zinc (below).*

the culture medium increased this activity at low concentrations of zinc: the optimal concentration for two of the specimens was 10^{-4} and 10^{-5} mol/l for the third specimen. There appeared to be some inhibitory effect at 10^{-3} mol/l (Table 1).

ANALYSIS OF TRACES

Because the strongest activity resided in the blood vessels it was possible that the values recorded, when a region was scanned, might have been influenced by the number of blood vessels in the selected region. Although a long scan was taken to avoid such sampling error, and the scans were made in histologically characteristic and comparable regions, this possibility of error had to be considered.

Consequently the traces obtained from such scans were analysed for maximal activity, such peaks being caused by the activity of blood vessels. Where possible the ten greatest peaks, attributable to blood vessels, were measured. The results (Table 2) agreed with those obtained by integrating the whole trace (Table 1). Thus at a particular concentration of zinc in the culture medium, which varied slightly between the specimens, there was a highly significant increase in peak activities recorded. The same inhibition at higher levels (10^{-3} mol/l) was also observed.

Discussion

The dose of zinc sulphate used by Simkin (1976) was 220 mg 3 times daily. Obviously not all of this will have been absorbed (Oelshlegel and Brewer, 1977). But it is possible to make some estimate of the upper limit of concentration of zinc achieved in the circulation if we do assume complete absorption, and take certain other variables into account. Thus if it mixed only with the blood (taken as 5 l), the dose would produce a concentration of either $2 \cdot 8$ or $1 \cdot 4 \times 10^{-4}$ mol/l, depending on whether he used the anhydrous or the hydrated ($Zn SO_4 \cdot 7H_2O$) zinc sulphate. If its mixing volume is that of extracellular fluid (taken as 17 l) the equivalent concentrations would be 9 or $4 \cdot 4 \times 10^{-5}$ mol/litre.

In the present study zinc, at between 10^{-5} and 10^{-4} mol/l, elevated the alkaline phosphatase activity in rheumatoid synovial tissue maintained *in vitro* for 20 hours (Table 1). This phenomenon was not confused by sampling error caused by the vascularity of the areas sampled because it applied even when the activity of the vessels alone was measured (Table 2). The enzyme has zinc at its active site (Ahlers, 1975) and it can be inhibited by substances, such as reduced glutathione, that bind to zinc (Ahlers, 1975; Shedden *et al.*, 1976). Thus it is possible that the alkaline phosphatase activity of rheumatoid synovial tissue is partially suppressed by such molecules and can be freed from such inhibition by exogenous zinc.

Although the function of alkaline phosphatase is not fully understood, it is implicated in absorption and transport mechanisms. Thus it is very active in the brush border of the proximal convoluted tubules of the kidney cortex, and of the villi of the small intestine. It follows that it may be involved in the absorption of fluid in the synovial tissue. The enhancement of activity produced by zinc might account for the significant improvement in joint swelling recorded by Simkin (1976) in patients treated with zinc sulphate.

Table 1 *The* in vitro *effect of various concentrations of zinc on alkaline phosphatase activity (integrated optical density/unit length of trace)*

Case No.	Normal culture	Culture plus zinc at mol/l					
		5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}
2039	154	—	207	—	504	—	424
2050	134	—	553	—	102	—	147
2056	608	758	—	762	—	826	—

Table 2 *The* in vitro *effect of various concentrations of zinc on the peak heights of alkaline phosphatase activity (mean ± standard error)*

Specimen No.	Normal culture	Culture plus zinc at mol/l					
		5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}
2039	$55 \cdot 4 \pm 8 \cdot 1$	—	$54 \cdot 1 \pm 13 \cdot 0$	—	$122 \cdot 7 \pm 12 \cdot 2*$	—	$76 \cdot 4 \pm 5 \cdot 8$
2050	$46 \cdot 0 \pm 7 \cdot 1$	—	$114 \cdot 6 \pm 9 \cdot 7*$	—	$57 \pm 13 \cdot 5$	—	$54 \cdot 1 \pm 6 \cdot 8$
2056	$95 \cdot 7 \pm 5 \cdot 9$	$130 \cdot 4 \pm 13 \cdot 6$	—	$134 \cdot 5 \pm 7 \cdot 7*$	—	$130 \cdot 1 \pm 6 \cdot 3*$	—

* $P < 0 \cdot 001$ compared with activity in normal culture (Student's *t* test).

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