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EFFECTS OF ANTIRHEUMATIC DRUGS ON SPONGE-INDUCED GRANULATION TISSUE, RHEUMATOID SYNOVIAL TISSUE, MATRIX-FREE TENDON CELLS AND FIBROBLAST PLASMA MEMBRANES *IN VITRO*

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Abstract—Slices of sponge-induced, mature granulation tissue were incubated in the presence of antirheumatic drugs (sodium acetylsalicylate, indomethacin and phenylbutazone at concentrations of 10^{-7}–10^{-2} M) and labelled precursors of nucleic acids, proteins and acid mucopolysaccharides. Analogous experiments were made with sliced human synovial tissue from rheumatoid patients and with matrix-free embryonic chick tendon cells. The synthetic functions were suppressed by 10^{-4}–10^{-3} M indomethacin and phenylbutazone but only by 10^{-2} M acetylsalicylic acid. At low concentrations (10^{-5}–10^{-6} M) there was an increase in the radioactivity in proteins during the early period of the incubation but this was reversed later. Indomethacin and phenylbutazone affected the production of collagen at two points: (i) the synthesis of the peptide chain and, more notably, (ii) the secretion of collagen to the extracellular space. Indomethacin affected the final content of cellular collagen only slightly. Preliminary experiments on the effect of indomethacin on the activities of enzymes in plasma membranes prepared from granulation tissue showed some effect on leucyl-β-naphthylamidase and Na^+,K^+-activated Mg^{2+}-adenosine triphosphatase. The use of such systems for the screening of new connective tissue-active drugs is discussed. The secretion of collagen from matrix-free embryonic tendon cells is the most sensitive target for indomethacin *in vitro*.

In an earlier paper [1] it was shown that the administration of certain antirheumatic drugs to rats suppresses the development of sponge-induced granulation tissue, and especially the metabolic capacities of the incubated granulation-tissue slices. At moderate doses, however, the formation of hydroxyproline, and hence of collagen, in slices of granulation tissue was apparently increased by indomethacin. The content of uronic acids in the tissue was not decreased whereas there was a notable diminution in the amount of hexosamine and in the synthesis of sulphated polysaccharides.

The purpose of this work was to find out whether related effects could be demonstrated *in vitro* with various preparations of connective tissue. The test systems were extended from granulation tissue slices to human synovial tissue slices and to detached tendon cells from chick embryos. Because evidence from the literature [reviewed in Refs. 2–4] suggests that the subcellular membranes are involved in rheumatoid disease and are targets of antirheumatic drugs [5], some functions of plasma membrane preparations from granuloma were also tested.

EXPERIMENTAL

Preparation, incubation and analysis of granuloma slices. Granulomas were induced subcutaneously with viscose cellulose-sponge in adult rats of Wistar strain. After 18–24 days the granulomas were harvested, sliced with a Stadie-Riggs microtome and incubated at 37° in Krebs–Ringer medium buffered to pH 7·4 with Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid; Sigma, St. Louis, U.S.A.) and containing 22·4 mM glucose. The gas phase was air. After preincubation for 30 min the precursors ([^{3}H]cytidine, [^{3}H]proline or [^{35}S]sulphate) and the drugs were added and the incubation continued for 3 hr.

The details are described in the previous paper [1] and in other publications [6–8]. One series of experiments was carried out with rat liver slices prepared and treated the same as the granulation tissue.

DNA was isolated as a nucleotide mixture after a modified Schmidt–Thannhäuser procedure [1]. For the analysis of proteins the washed and dried material was gelatinized in water and then both collagenous and non-gelatinizable protein fractions were hydrolyzed separately. Imino acids in the hydrolyzed gelatinized fraction were oxidized to pyrroles and the derivative of hydroxyproline was isolated for the determination of radioactivity. The sulphated mucopolysaccharides (AMPS) were liberated by hydrolysis with papain. The solubilized carbohydrates were precipitated first with ethanol and then with cetylpyridinium chloride (CPC) [1].

The details of the analytical procedures are described in the earlier papers [1, 6–8].

In one experiment the slices were pretreated with neuraminidase [9]. They were preincubated for 15 min in Krebs–Ringer–Hepes medium, pH 6·5, which did not contain glucose or proline. Then neuraminidase solution (Behringwerke AG, Marburg/Lahn) was added to a final concentration of 10 U/ml medium and the slices incubated with shaking for 60 min. The incubation was stopped by the immersion of the bottles in ice and the slices washed four times

with the cold complete medium, pH 7·4. The control samples were treated similarly but without enzyme. After preincubation for 30 min in the final medium which contained indomethacin also, [^{3}H]proline was added and the incubation continued for 3 hr.

Incubations with human synovial tissue slices. The samples of human synovial tissue were obtained from the University Hospital in Turku. They originated from various joints of seven rheumatoid patients aged 8 to 70 yr, with rheumatoid disease. The patients had been pretreated with various drugs. The dissected tissue was immersed in ice-cold Krebs–Ringer–Hepes medium (pH 7·4) and slices were prepared as soon as possible, usually within one hour. Each incubation was performed in duplicate with ^{3}H-proline and indomethacin as described above for the granulation-tissue slices. The results were calculated in per cent, taking the indomethacin-free sample as the reference.

Incubations with matrix-free tendon cells of chick embryos. The procedure was adopted from Dehm and Prockop [9, 10]. The leg tendons of about twenty 17-day Leghorn-chick embryos were placed into siliconized glass vessels containing 3 ml of Krebs–Ringer, buffered with 20 mM Hepes, pH 7·4, and containing 22·4 mM glucose as well as 0·33 ml of 5% trypsin (Type III, Sigma) in 154 mM NaCl and 3·3 mg of bacterial collagenase (Type I, Sigma). After incubation at 37° for 60 min with vigorous shaking, the mixtures were filtered through lens paper and centrifuged at 50 *g* for 12 min at room temperature. The sedimented cells were suspended in 6 ml of 0·1% soybean trypsin inhibitor (Serva Feinbiochemica GmbH, Heidelberg, Germany) in Krebs–Ringer medium. The cells were washed twice with the medium by centrifugation.

About 10^6 tendon cells were suspended in 3 ml of Krebs–Ringer medium which contained 0·1 mM ascorbate and 14·5 μM proline. After a 15-min preincubation 5 μCi of [^{3}H]proline and various amounts of the drugs were added and the incubation continued for 2 hr if not stated otherwise. The incubation was stopped by adding 270 μg of cycloheximide (Sigma) and 400 μg of α,α′-dipyridyl (Fluka AG, Buchs, Switzerland) in 0·2 ml of Krebs–Ringer medium. Aliquots were taken for the assays with an electronic cell counter (Celloscope 401, Linson Instrument AB, Stockholm, Sweden) and for viability measurements with the trypan-blue test [11].

The medium was separated from the cells by centrifuging the samples once and the cells were suspended with the same medium containing 100 mg/l. cycloheximide and 155 mg/l. of α,α′-dipyridyl, and recentrifuged. This supernatant was discarded. The cells were sonicated in 5 ml of water (Disontegrator®, System Forty, Ultrasonic Industries Inc., Plainview, N.Y., U.S.A.). The medium and the cell suspension were dialyzed separately for 24 hr with 0·05 mg of unlabelled proline against running tap water. The dialyzates were hydrolyzed in 6 N HCl at 130° for 3 hr. Hydrochloric acid was evaporated on a boiling water bath. Aliquots were taken from the hydrolyzates for the assay of total radioactivity and of [^{3}H]hydroxyproline [12]. The standard error of the mean varied in the range of 7–9% (n = 5–6).

Preparation and analysis of plasma membranes from sponge-induced granuloma. Three-week old sponge-induced granulomas were pressed through a perforated plate [13] and the homogenization completed in a tight Dounce homogenizer (0·075 mm radial clearance). After centrifugation (7000 *g*, 30 min) the sediment was rehomogenized in 8·54% sucrose and centrifuged in a discontinuous sucrose gradient (layers 20%, 28% and 38%) for 4 hr at 100,000 *g* in a swing-out rotor. The fraction at the 28/38% sucrose interphase contains the bulk of the plasma membranes. A detailed account on the procedure has appeared [14].

The plasma membranes were suspended in isotonic sucrose and diluted with 3·6 mM Tris–buffer, pH 7·4. The drugs were allowed to act in various concentrations on the plasma membrane suspension for 60 min in water bath at 37°. Then the substrates for the various enzymes were added. Leucyl-β-naphthylamidase was determined according to Goldbarg and Rutenburg [15], Na^+,K^+-activated Mg^{2+}-ATPase according to Swanson *et al.* [16], 5′-nucleotidase according to Michell and Hawthorne [17], and adenylyl cyclase according to Krishna *et al.* [18]. The phosphorus determinations were carried out as described by Martin and Doty [19].

RESULTS

Effects of added antirheumatic drugs on the synthesis of DNA, proteins and acid mucopolysaccharides in tissue slices. Figs. 1–3 show that the addition of antirheumatic agents to the incubation medium suppressed the incorporation of connective tissue precursors depending on the concentration of the drugs. About 10-fold concentrations of sodium acetylsalicylate were required for the same effect as with indomethacin and phenylbutazone which were active at 0·1–1 mM. The synthesis of acid mucopolysaccharides was especially sensitive.

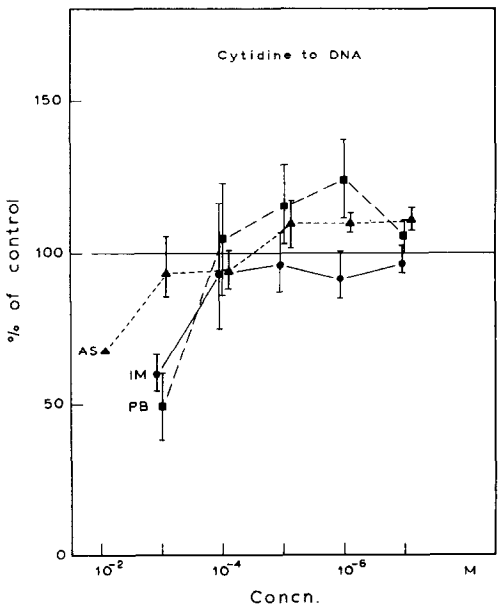

Fig. 1. Effect of various concentrations of antirheumatic drugs on the incorporation of [^{3}H]cytidine into DNA in incubated granulation-tissue slices. The points are averages of 2–4 independent experiments with the dispersion calculated as S.E.M. shown by the vertical bars. ▲, sodium acetylsalicylate (AS); ●, indomethacin (IM); ■, phenylbutazone (PB).

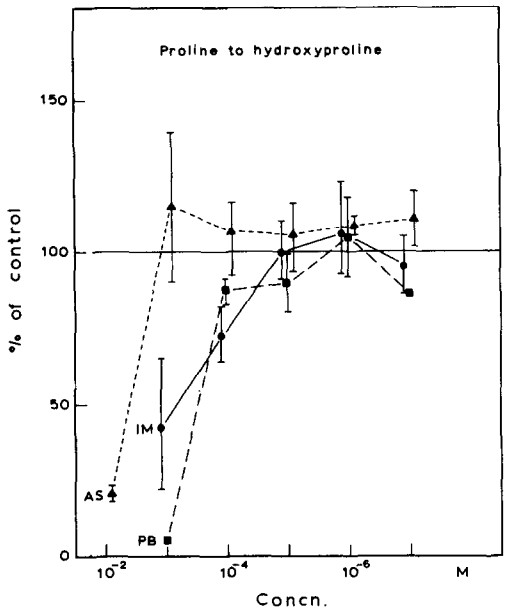

Fig. 2. Effect of various concentrations of antirheumatic drugs on the incorporation of [^{3}H]proline into collagen-hydroxyproline (n = 2–4). The dispersion is calculated as S.E.M. shown by vertical bars. ▲, sodium acetylsalicylate (AS); ●, indomethacin (IM); ■, phenylbutazone (PB).

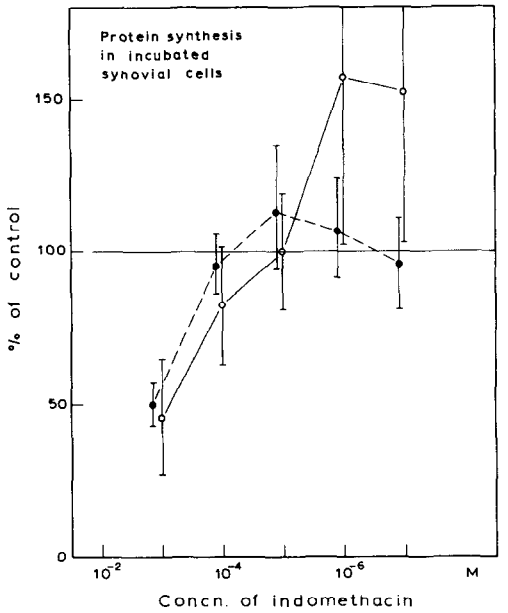

Fig. 4. Effect of indomethacin on the incorporation of [^{3}H]proline into proteins in slices of synovial tissue from rheumatoid patients (n = 7). S.E.M. is shown by vertical bars. ○ collagen (hydroxyproline), sp. act.; ●, non-collagenous protein.

There was an apparent stimulatory effect of phenylbutazone on the synthesis of DNA (Fig. 1) and of sodium acetylsalicylate on the synthesis of collagen (Fig. 2), both at the low concentrations resembling the plasma levels in treated patients.

When liver slices were incubated in the presence of sodium acetylsalicylate (10^{-2} M), phenylbutazone (10^{-3} M) or indomethacin (10^{-3} M), there was about 40–50% decrease in the incorporation of [^{3}H]proline into non-collagenous protein. This shows that these effects of the antirheumatic drugs are not limited to connective tissue.

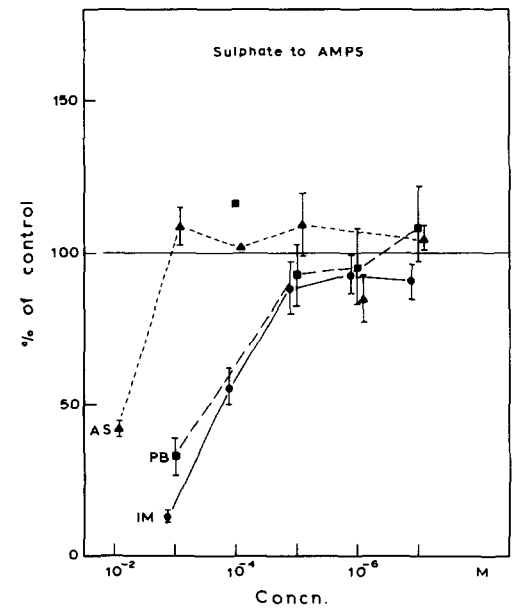

Fig. 3. Effect of various concentrations of antirheumatic drugs on the incorporation of [^{35}S]sulphate into acid mucopolysaccharides (AMPS) (n = 2–4). The dispersion is calculated as S.E.M. shown by vertical bars. ▲, sodium acetylsalicylate (AS); ●, indomethacin (IM); ■, phenylbutazone (PB).

Synovial tissue from rheumatoid patients is the most relevant for experimentation with antirheumatic drugs *in vitro*. The effect of indomethacin on the incorporation of [^{3}H]proline into proteins, including collagen, was marked (Fig. 4). The variation in the test tissue accounts for the high dispersion of the results, but the addition of indomethacin (10^{-7}–10^{-4} M) seemed to increase the synthesis of collagen significantly ($P < 0.025$; $n = 7$). There was general agreement with the findings obtained above for granulation-tissue slices.

Figure 5 shows that the hourly increments in the specific activities of collagen-bound hydroxyproline were positive during the two first hours of incubation and that during the 2nd hr they were significantly higher in the presence of indomethacin ($P < 0.05$; $n = 6$). In the type A experiments [^{3}H]proline remained in contact with the slices during the whole experiment. In the type B experiments the medium, containing both the isotope and indomethacin was changed after the first hour. The slices were washed five times with 5 ml of cold complete medium until the last washing did not contain any measurable radioactivity. After this interruption of about 30 min the incubation was continued without isotope but in the presence of indomethacin for a further 3 hr.

Effect of added antirheumatic drugs on the synthesis and secretion of collagen in matrix-free embryonic tendon cells. In addition to the synthesis of collagen, the secretion of collagen to the medium could also be measured. Figure 6 confirms the effect of indomethacin and phenylbutazone on the synthesis of collagen

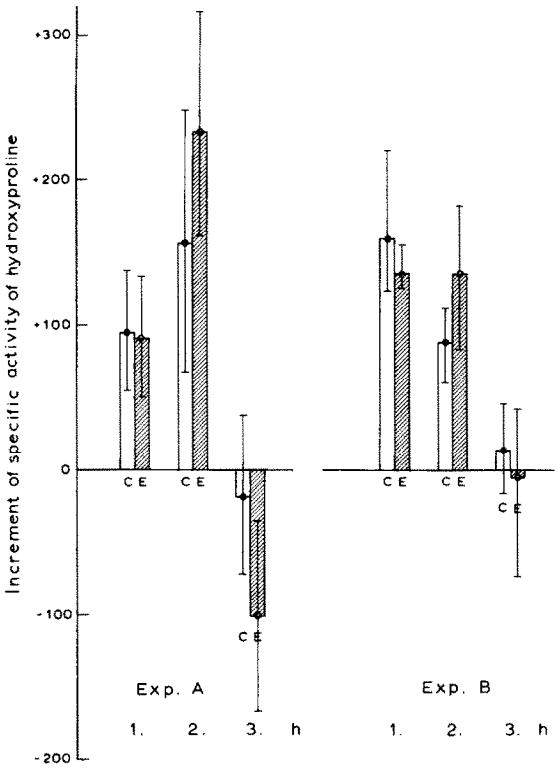

Fig. 5. Effect of indomethacin on the formation of collagen in incubated slices of granulation tissue during subsequent time intervals. Exp. A, cumulative experiment with a sustained presence of [^{3}H]proline. Exp. B, pulse experiment with [^{3}H]proline present during the first hour of the incubation whereafter the washed slices were transferred to fresh medium with indomethacin. C, control; E, respective experimental average hourly increment in the presence of 10^{-5} M indomethacin (in one experiment 5×10^{-5} M). The ranges of triplicate determinations are shown by vertical bars.

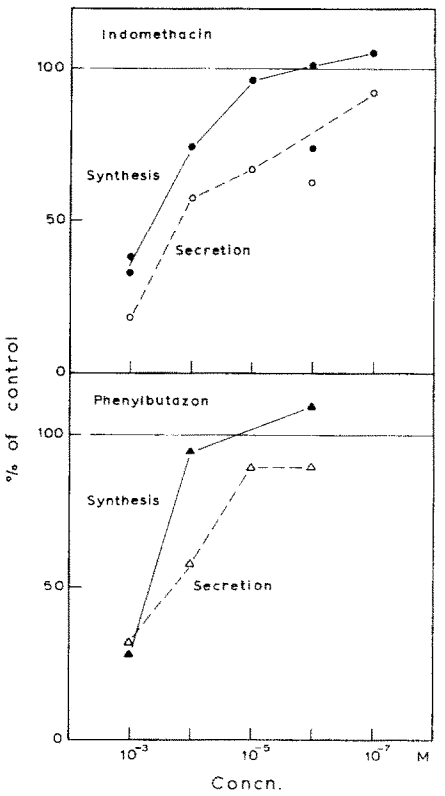

Fig. 6. Effect of antirheumatic drugs on the synthesis and secretion of collagen in incubated matrix-free embryonic tendon cells. Collagen synthesis: ●, with indomethacin; ▲, with phenylbutazone; Collagen secretion (labelled hydroxyproline in medium vs cells); ○, with indomethacin; △, with phenylbutazone.

but it shows that the secretion of collagen, i.e. the ratio of collagen in medium vs cells, was affected still more. This is seen directly in the top part of Fig. 7. The lower part of Fig. 7 shows explicitly that indomethacin affected mainly the secreted portion of collagen. In the cells the amount of collagen was almost constant at the various concentrations of indomethacin. The ratio of collagen in medium vs cells was very sensitive to indomethacin. The secretion was inhibited almost entirely at 10^{-3} M but affected already at 10^{-7} M indomethacin.

The amount of radioactive collagen in the incubated matrix-free cells remained rather steady after 90 min but increased in the medium almost linearly for the first 5 hr at least (Fig. 8). The effect of varying the concentration of indomethacin was more marked in the amounts of labelled collagen in the medium than in the respective concentrations in the cells where the three curves reached almost the same level after initial lagging in the presence of indomethacin. A more detailed analysis of the data shows (Fig. 9) that the effect of indomethacin on the secretion of collagen became more marked with time, at least to 180 min.

Figs. 10 and 11 show an experiment on the passage of collagen from the cells to the medium after 20-min incubation with ^{3}H-proline. After this pulse the cells

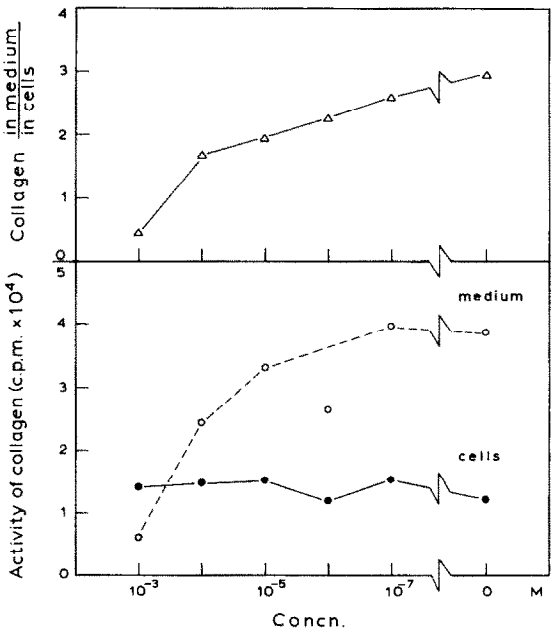

Fig. 7. Effects of indomethacin on collagen in cells vs medium of incubated matrix-free embryonic tendon cells. c.p.m. activity in the whole sample. Total activity of collagen in ● cells, ○ medium; collagen in medium vs cells △.

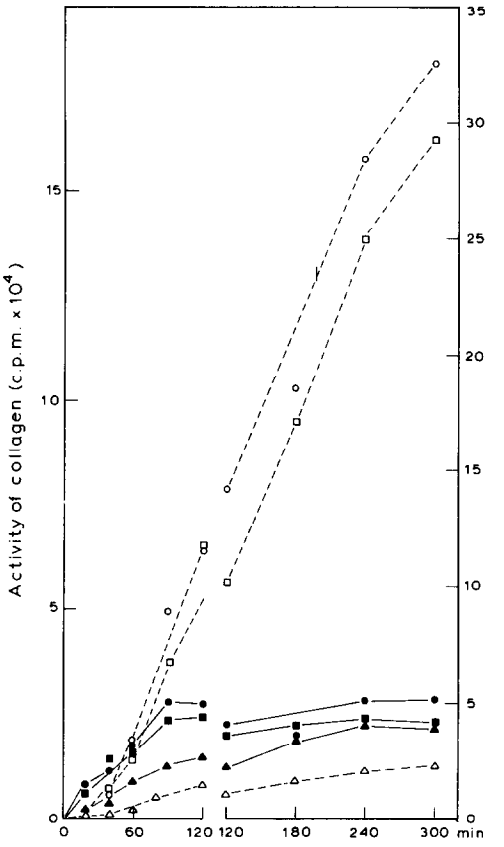

Fig. 8. The appearance of labelled collagen during the incubation of matrix-free embryonic tendon cells with [^{3}H]proline in the presence of indomethacin. The figure shows the combined results of two experiments (0–120 min, left ordinate; 120–300 min, right ordinate). No indomethacin; cells ●, medium ○; 10^{-5} M indomethacin; cells ■, medium □; 10^{-3} M indomethacin; cells ▲, medium △.

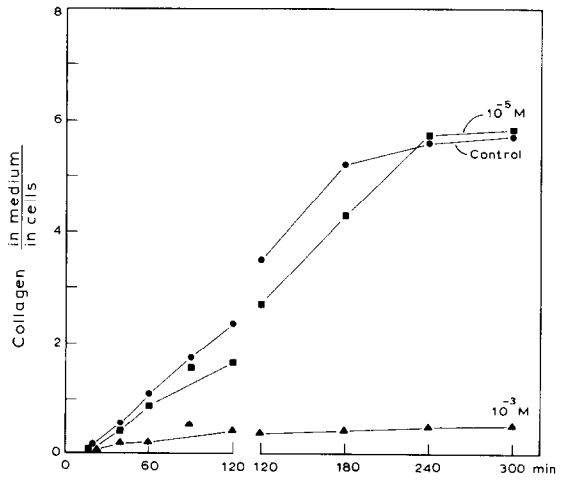

Fig. 9. Secretion of collagen by matrix-free embryonic tendon cells in the presence of indomethacin. The figure is based on the same two experiments as Fig. 8. No indomethacin ●, 10^{-5} M indomethacin ■, 10^{-3} M indomethacin ▲.

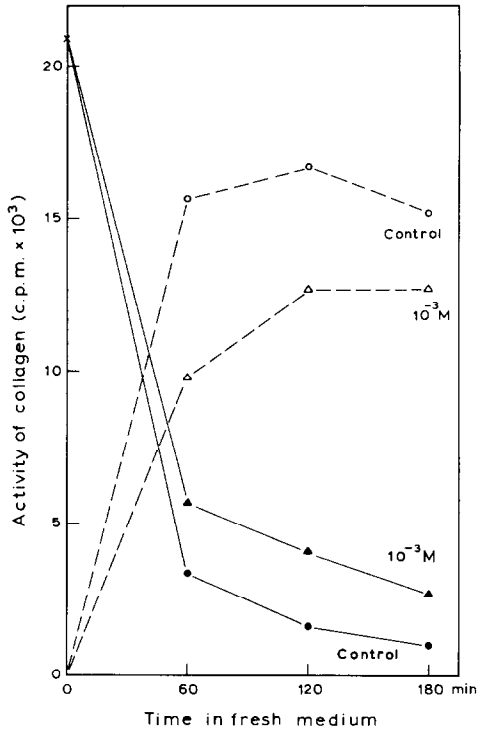

Fig. 10. The transfer of labelled collagen from the cells to the medium of incubated matrix-free embryonic tendon cells in the presence of indomethacin. After a 20-min incubation in the presence of [^{3}H]proline the cells were collected, washed and transferred to fresh medium which contained 10^{-3} M indomethacin and the incubation continued for 180 min. Activities in the cells ●, ▲; in the medium ○, △.

were transferred to fresh medium which contained various concentrations of indomethacin. The content of collagen decreased rapidly in the cells, less if indomethacin was present, and appeared in the medium. The transfer of collagen to the fresh medium was slower during the first 60 min but thereafter increased slightly (Fig. 11 bottom). Collagen was secreted more rapidly than the other proteins and indomethacin affected the secretion of collagen especially (Fig. 11 top). The experiment made with a 90-min incubation with the labelled precursor is not presented in detail because the results confirmed those described above. After the transfer of the cells to fresh medium, the ratio of collagen in the cells to that in the medium rapidly assumed the value reached in the original incubation mixture.

Antirheumatic drugs and the plasma membranes. Pretreatment with neuraminidase has an inhibiting effect on the synthesis of collagen in granuloma slices [9]. When combined with the action of indomethacin, the influences seemed independent and the results do not suggest that indomethacin would be bound on the cell surface through sialic acid-containing glycoproteins as suggested for another indole derivative, serotonin [20].

Prompted by the suggestion that antirheumatic drugs affect the function of membranes [2–5], the activities of certain characteristic enzymes from isolated plasma membranes of fibroblasts were determined

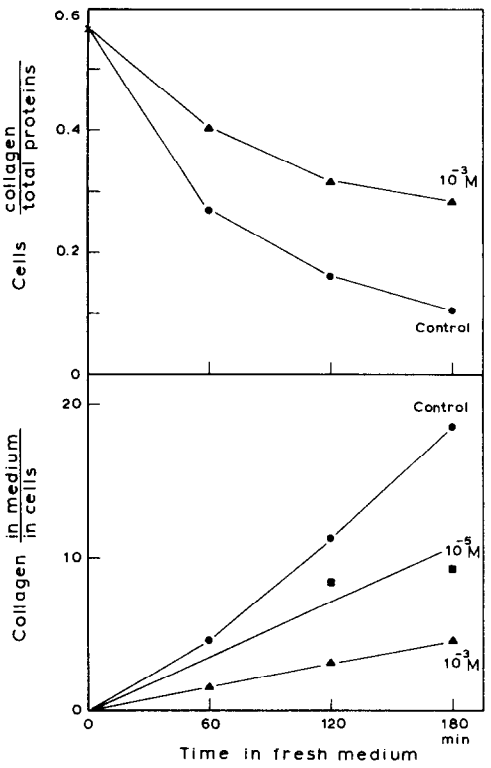

Fig. 11. Time course of the transfer of labelled collagen from cells to medium in the presence of indomethacin. The data refer to the same experiment as described in the legend to Fig. 10. The top part shows the change in the mutual relations of cell proteins, the lower part the appearance of collagen in the fresh medium. Indomethacin; none ●, 10^{-5} M ■, 10^{-3} M ▲.

(Fig. 12). The effects of antirheumatic drugs were small if any. At high concentrations phenylbutazone and sodium acetylsalicylate inhibited both Na^+,K^+-activated Mg^{2+}-ATPase and leucyl-β-naphthylamidase slightly. Indomethacin was more active but first at a concentration of 10^{-4}–10^{-3} M. To ascertain whether the drugs would produce changes in the plasma membranes of the intact cells, granulation-tissue slices were incubated, as described above, in the presence of 10^{-3} M indomethacin, homogenized and centrifuged. The activity of leucyl-β-naphthylamidase was decreased by 14% and that of Na^+,K^+-activated Mg^{2+}-ATPase by 11%. The antirheumatic drugs had no measurable effect on adenylyl cyclase with either type of experiment. Because the concentration of 10^{-3} M indomethacin was not effective, the eventual actions at lower, more relevant concentrations, can be excluded.

DISCUSSION

Relation of the effects in vitro *to those* in vivo. The effects of the antirheumatic drugs on the metabolism of DNA, proteins and acid mucopolysaccharides in slices seem to be two-fold: (i) at the concentration 10^{-4}–10^{-2} M there is a general depression of the syntheses, but (ii) at lower concentrations there are indications of a temporary stimulation. Both these findings have their counterparts in the results obtained when the drugs were administered *in vivo* [1].

Phenylbutazone is reported to inhibit the incorporation of glucosamine into proteins in rat intestinal slices [21]. The effect of phenylbutazone on the synthesis of DNA is interesting because it has been shown [22] that phenylbutazone interferes with mitosis, that the Ehrlich ascites tumor cells are enlarged under its influence and that it depresses DNA synthesis in lymphocytes [23]. The suppressing effect of anti-inflammatory drugs on the synthesis of sulphated mucopolysaccharides was observed before by Whitehouse and Boström [24] with slices from cartilage. The subject is reviewed by Domenjoz [pp. 537–544 in Ref. 4]. All the connective tissue-suppressing substances are potential antirheumatic drugs.

From the experiments *in vivo* [1] we know that the net amount of collagen accumulated in experimental granulomas is decreased as the effect of even moderate treatment with indomethacin, although the total

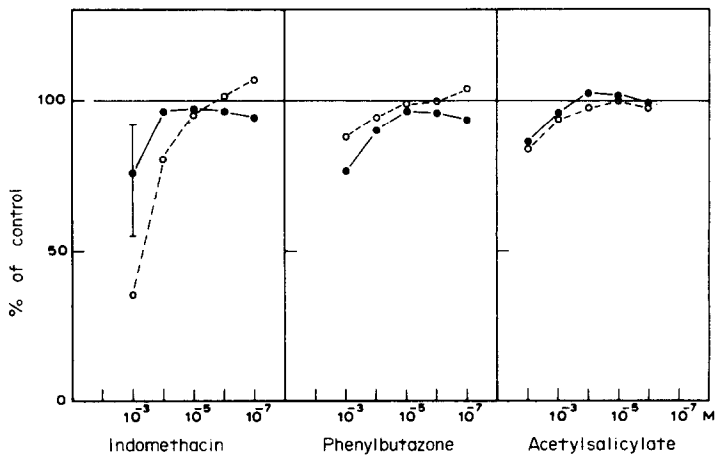

Fig. 12. Effect of antirheumatic drugs on the activities of some enzymes in isolated plasma membranes from granulation tissue. ●, Na^+, K^+-activated Mg^{2+}-adenosine triphosphatase; ○ leucyl-β-naphthylamidase.

radioactivity of collagen, originating from [^{3}H]proline in incubated slices from similar granulomas, may be increased. The present and previous results suggest that indomethacin stimulates the incorporation of labelled proline into proteins of the incubated slices so that a higher level of radioactivity in collagen is reached in treated than in control samples. However, the degradation of collagen also occurs sooner in the presence of indomethacin, and the total amount of collagen actually remains smaller. The incubated slices are slowly dying and it is not known how the decay rate of various subcellular structures and metabolic functions, including protein synthesis, depends on the rheumatoid disease and on the drugs added, e.g. on their stabilizing effects [Ref. 3, p. 577]. The explanation of the apparent stimulation of collagen synthesis by indomethacin cannot lie in the hydroxylation of collagen proline because increased activity is observed also in noncollagenous proteins. It is possible that granulation tissue contains two types of collagen [25], but it is not known whether the drugs affect the turnover of these two collagens to a different degree.

The work with the matrix-free tendon cells fully confirms the findings for the administration of indomethacin *in vivo* as well as those obtained with granulation-tissue slices. Labelled collagen in the extracellular compartment is more drug-dependent. The most sensitive parameter for small doses of indomethacin and phenylbutazone seems to be the ratio of extra- vs intracellular collagen, i.e. its secretion.

The cell wall function is necessary for collagen synthesis as exemplified by the effects of neuraminidase [9] and ouabain [7]. It is natural that connective tissue-suppressing antirheumatics can act on different and multiple levels. A general target in membranes would involve the synthesis of collagen in the endoplasmic reticulum but also its intracellular transport and secretion in the various membranous subcellular organs. It is not clear whether the secretion is related to the postribosomal modifications (hydroxylations, glycosylations, conversion of procollagen) of collagen. Antirheumatic drugs are assumed to stabilize the lysosomal membranes [reviewed in Refs. 3, 4; 2, 26].

Metabolic systems in the assessment of antirheumatic drugs in vitro. The following systems, at least, can be applied to the metabolic study of the antirheumatic drugs *in vitro* as listed in the order of decreasing relevancy: (i) human rheumatoid synovial tissue; (ii) slices of experimental granulation tissue or of artificially irritated synovial tissue [22, 27]; (iii) integrated normal or embryonal tissues (calvaria [28], tibia, skin); (iv) suspensions of detached matrix-free fibroblasts [10, 29]; (v) cultured fibroblasts either from biopsy samples [30] or established cell lines; (vi) nonfibroblastic cultured cells [31, 32], and (vii) subcellular parts or enzyme proteins [4, 27].

Because there may be cytological differences between the fibroblasts in normal and rheumatoid tissues it is not certain whether antirheumatic agents can be assessed properly with a non-rheumatoid system. Human rheumatoid synovial tissue may be specifically sensitive to the antirheumatic drugs and it is therefore the first choice, but its supply is limited and its composition variable. Experimental granulation tissue is the second choice.

The use of incubated matrix-free tendon cells introduces a new parameter: secretion of collagen. Nevertheless, this system is less relevant to rheumatoid disease than granulation tissue or human synovial tissue. The granulation tissue and rheumatoid synovial tissue presumably differ from the embryonic tendon in their biological function and metabolic state and the collagen produced may be different [33].

In the previous paper [1] it was stated that the administration of a supposed antirheumatic drug *in vivo* and the subsequent study of the developing granulation tissue is not a suitable method for screening. However, if the insolubility of the test substance does not limit the work *in vitro*, granulation-tissue slices and detached matrix-free cells could be used to screen the connective tissue-suppressing effect of various compounds because only mg-quantities are needed, the scatter is smaller and large-scale work is easier to organize. Their sensitivity can be compared with that of the pharmacological models, for example, the prevention of oedema [34].

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