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EFFECT OF THE ADMINISTRATION OF ANTIRHEUMATIC DRUGS ON EXPERIMENTAL GRANULOMA IN RAT

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Abstract—Subcutaneous granulomas were induced in rats by viscose cellulose-sponge and allowed to develop for 7 or 21 days. Before sacrificing the rats were treated for 5 days with antirheumatic drugs: sodium acetylsalicylate 300 mg/kg per day, prednisolone 1 and 10 mg/kg per day, indomethacin 0·1 and 10 mg/kg per day or phenylbutazone 100 mg/kg/day. The contents of DNA and especially of RNA in the granulomas were decreased, and the incorporation of ^{3}H-cytidine into DNA *in vitro* was suppressed. The amounts of total nitrogen and of hydroxyproline (and consequently of collagen) were decreased. The incorporation of ^{3}H-proline into proteins of granuloma slices was also decreased, except after treatment with the moderate dose of indomethacin, which apparently increased the incorporation of proline into collagenous proteins. This paradoxical effect of indomethacin is discussed. The content of total hexosamine was also decreased but that of uronic acids was increased. It is suggested that by multiple mechanisms the antirheumatic drugs retard the development of granulation tissue, but not the synthesis of acid mucopolysaccharides. At the proliferative phase the granulomas are more sensitive to the drugs than at the mature phase, and the incorporation rates are more affected than the total amounts of nitrogenous components.

Recent studies indicate that antirheumatic drugs act through multiple and varied mechanisms [1–4]. The purpose of this work was to elaborate the effects of certain antirheumatic drugs on sponge-induced granulation tissue. To ensure relevancy to the phenomena *in vivo*, the comprehensive effects on the composition of the granulation tissue *in vivo* were defined first, though more fundamental knowledge is to be found through assessment of the metabolic capacities of the tissue.

An important element in rheumatoid disease is the proliferation of the cells and of the non-fibrous components of the intercellular matrix. Thus the experimental granulation tissue, especially in the proliferation stage [5–6], may serve as a useful model in the study of rheumatic disease, at least with regard to its reactive and anabolic features. Studies on the pathogenesis of rheumatoid disease and on the effects of antirheumatic drugs are closely related [3].

EXPERIMENTAL

Treatment of the animals

Male Wistar rats (average age 2·5 months and average weights in experimental groups varying from 170·1 ± 9·6 (S.E.M.) to 220 ± 2·5 g) were kept in individual steel wire-bottomed cages and fed our standard laboratory diet (a thick porridge prepared from wheat flour, fat-free milk powder, salt mixture and water) supplemented with vegetables.

The granulomas were induced subcutaneously with viscose cellulose-sponge (10 × 10 × 20 mm) as described by Viljanto and Kulonen [5], four pieces to each rat. The rats were sacrificed after 7 or 21 days (corresponding to proliferation and mature phases, respectively [6], and the granulomas were prepared free from the capsules and used, after storage in a frozen state, for the analysis of their composition, or immediately for the incorporation experiments [6, 7] as described below.

Five days before the sacrificing the rats were treated with antiphlogistic drugs as follows: sodium acetylsalicylate, 300 mg/kg per day, orally; indomethacin, both 0·1 mg/kg per day and 10 mg/kg per day, orally; phenylbutazone, 100 mg/kg per day, orally; prednisolone, both 1 mg/kg per day and 10 mg/kg per day, s.c. Prednisolone was obtained from E. Merck AG (Darmstadt), indomethacin from Dumex A/S (Copenhagen), and the other drugs from Hoffman–La Roche (Basle). The larger doses of indomethacin and prednisolone decreased the body weight by about 8 g/day, compared with the control rats; the other treatments retarded it by 1–2 g/day.

Each morning the rats received the daily dose of the drug (except prednisolone) mixed with such an amount of food such that all was eaten by the late afternoon. A new portion of unmixed food was given for the night, and any that remained was taken away the next morning. Thus it was ensured that the whole dose was ingested and that unmixed food was also available to each rat. Despite some diarrhoea, the animals tolerated the treatment well.

Analysis of the composition of granulomas

Nucleic acids. The minced tissue was homogenized (10 sec) in 5 volumes of cold 0·3 N KOH with an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen i. Breisgau). For the hydrolysis of RNA, the material was kept at 37° for 20 hr. The cooled digest was adjusted with cold 1 N perchloric acid (PCA) to 0·2 N and the pH was checked to be below 1. After standing for 30 min at 0° the mixture was centrifuged at 23,000 *g* for 30 min in a refrigerated centrifuge. The precipitate was washed twice with 0·2 N PCA and the supernatants combined with the RNA fraction. The standard deviation (S.D.) for RNA-ribose was 11·9 per cent in the control and 18·9 per cent in the experimental groups, respectively.

The insoluble residue was suspended in 10 ml of 0·5 N PCA and hydrolyzed for a further 30 min at 90°. After cooling, the mixture was centrifuged and washed as above and the supernatants were combined to form the DNA fraction. The S.D. of this assay was on average 18·1 per cent in the control and 21·4 per cent in the experimental groups.

RNA-ribose was estimated by the orcinol reaction [8] and DNA by the diphenylamine reagent [9].

Uronic acid, hexosamine, nitrogen and hydroxyproline. The granulation tissue was minced and hydrolyzed in approx. 2·5 volumes of 1 N HCl (5 ml) at 105° for 3 hr. The hydrolysate was centrifuged for 30 min at 1000 *g*. A 1 ml sample was taken for the determination of uronic acids. For the liberation of hexosamine, 1 ml of 5 N HCl was added to the remaining hydrolysate and the hydrolysis continued for 16 hr at 105° when a further 1 ml sample was taken. For the analyses of hydroxyproline and nitrogen, 4 ml of 10 N HCl was added to the remaining hydrolysate which was then kept for 3 hr at 130°. The sample was filtered through charcoal-coated paper. The filtrate was evaporated to dryness over a boiling-water bath. The final residue was dissolved in water for the determination of nitrogen and hydroxyproline.

Uronic acids were determined by the carbazole reaction [10], a separate blank being made for each sample (S.D. 9·6 per cent).

Hexosamine was determined by the Elson–Morgan reaction [11]. The samples were pretreated with Dowex-50W × 8 (100–200 mesh in H^+ form) in 10 × 1 cm columns (S.D. 9·8 per cent).

Nitrogen was determined with Nessler's reagent [12] after Kjeldahl combustion in an aluminium block which had been heated to 250–300° for at least 30 min. Hydrogen peroxide was added as a catalyst (S.D. 8·6 per cent).

Hydroxyproline was assayed according to Stegemann [13] and Woessner [14]. The method is based on the oxidation of hydroxyproline to pyrrole by chloramine-T (the average S.D. was 14·4 per cent in the control and 21·7 per cent in the experimental groups, respectively).

Assessment of the metabolic capacities of granuloma slices

Incubation. The granulomas were sliced to 0·5 mm with a Stadie–Riggs microtome. Each slice was cut into four identical parts which were immersed into cold Krebs–Ringer–phosphate solution containing neither glucose or proline. The slices were dried with tissue paper, weighed with a torsion balance and pooled to 500 mg samples which were placed into 5 ml of Krebs–Ringer–phosphate, buffered with 20 mM-2-(*N*-2-hydroxyethyl-piperazin-*N'*-yl)ethane sulphonic acid (Hepes) [15] at pH 7·4, in 25 ml erlenmeyer flasks [6]. The incubation medium contained 2·87 mM inactive proline (to avoid the effects of variable extracellular concentrations of proline) and 22 mM glucose. After preincubation for 30 min at 37°, the labelled precursor (varying amounts, usually 10 μCi of ^{3}H-cytidine (G), 20 μCi of ^{3}H-proline (G) or 10 μCi of ^{35}S-sulphate, all obtained from the Radiochemical Centre, Amersham, Bucks.) was added and the incubation continued for 3 hr at 37° in a Gallenkamp IH 350 metabolic shaking incubator.

The presence of Hepes increases the incorporation two-fold (cf. Uitto [15]), almost to the same level as Eagle's medium with O_2 + CO_2. For practical reasons the Krebs–Ringer–phosphate–Hepes medium was chosen.

Incorporation of cytidine. The incubation was stopped by the addition of cold 1 N PCA to a final concn of 0·6 N with the flasks immersed in crushed ice: the contents were then centrifuged at 300 rev./min for 15 min. The insoluble material was washed once with 5 ml of 0·6 N PCA and then suspended in 5 ml of 0·6 N PCA. The insoluble matter was harvested by centrifugation, washed 6 times with 80 per cent (v/v) ethanol and finally with diethyl ether. The last ethanol-washing did not contain any measurable radioactivity. For the isolation of RNA and DNA the method described above was applied [16]. The average S.D. for the incorporation into DNA was 9·0 per cent. To obtain reliable RNA analyses, cytidine should be separated by chromatography.

To measure radioactivity, 200 μl of the respective supernatant was mixed with 10 ml of hydrophilic scintillation fluid (6 ml of methylcellosolve and 10 ml of scintillation mixture, which contained 15 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) in 1000 ml of distilled toluene) in the counting flasks and counted in a Packard Model 3320 Liquid Scintillation Spectrometer.

Incorporation of proline. The incubation was terminated by the addition of ethanol to a final concn. of 80 per cent (v/v). The slices were collected by centrifugation and homogenized in 80 per cent ethanol. The insoluble material was washed 5 times with 80 per cent ethanol and with diethyl ether. The air-dried material was gelatinized in water at 130° for 120 min. The samples were filtered while hot through gauze into 50 ml tubes and the insoluble material washed with 2 × 5 ml

of boiling water. Both the gelatinized and the insoluble, non-collagenous protein fractions were hydrolyzed at 130° for 3 hr in 6 N HCl. After the hydrolysis the non-collagenous protein fractions were filtered through charcoal-treated paper. The samples were evaporated to dryness on a water bath and dissolved in 8 ml of water. Aliquots were taken for the determination of nitrogen and total radioactivity [6, 7].

The remaining part of the sample was used for the assay of the activity of hydroxyproline according to Juva and Prockop [17]. Imino acids are oxidized by chloramine-T, first at room temperature, when proline is converted into Δ-pyrroline which is extracted with toluene, and then at boiling temperature, when hydroxyproline forms pyrrole which is in turn extracted with toluene. The eventual degradation products of proline in this toluene extract are removed by a silica gel column. Pyrrole is estimated with *p*-dimethylaminobenzaldehyde for the calculation of the specific activity of pyrrole. For the total radioactivity, and to ascertain the course of the oxidation, the recovery was determined for each series by means of an internal standard of hydroxyproline. The yield of hydroxyproline as pyrrole was checked in each series and found to be about 40–50 per cent but consistent. The radioactivity in the toluene solutions was measured after addition of 1 ml of the scintillation fluid to 16 ml of the toluene solution. The details of the procedure and its reliability were checked thoroughly, as described elsewhere [7].

The average S.D. for the incorporation of proline into noncollagenous protein and into collagen were 18·0 and 19·6 per cent, respectively.

Incorporation of sulphate. The incubation was stopped after 3 hr by the addition of 20 ml of acetone. After standing overnight in the cold, the insoluble material was collected by centrifugation. The samples were homogenized in acetone, left in the cold overnight, centrifuged and the pelleted material was air-dried overnight at 60°. After hydrolysis with papain [18] the digests were precipitated with ice-cold 50% trichloroacetic acid (TCA) (final concn. 10%) and after 30 min centrifuged for 60 min at 1800 *g*. The supernatants were dialyzed against tap water for 48 hr in special chambers (designed by K. von Berlepsch). The retentates were precipitated with 6 volumes of 96% ethanol which contained 0·5% of potassium acetate. The precipitates were washed twice with cold 80% ethanol and then dissolved in water. After reserving an aliquot for the determination of the radioactivity, the acid mucopolysaccharides (AMPS) were precipitated with 1% cetylpyridinium chloride (CPC); the precipitates were collected by centrifugation and dissolved in 1·2 M $MgCl_2$.

When the incubated slices had been washed with 80% ethanol, the activities in the washings declined to 4 per cent of the first washing in the fifth and to 0·1 per cent in the tenth. However, all free sulphate could not be removed by this washing. When, after papain hydrolysis and TCA-precipitation, the acid mucopolysaccharides were repeatedly precipitated with ethanol, the activities in the subsequent precipitates decreased by about 25 per cent with each precipitation; 1% CPC precipitated only about one-fifth of the original activity in the third ethanol precipitate. The activities of the CPC-precipitates were the most consistent and therefore considered to be the most reliable (S.D. 23·2 per cent).

RESULTS

Effects of individual drugs

The results of drug effects are summarized in Tables 1–4, but discussed in full in the following sections. The averages are calculated from the percentage effects of the treatments obtained in the individual experiments; their number is indicated.

Sodium acetylsalicylate. Considering the data in Table 1 as a whole, the administration of sodium acetylsalicylate caused a suppression of almost all the parameters, although only a few changes were statistically significant, partly because the groups were small. The various nitrogen-containing components decreased by 11·9 ± 3·9% (n = 9) (2P < 0·02) and the incorporation rates of the different precursors by 22·4 ± 6·9% (n = 8) (2P < 0·01). The average effect was larger on the 7-day (n = 10) than on the 21-day (n = 9) samples (− 19·7% and − 6·7%, respectively) but the difference was not statistically significant. The content of uronic acids increased as a result of sodium acetylsalicylate administration, especially if calculated per DNA.

Indomethacin. At the 10 mg dose the nitrogenous constituents decreased by 16·1 ± 4·6% (n = 5) (2P < 0·05) and the incorporation rates by 35·5 ± 9·3% (n = 6) (2P < 0·02) (Table 2). The 7-day samples (n = 4) were significantly more sensitive than the 21-day samples (n = 7) (2P < 0·05) (effects − 46·4 and − 15·4%, respectively).

At the moderate dose (0·1 mg/kg per day) there was a modest but significant decrease in the nitrogenous constituents (− 9·6 ± 2·5%, n = 10; 2P < 0·01). Surprisingly, the incorporation of proline into collagen was increased (Table 2). If the 7- and the 21-day samples are considered together, the incorporation was 143·2 ± 4·0% (n = 4) compared with the respective controls (2P < 0·01). The contents of RNA-ribose and hexosamine were both slightly but significantly decreased (each 2P < 0·01).

Phenylbutazone. All the parameters measured were suppressed by phenylbutazone administration, (Table 3), the nitrogenous components by 18·4 ± 3·5% (n = 9) (2P < 0·001) and the incorporation rates by 20·0 ± 4·6% (n = 6) (2P < 0·005). The 7-day samples (n = 9) were more sensitive than those from the 21-day granulomas (n = 8) (−24·2 and −8·9%, respectively; 2P < 0·005).

Prednisolone. The effects of the 1 mg dose was small (< 10%) on all the parameters except on the synthesis of AMPS (− 35·1%) which seems especially sensitive.

Table 1. Effect of sodium acetylsalicylate on sponge-induced granulomas

Parameter observed	Growth (days)	Control	Experimental	Effect of drug
Weight gain of rats (g/day)	21	+2·30‡	−0·20‡	—
DNA (mg/g)	7	2·20 ± 0·20 (4)	2·08 ± 0·25 (5)	−5%
RNA-ribose (mg/g)	7	0·68 ± 0·03 (4)	0·48 ± 0·06 (5)	−29%*
	21	0·72 ± 0·03 (5)	0·60 ± 0·03 (6)	−17%†
Synthesis of DNA	7	30,500 ± 1960 (3)	22,270 ± 3020 (3)	−27%
(count/min/500 mg)	21	11,230 ± 290 (3)	10,600 ± 270 (3)	−6%
Nitrogen (mg/g)	7	11·23 ± 0·50 (3)	12·20 ± 0·81 (5)	+9%
	21	14·38 ± 0·09 (4)	13·87 ± 0·87 (6)	−4%
Hydroxyproline (mg/g)	7	0·94 ± 0·12 (3)	0·69 ± 0·08 (5)	−27%
	21	2·13 ± 0·05 (4)	1·83 ± 0·18 (6)	−14%
Synthesis of noncollagenous proteins	7	44·7 ± 7·7 (3)	27·7 ± 4·4 (3)	−38%
(count/min/mg)	21	89·0 ± 8·7 (4)	81·3 ± 2·1 (4)	−9%
Synthesis of collagen (hydroxyproline)	7	2·90 ± 0·20 (2)	1·20 ± 0·20 (2)	−59%*
(count/min/mg)	21	5·70 ± 0·47 (3)	5·63 ± 0·43 (3)	−1%
Uronic acid (μg/g)	7	410 ± 18 (3)	480 ± 30 (5)	+17%
	21	330 ± 17 (4)	370 ± 21 (6)	+12%
Hexosamine (μg/g)	7	740 ± 18 (3)	680 ± 17 (5)	−8%
	21	670 ± 55 (3)	590 ± 21 (5)	−12%
Synthesis of AMPS (CPC-precipitable sulphate)	7	7,770 ± 1530 (3)	5,470 ± 350 (3)	−30%
(count/min/500 mg)	21	11,570 ± 980 (3)	10,300 ± 650 (3)	−11%

The dose was 300 mg/kg per day. Results are expressed as mean ± S.E.M.; number of rats in parentheses. The weights refer to fresh tissue.

Statistical significance: *2P < 0·05; †2P < 0·01. ‡Calculated from the group averages.

Table 2. Effect of indomethacin on sponge-induced granulomas

Parameter observed	Growth (days)	Control	Experimental	Effect of drug
Weight gain of rats (g/day)	21	+3·17§	−4·70§	—
DNA (mg/g)	21	1·53 ± 0·07 (4)	1·48 ± 0·08 (5)	−3%
Synthesis of DNA	7**	12,630 ± 270 (3)	8,850 ± 740 (3)	−30%†
(count/min/500 mg)	21**	10,330 ± 690 (3)	8,530 ± 490 (2)	−17%
	21	9,350 ± 380 (4)	7,450 ± 570 (4)	−20%
Nitrogen (mg/g)	21**	12·44 ± 0·01 (2)	10·49 ± 0·24 (2)	−16%*
	21	13·49 ± 0·39 (5)	11·96 ± 0·61 (6)	−11%
Hydroxyproline (mg/g)	21**	1·84 ± 0·05 (2)	1·50 ± 0·10 (2)	−18%
Synthesis of noncollagenous proteins	21**	81·3 ± 5·4 (3)	91·3 ± 9·8 (3)	+12%
(count/min/mg)	21	137·5 ± 4·6 (4)	99·5 ± 7·1 (4)	−28%†
Synthesis of collagen (hydroxyproline)	7**	3·90 ± 0·38 (3)	1·73 ± 0·21 (3)	−56%†
(count/min/mg)	21**	8·31 ± 1·16 (3)	7·16 ± 1·00 (3)	−14%
	21	13·43 ± 0·31 (4)	6·55 ± 0·30 (3)	−51%‡
	7††	5·70 ± 0·50 (3)	8·50 ± 0·66 (3)	+49%*
	8††	0·90 ± 0·15 (4)	1·33 ± 0·10 (4)	+48%
	21††	1·73 ± 0·28 (4)	2·50 ± 0·38 (4)	+45%
	21†††	11·40 ± 0·93 (3)	15·00 ± 1·70 (2)	+32%
Hexosamine (μg/g)	7**	790 ± 36 (3)	540 ± 23 (3)	−32%†
	21**	820 ± 46 (3)	710 ± 26 (3)	−13%
Synthesis of AMPS (CPC-precipitable sulphate) (count/min/500 mg)	7**	17,400 ± 4090 (3)	5480 ± 1420 (3)	−69%

The dose was 10 mg/kg per day, except in experiments marked †† when it was 0·1 mg/kg per day. Results are expressed as mean ± S.E.M.; number of rats in parentheses. The weights refer to fresh tissue.

Statistical significance: *2P < 0·05; †2P < 0·01; ‡2P < 0·001. §Calculated from the group averages. **Treated only for 3 days before killing.

Table 3. Effect of phenylbutazone on sponge-induced granulomas

Parameter observed	Growth (days)	Control	Experimental	Effect of drug
Weight gain of rats (g/day)	7	+2·28 ± 0·85 (3)	−0·36 ± 0·31 (4)	–
RNA-ribose (mg/g)	7	0·63 ± 0·09 (3)	0·42 ± 0·03 (5)	−33%*
	21	0·84 ± 0·04 (5)	0·80 ± 0·07 (5)	−5%
Synthesis of DNA (count/min/500 mg)	7	7610 ± 560 (3)	5380 ± 180 (3)	−29%*
	21	8830 ± 220 (2)	7620 ± 340 (3)	−14%
Nitrogen (mg/g)	7	9·30 ± 0·31 (3)	7·98 ± 0·26 (5)	−14%*
	21	12·62 ± 0·36 (4)	10·81 ± 0·24 (5)	−14%†
Synthesis of collagen (hydroxyproline) (count/min/mg)	7	4·60 ± 0·30 (4)	3·27 ± 0·12 (3)	−29%*
	21	8·20 ± 0·33 (4)	6·13 ± 1·68 (3)	−25%
Hexosamine (μg/g)	7	810 ± 61 (3)	610 ± 73 (5)	−25%*
	21	700 ± 4 (4)	660 ± 33 (5)	−6%

The dose was 100 mg/kg per day. Results are expressed as mean ± S.E.M.; number of rats in parentheses. The weights refer to fresh tissue.

Statistical significance: *2P < 0·05; †2P < 0·01.

The larger dose (10 mg/kg per day) was very effective (Table 4): the nitrogenous constituents decreased by 21·6 ± 6·7% (n = 10) (2P < 0·02) and the incorporation rates by 77·7 ± 4·9% (n = 8) (2P < 0·001). Again, the 7-day granulomas (n = 9) were more affected than the mature ones (n = 9) (−53·7 and −39·9%, respectively; 2P < 0·02).

General effects on various parameters

Nucleic acids. Considering all the drugs and concentrations together there was a decrease in the contents of both DNA (mean − 13·1 ± 4·5; n = 9) and RNA (− 19·8 ± 5·0%; n = 11; 2P for paired samples < 0·01). This trend is supported by the values for the incorporation of cytidine.

Proteins, including collagen. There was a small general decrease in total nitrogen, containing imbibing plasma proteins (− 6·6 ± 3·0%; n = 11), and a larger decrease in hydroxyproline (− 21·3 ± 3·5%; n = 10; 2P < 0·01) and hence also in collagen. All the drugs

Table 4. Effect of prednisolone on sponge-induced granulomas

Parameter	Growth (days)	Control	Experimental	Effect of drug
Weight gain of rats (g/day)	21	+2·48§	−5·86§	–
DNA (mg/g)	7	1·70 ± 0·24 (5)	1·08 ± 0·04 (4)	−36%
	21	1·63 ± 0·14 (5)	1·39 ± 0·18 (5)	−15%
RNA-ribose (mg/g)	7	0·52 ± 0·04 (5)	0·22 ± 0·02 (4)	−58%‡
	21	0·86 ± 0·04 (5)	0·58 ± 0·08 (5)	−33%‡
Synthesis of DNA (count/min/500 mg)	7	19,200 ± 1230 (3)	7640 ± 560 (3)	−60%†
	21	19,100 ± 1370 (3)	8700 ± 610 (3)	−54%†
Nitrogen (mg/g)	7	9·76 ± 0·36 (4)	7·58 ± 0·64 (4)	−22%
	21	12·59 ± 0·34 (5)	12·61 ± 1·08 (5)	±0%
Hydroxyproline (mg/g)	7	0·43 ± 0·05 (4)	0·26 ± 0·04 (4)	−40%*
	21	1·99 ± 0·09 (5)	1·61 ± 0·06 (5)	−19%†
Synthesis of non collagenous proteins (count/min/mg)	7	28·0 ± 3·9 (4)	6·0 ± 0·6 (3)	−79%†
	21	48·0 ± 2·9 (4)	9·8 ± 0·3 (4)	−80%‡
Synthesis of collagen (hydroxyproline) (count/min/mg)	7	4·05 ± 0·91 (4)	0·57 ± 0·11 (3)	−86%*
	21	11·64 ± 1·42 (4)	0·95 ± 0·10 (3)	−92%†
Uronic acid (μg/g)	21	240 ± 7 (5)	280 ± 12 (5)	+17%*
Hexosamine (μg/g)	7	750 ± 3 (4)	670 ± 15 (4)	−11%†
Synthesis of AMPS (CPC-precipitable sulphate) (count/min/500 mg)	7	35,900 ± 2340 (3)	2870 ± 320 (3)	−92%‡
	21	14,730 ± 1350 (3)	3060 ± 370 (3)	−79%†
	10**	4050 ± 430 (3)	2630 ± 280 (3)	−35%

The dose was 10 mg/kg per day, except in the experiment marked ** when it was 1 mg/kg per day. Results are expressed as mean ± S.E.M.; number of rats in parentheses. The weights refer to fresh tissue.

Statistical significance: *2P < 0·05; †2P < 0·01; ‡2P < 0·001. §Calculated from the group averages.

except indomethacin decreased the incorporation of proline into non-collagenous protein ($-33{\cdot}9 \pm 12{\cdot}7\%$; n = 7). Severe treatment both with prednisolone and indomethacin strongly inhibited hydroxyproline formation ($-50{\cdot}5 \pm 14{\cdot}7\%$; n = 6), but at a moderate dose the effect of indomethacin was quite different from the other drugs. Whereas indomethacin seemed to promote the incorporation of proline by $43{\cdot}5 \pm 3{\cdot}9\%$ (n = 4), the others decreased it by $23{\cdot}6 \pm 10{\cdot}4\%$ (n = 5). The specific activities of hydroxyproline (not presented) were in agreement with the exception that the treatments with phenylbutazone and prednisolone had effects similar to those associated with the lower dose of indomethacin. With the exception of phenylbutazone, this result was even accentuated when the results were calculated as total activity of hydroxyproline.

Hexosamine, uronic acids and incorporation of sulphate. The effects of the drugs on the content of uronic acids were variable but the general trend was an increase ($+3{\cdot}9 \pm 4{\cdot}5\%$; n = 7), especially if calculated per DNA ($+16{\cdot}4 \pm 8{\cdot}3\%$; n = 5). The total hexosamines decreased ($-10{\cdot}3 \pm 2{\cdot}1\%$; n = 9; $2P < 0{\cdot}01$) after all treatments, also if calculated per uronic acid ($-11{\cdot}5 \pm 3{\cdot}9\%$; n = 6; $2P < 0{\cdot}05$). All the treatments, except the lower dose of indomethacin, decreased the incorporation of sulphate very markedly.

DISCUSSION

Granuloma as a model for rheumatoid disease. Many experimental systems have been suggested for the evaluation of antirheumatic drugs [1–4]. They range from granulomas and other tissue reactions to cell cultures, and even to cellular subparticles and individual components, especially enzymes. The rheumatoid process is envisaged as a sequence from local damage to a reactive phase, and finally to the cellular proliferation and synthesis of connective tissue components. The sponge-granuloma serves mainly as a model of the last phases. The known ages and definite volumes, as well as the formation of integrated solid connective tissue, favour the use of sponge-granulomas.

It is inherent to any biological reaction that there will be scatter in the various parameters being measured. The reproducibility of granuloma can be improved by standardizing the breeding and feeding of animals and the implantation technique and by using sufficiently large groups; the control and experimental series must be matched carefully. It should be noted that the proliferation phase of the granulomas is more sensitive to antirheumatic drugs than the latter phase. For routine screening, the administration of test substances *in vivo* is too laborious and expensive.

Some of the observed suppressive effects may have been enhanced by the simultaneous retardation of growth. However, the positive effects (*e.g.* in the incorporation of proline to collagen and the content of uronic acid) cannot depend on inanition.

Suggested effects of antirheumatic drugs. The common feature of the present experiments is the retardation of granuloma formation, as seen in the contents of DNA, RNA, collagen, non-collagenous protein, protein-bound hexosamine as well as in the incorporation of labelled precursors of DNA, non-collagenous proteins and sulphated mucopolysaccharides. The decrease was most pronounced in the content of RNA and the incorporation of sulphate. Apparent exceptions are the incorporation of proline into hydroxyproline after a therapeutic dose of indomethacin and the contents of uronic acids. Dr. L. Strauch (Basle) has also observed the increase of uronic acids in cotton pellet granulomas as the effect of indomethacin administration (personal communication).

Domenjoz [2] found that antirheumatic drugs have cytostatic effects in cell cultures. On the hematopoetic system indomethacin had no effect [19]. Fukuhara and Tsurufuji [20] and Ohno *et al.*, [21] concluded that the antirheumatic drugs inhibited the formation of carrageenin granuloma.

Since the proliferation of connective tissue is an essential feature of rheumatoid disease [3], it is inferable that antirheumatic drugs suppress the proliferation of granulation tissue. The formation of granuloma and the immunologic response are analogous in the cell proliferation and synthesis of specific proteins.

The sharp decrease in the incorporation of sulphate is in agreement with many reports in the literature [1, 2, 22–26] that the synthesis of mucopolysaccharides is more vulnerable to drugs than the proliferation of cells. There is an apparent contradiction between the slightly increased concentration of uronic acids and the decreased formation of sulphated mucopolysaccharides. There is either a discrepancy between sulphated and non-sulphated mucopolysaccharides or the turnover of the latter is slowed down as the effect of the drugs. Seppälä *et al.* [27–29] have shown that intra-articular treatment with hydrocortisone causes an increase in the concentration of hyaluronic acid and a decrease in the concentration of protein glucosamine in rheumatoid synovial fluid. The decrease in the content of hexosamine presumably depends on depressed synthesis of glycoproteins [27, 30].

The paradoxical stimulatory effect of indomethacin. After the administration of the therapeutic dose of indomethacin, which results in the concentration of 1–2 μg/ml [31] or about 5×10^{-6} M in the human synovial fluid, there was a clear increase in the observed incorporation of proline into hydroxyproline (Table 2). This is in agreement with our earlier results [32]. Struck and Hernández-Richter [33] mention an increase in the tensile strength of skin-wound tissue after treatment with indomethacin. When Trnavsky and Trnavska [34] treated rats with phenylbutazone and administered ^{14}C-proline, they found that, in the acid soluble fraction of skin collagen the activity was initially lower but higher during the 10th to 28th days.

In granuloma slices from indomethacin-treated rats both the synthesis and turnover of collagen seem to be

stimulated, but the increase in the incorporated radioactivity must be only transitory.

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