Anti-inflammatory substance in human serum

It has been proposed (McArthur, Dawkins & others, 1971) that human serum contains peptide-like substances in protein-bound and free forms, and that the abnormally low concentrations of the free forms are an important factor in the development of the rheumatic diseases in susceptible individuals. We wish to report the isolation of a substance from human serum, that yields a consistent pattern of amino-acids on hydrolysis and shows marked activity in the carrageenan oedema test in rats.

Pooled human serum (500 ml) was mixed with 25 ml of 100% w/v trichloroacetic acid and the precipitated proteins were removed by centrifugation at 3500 *g* for 25 min at 4°. Two methods of isolation were used on the separated supernatant. In the first, the excess trichloroacetic acid was removed by ether extraction (3 × 500 ml portions of ether), the aqueous phase adjusted to pH 7·0 with M NaOH and its volume reduced to 30 ml at 37° under reduced pressure. The concentrate was applied to a 33 × 5 cm column containing Sephadex G25 fine and eluted with distilled water. Two % of the eluate was monitored for ninhydrin-positive fractions, using a Technicon Autoanalyser, and the remainder collected for further analysis. Three peaks were distinguished, the first (I) was identified as α_1 acid glycoprotein, the second (II) consisted of a mixture of amino-acids, and the third (III) contained tryptophan plus the active substance. Fraction III from four similar procedures were combined, reduced to 30 ml, and applied to the G25 fine Sephadex to remove traces of amino-acids and protein and the corresponding fraction III (200 ml) of the eluate was cleared of tryptophan by ultrafiltration in an Amicon 65 ml cell using a Diaflo UM2 membrane. The volume of fraction (IV) remaining inside the cell after two washes with distilled water was adjusted to 40 ml with distilled water and used for amino-acid analysis and anti-inflammatory testing. The whole procedure took 60 h and used large quantities of reagents, e.g. 3 litre of 1% (w/v) ninhydrin.

In the second method, the supernatant from 2 litre serum to which trichloroacetic acid had been added, was adjusted to pH 7·0 with M NaOH, made up to 4 litre with distilled water and continuously ultrafiltered using a Diaflo UM2 membrane until the volume of solution inside the cell was reduced to 50 ml. This concentrate was applied to a 33 × 5 cm column containing Sephadex G25 fine and the three separate peaks eluted and distinguished as described above. This procedure, which could be completed within 20 h using a 2 litre ultrafiltration cell, obviated the ether extraction. The volume of the third peak was reduced to 40 ml at 37° under reduced pressure (equivalent to fraction IV of the first method).

Anti-inflammatory activity of six independent preparations of fraction IV was assessed by the carrageenan paw oedema test in the rat (Winter, Risley & Nuss, 1962) using groups of five female animals with body weights 150–200 g. In the control groups each rat received 1 ml of 0·9 g/100 ml (w/v) saline, in the reference drug group each rat received 1 ml of a solution containing sufficient sodium salicylate to give a dose of 50 mg/kg and in the experimental groups each animal was given 1 ml of the serum fraction to which sufficient NaCl was added to give a final concentration of 0·9 g/100 ml (w/v). All the injections were given intravenously into a tail vein 30 min before the injection of 0·1 ml of 1·0 g/100 ml carrageenan (Viscarin Marine Colloids) in 0·9 g/100 ml (w/v) NaCl in the plantar region of the right hind foot. Foot volumes were measured using a mercury plethysmograph (Arnold R. Horwell, Ltd., London) immediately after the injection of the carrageenan (0 h) and at hourly intervals for 6 h. The results were calculated, and are expressed in Table 1, as mean percentage increases in the volume of the injected paw compared to the value observed at 0 h. The results (Table 1) show that the group injected with salicylate exhibited a significant inhibition of paw swelling indicating that the test system was sensitive

to a known anti-inflammatory drug and that the fraction IV also caused a significant inhibition of carrageenan oedema at the time intervals studied.

Hydrolysis of several preparations of the active fraction with 6M HCl for 24 h at 100° under an atmosphere of N_2 reproducibly gave the following amino-acid residues, the value for methionine having been taken as unity; aspartate 9, threonine 4, serine 5, glutamate 12, proline 5, glycine 8, alanine 9, valine 5, methionine 1, isoleucine 2, leucine 7, tyrosine 2, phenylalanine 7, lysine 8, histidine 3 and arginine 5. This amino-acid composition is consistent with a minimum molecular weight of 12 000 for the peptide-like substance. Acid hydrolysis of the serum fraction which showed anti-inflammatory activity (see Table 1) consistently yielded 1 nmole of

Table 1. *Anti-inflammatory effects on carrageenan paw oedema test.* (The results, given as means ± standard deviations, have been analysed by the *t*-test and those marked * show a significant difference, $P < 0.01$, from the percentage increase in foot volume observed in the corresponding control experiments. The total number of rats used is given in brackets.)

Time after carrageenan injection (h)	Control (20)	Fraction IV (30)	Sodium salicylate 50 mg/kg (5)
1	16·3 ± 5·4	15·0 ± 4·7	8·3 ± 5·0
2	33·0 ± 7·2	18·9 ± 6·5*	13·4 ± 4·6*
3	47·9 ± 7·9	23·3 ± 4·1*	13·3 ± 5·3*
4	57·2 ± 8·0	33·9 ± 5·0*	21·1 ± 5·4*
5	60·2 ± 8·0	43·7 ± 5·3*	39·3 ± 5·5*
6	62·1 ± 8·6	48·4 ± 7·6*	49·9 ± 6·2*

methionine per ml. Assuming a molecular weight of 12 000 for the active substance in this fraction then its concentration is also 1 nmol/ml, which is approximately equivalent to 12 μg/ml. L-Tryptophan, acidic antirheumatic drugs and certain dipeptides (McArthur, Dawkins & Smith, 1971) which are known to bind to serum proteins are retarded on Sephadex when eluted with distilled water. The elution of the active fraction is also retarded compared to amino-acids on G25 and G50 Sephadex despite its high molecular weight. This finding strongly suggests that it is also bound to serum proteins.

The present work shows that human serum contains a substance which possesses anti-inflammatory activity, is peptide-like, and binds to circulating proteins. It fulfils several of the criteria proposed for the hypothetical antirheumatic peptides of McArthur, Dawkins & others (1971) but it remains to be shown if its free concentration is abnormally low in patients with active rheumatoid disease and if it is displaced from binding to proteins by antirheumatic drugs.

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REFERENCES

McARTHUR, J. N., DAWKINS, P. D., SMITH, M. J. H. & HAMILTON, E. B. D. (1971). *Br. med. J.*, **2**, 677–679.

McARTHUR, J. N., DAWKINS, P. D. & SMITH, M. J. H. (1971). *J. Pharm. Pharmac.*, **23**, 393–398.

WINTER, C. A., RISLEY, E. A. & NUSS, G. W. (1962). *Proc. Soc. exp. Biol. Med.*, **111**, 544–547.

The recovery of *Bacillus megaterium* spores from white soft paraffin

The isolation and enumeration of viable contaminant organisms from topical non-sterile preparations is particularly difficult. The efficiency of recovery of micro-organisms will depend upon the solvent or dispersal system used to liberate the cells from the fatty base. The following systems have been reported: isopropyl myristate (Sokolski & Chidester, 1964), n-hexane (White, Bowman & Kirshbaum, 1968), Tween 80 (Buhlmann, 1968), polyethylene glycol-ether (Millipore Corporation, 1969) and a liquid growth medium (Woodward & McNamara, 1971). A polyethylene glycol, aqueous buffer 2 phase system has also been used to purify bacterial spore suspensions (Sacks & Alderton, 1961). This report describes the relative merits of five solvent or dispersal systems in the recovery of spores from white soft paraffin experimentally contaminated with *Bacillus megaterium*.

Spores of *B. megaterium* ATCC 8245 were heat activated at 80° for 10 min and 2 ml of a diluted aqueous suspension was added to 18 g of molten white soft paraffin held at 50° in a glass screw-capped jar. The mixture was shaken mechanically until cold to disperse the spores in the ointment base. The concentration of spores in the ointment base was about 2000/g. A comparison between the total count of the spores by the haemocytometer counting chamber method (Marshall & Rigby, 1970) and colony counts on membrane filters indicated that at least 90% of the heat activated-spores could produce colonies.

The following solvent or dispersal systems were tested: isopropyl myristate, n-hexane and light petroleum (b.p. 60–80°), which were filter sterilized before use; a 1·0% v/v Tween 80 solution in peptone water and a 50% v/v mixture of polyethylene glycol 200 in peptone water, which were sterilized by autoclaving. Each solvent and dispersal system was tested for efficiency by placing about 0·5 g of white soft paraffin in 10 ml of the system at 37° and shaking mechanically for up to 10 min. Where solution or dispersal took place, the resultant mixtures were tested for their ability to pass through a 0·45 μm pore size membrane filter.

Neither the Tween 80 nor polyethylene glycol systems dissolved the base. Isopropyl myristate, n-hexane and light petroleum rapidly dissolved up to 5·0% w/v of base but only the isopropyl myristate and n-hexane solutions filtered sufficiently rapidly to be of practical use.

Recovery of spores from the base was achieved by the following method. Approximately 0·5 g of contaminated ointment, accurately weighed, was added to 20 ml of solvent or dispersal system at 37°. This was then either homogenized for 1 min in a blender or shaken mechanically,with the addition of 1 g of 0·5 mm sterile glass beads, for 5 min. Samples of the resulting mixture were added to 100 ml of a sterile filtration vehicle consisting of 0·1% v/v Tween 80 in peptone water, and the whole filtered through a 0·45 μm pore membrane filter followed by 100 ml of peptone water, then the membrane was transferred to a Petri dish containing an absorbent pad moistened with nutrient broth enriched with 1·0% w/v Difco yeast extract and incubated for