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STUDIES OF ANTIPYRETIC COMPONENTS IN THE JAPANESE EARTHWORM

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Abstract—Antipyretic components in the Japanese earthworm (*Lumbricus Spencer, Perichaeta communishima, Goto and Hatai*) were investigated through a study of the antipyretic effect on rabbits having pyrogen-induced fevers caused by *Esherichia coli* pyrogen and chromatographic separations. The main proven antipyretic components are considered to be all-*cis*-5,8,11,14-eicosatetraenoic acid and all-*cis*-5,8,11,14,17-eicosapentaenoic acid.

DECOCTIONS of the Japanese earthworm (*Lumbricus spencer, Perichaeta communishima, Goto and Hatai*) have been used as an antipyretic in folk medicine. It is generally reported that most antipyretics have an anti-inflammatory, analgetic or other function.[1] Although several reports on antipyretics in the earthworm have been presented, none seems to clarify the antipyretic components. Some properties, e.g. solubilities in water, ethyl alcohol, ethyl ether, etc., and the effects of precipitants such as mercury chloride and lead acetate were also reported.[2–5] Some speculations on the component being a kind of derivative of tyrosine[6] and a limited digest of a protein[7,8] have also been reported, but without experimental substantiation.

To identify the antipyretic components in the earthworm, the authors extracted these components with organic solvents and separated them chromatographically by means of an antipyretic test on rabbits in which fever had been caused by *Escherichia coli* pyrogen.

EXPERIMENTAL

Materials. Dried earthworms were purchased from a Chinese-drug store. All reagents used were purchased from Wako Pure Chemical Industry, except the arachidonic acid from Mann Research Lab., New York; the Silica gel-0·08 mm from E. Merck; the Amberlite XAD-2 resin from Rohm & Haas, and Gaschrom Q from Applied Sci.

Extraction and separation. Dried earthworms were cut and crushed with a mixer (Broun, Germany). The crushed sample (31·6 kg) was extracted with 50 l. of ethyl ether (twice), ethanol (twice) and water (twice) successively by soaking and filtration (Fig. 1). The three kinds of extracts were concentrated *in vacuo* and the residues were tested for antipyretic activity. The biologically active extract (ethyl ether extract) was initially separated by column chromatography using Silica gel 0·08 mm (5 × 13 cm).[9] The active fraction (fraction IV_1 in Fig. 2) was separated with an Amberlite XAD-2 column (5 × 75 cm) with the concentration of solvent (ethanol) being varied stepwise. The active fractions (fractions 2 and 3 in Fig. 1) were again separated with an

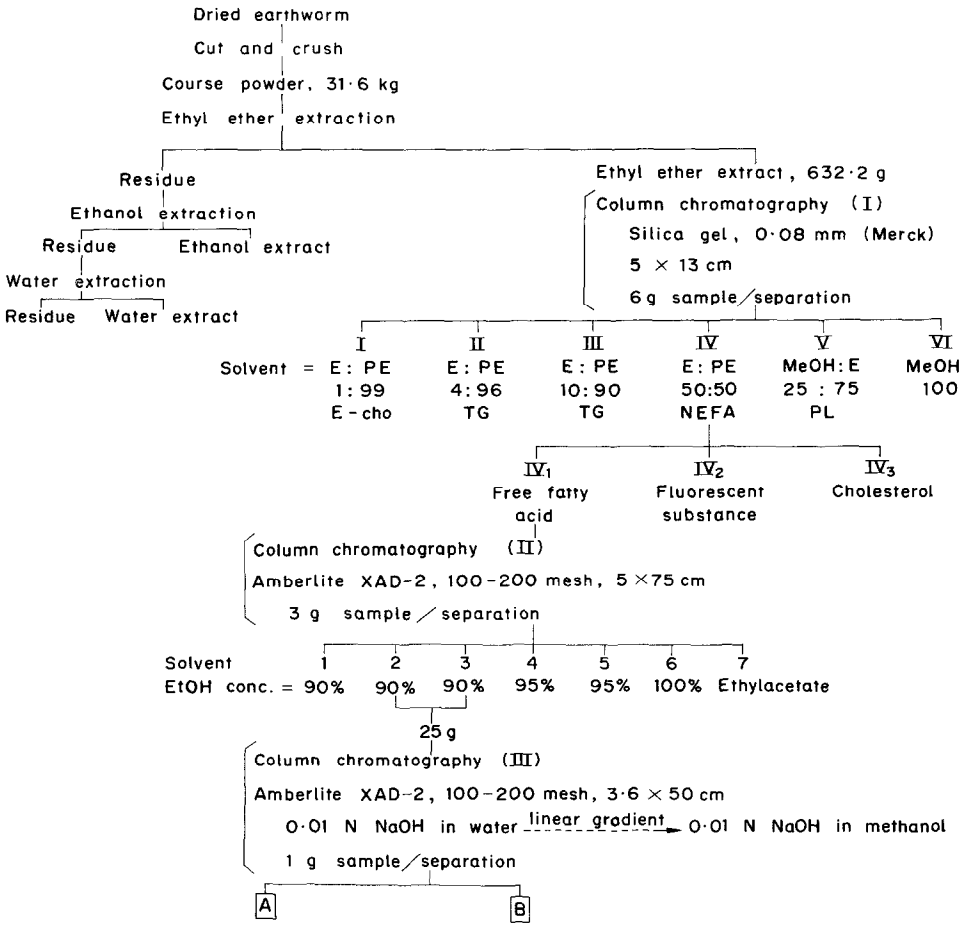

FIG. 1. Separation process for antipyretic substances. E; ethyl ether; PE, petroleum ether; MeOH, methanol; EtOH, ethanol; E-cho, cholesterol ester; TG, triglyceride; Cho, cholesterol, NEFA, nonesterified fatty acid; PL, phospholipid.

Amberlite XAD-2 column (3·6 × 50 cm) with a linear gradient elution system. By these methods, the antipyretics A and B in Fig. 1 were obtained.

Determination of antipyretic activities. Three male albino rabbits weighing between 2·5 and 3·0 kg were used for each determination and the results were indicated in the mean value. The rectal temperatures were recorded by an electric thermometer. The dose of 0·01 μg/kg of *E. coli* pyrogen, which was extracted by the method of Westophal and Luderitz[10] and the concentration of which was 0·01 μg/kg was administered intravenously. After 1 hr, the test sample (1–100 mg/kg), suspended with *gummi arabicum pulveratum* in 0·9% (w/v) saline (2 ml), was administered to the pyrogen-induced rabbit intraperitoneally. Aminopyrine (100 mg/kg) was used as the positive control in the form of aqueous solution (100 mg/3 ml). Temperature readings just prior to administration of pyrogen were used as the baseline from which changes were measured.

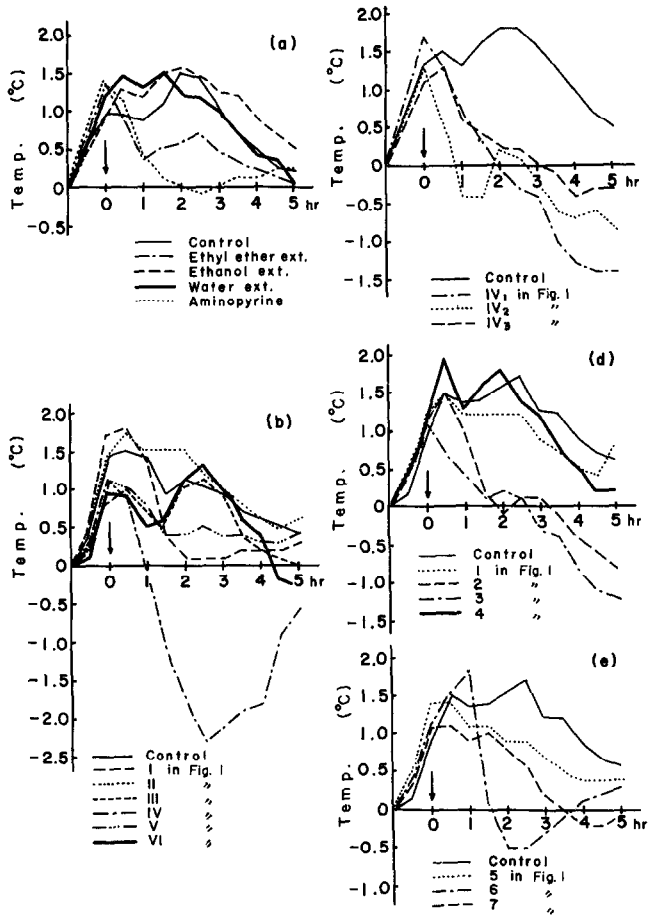

FIG. 2. Antipyretic effects of partially purified fractions (100 mg/kg) of the earthworm against fever produced by intravenous injection of pyrogen (0·01 μg/kg). Numbers of each sample refer to those in Fig.1. Pyrogen was administered i.v. at start (left side) and the fractions (100 mg/kg) were added i.p. at zero hr (arrow).

Determination of double bonds. The samples were cleaved by ozonolysis at $-50°$ in a methylene dichloride solution[11] or oxidized with potassium permanganate in an acetic acid solution.[12] The resulting carboxylic acid fragments were determined by gas chromatography using Porapak Q (for free acids) or diethyleneglycolsuccinate (DEGS), after being methylated with methanolic hydrochloric acid.

RESULTS

Separation. Ethyl ether extracts of the earthworm undoubtedly showed an antipyretic activity in the biological test (Fig. 2a). These active compounds were separated effectively with Silica gel column chromatography (Fig. 2b). The antipyretically active fraction IV in Fig. 1 was separated into three fractions, IV_1, IV_2 and IV_3, i.e. fatty acids, fluorescent substance and cholesterol respectively. All of these fractions showed antipyretic activities (Fig. 2c). However, IV_2 and IV_3 were not investigated further because these fractions consisted mainly of cholesterol. Since the quantity of

the fluorescent substance was very small, no further biological testing could be done. The fluorescent substance has been identified recently as ergosta-4,6,8(14), 22-tetraen-3-on.* Consequently, the authors examined only the fatty acid fraction.

The antipyretic substances in fraction IV_1 were anticipated to be fatty acids because the active substances were soluble in aqueous 2 N sodium carbonate and were extracted into ethyl ether from the acidic solution.

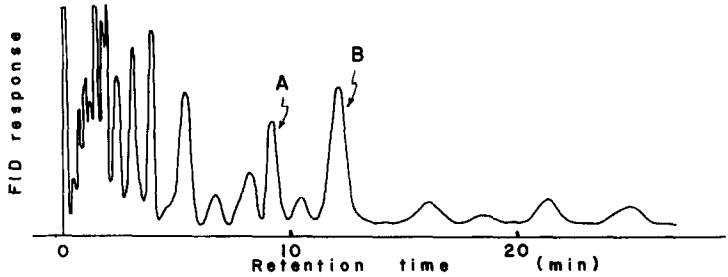

FIG. 3. Gas chromatogram of the free fatty acid fraction (IV_1 in Fig. 1). Column, DEGS (15%) on Gaschrom Q (60–80), 1·2 m × 2·3 mm, i.d; carrier gas, He (30 ml/min); temperature, 191°; apparatus, Yanagimoto GCG-550 TF.

Gas chromatographic separation of these fatty acids after methylation suggested that the fraction included many saturated and unsaturated fatty acids; two characteristic peaks were found (peaks A and B in Fig. 3). These fatty acids were separated with an Amberlite XAD-2 column using as the solvent changing stepwise concentrations of ethanol (column chromatography II in Fig. 1). In this separation, the antipyretic substances eluted in fractions 2 and 3 with 90% (v/v) ethanol (Fig. 2d and e), the volume of which was between 3 and 4·5 times the column volume. All fatty

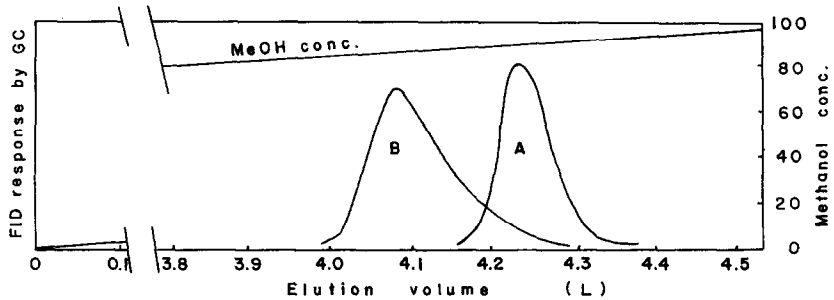

FIG. 4. Column chromatogram of the fatty acid fraction (2 + 3 in Fig. 1). Column, Amberlite XAD-2 (100–200 mesh), 3·6 × 50 cm. Solvent 1: 0–3·7 l., 0·01 N NaOH linear gradient → 0·01 N NaOH in 80% MeOH. Solvent 2: 3·7–4·8 l., 0·01 N NaOH in 80% MeOH linear gradient → 0·01 N NaOH in MeOH. Detection, gas chromatography.

acids of less than C-16 and a part of C-18 were removed in this process. The above fraction with an antipyretic effect was again separated with an Amberlite XAD-2 column with a gradient elution system in which the methanol concentration in a 0·01 N sodium hydroxide solution was changed as shown in Fig. 4. Two main peaks shown in Fig. 4 (A and B) corresponded to peaks A and B of Fig. 3. The fractions of A and B were acidified with 2 N hydrochloric acid and extracted with ethyl ether,

* I. Imada and H. Morimoto, unpublished data.

TABLE 1. ANALYTICAL DATA FOR A AND B

		A	B
Elemental analysis	Calc.	C,78·9, H,10·6, O, 10·5	C,79·4, H,10·0, O,10·6
	Found:	C,78·5, H,10·9, O, 10·4	C,78·9, H,10·3, O,10·7
Mol. wt (MS)	Free-	m/e = 304	m/e = 302
	Me-	m/e = 318	m/e = 316
t_R Of methylate (GC)		Coincident with $C_{20:4}$	coincident with $C_{20:5}$
Catalytic hydrogenation		3·53 mole equiv.	4·81 mole equiv.
t_R Of red. subst. (GC)		Coindicent with $C_{20:0}$	Coincident with $C_{20:0}$
Ozonolysis and $KMnO_4$ oxidation		Caproic, malonic, glutaric	Propionic, malonic, glutaric
NMR spectra		δ = 0·88: $-C\underline{H}_3$	δ = 0·95: $-C\underline{H}_3$
		δ = 1·29: $-(C\underline{H}_2)_n-$	δ = 1·27: $-(C\underline{H}_2)_n-$
		δ = 1·70: $-C\underline{H}_2-$	δ = 1·68: $-C\underline{H}_2-$
		δ = 2·10: $>CH-C\underline{H}_2-$	δ = 2·06: $>CH-C\underline{H}_2-$
		δ = 2·36: $-C\underline{H}_2-COOH$	δ = 2·32: $-C\underline{H}_2-COOH$
		δ = 2·80: $>C\underline{H}-C\underline{H}_2-CH<$	δ = 2·80: $>C\underline{H}-C\underline{H}_2-CH<$
		δ = 5·36: $>C\underline{H}-$	δ = 5·36: $>C\underline{H}-$
i.r. spectra		1715 cm^{-1}: = CO	1715 cm^{-1}: = CO
		700–710 cm^{-1}: *cis*-olefine	700–710 cm^{-1}: *cis*-olefine

then the ethyl ether was evaporated to obtain oily compounds. The purities of these compounds determined by gas chromatography were considered to be over 90% (w/w) for A and over 95% (w/w) for B respectively; the weights were 0·014% (w/w) for A and 0·017% (w/w) for B of the initial dried earthworms.

Structure. The physicochemical data are tabulated in Table 1. The elemental analyses considerably resembled those of the calculated values of $C_{20}H_{32}O_2$ for A and $C_{20}H_{30}O_2$ for B. Therefore, the molecular weight of each substance was determined with mass spectrometry on both the free fatty acid and the methylated fatty acid. The result showed that the m/e of M-peaks are: A = 304 and B = 302 for the free fatty acids; and A = 318 and B = 316 for the methylates. These results correspond to $C_{20:4}$ (A) and $C_{20:5}$ (B).

The retention times of gas chromatographic separation coincided with $C_{20:4}$ (A) and $C_{20:5}$ (B). Gas chromatographic separation was also performed after a catalytic hydrogenation. The resulting peaks coincided with $C_{20:0}$ in both cases.

Hydrogen absorption by catalytic reduction in an ethanol solution using platinum black as a catalyst was 3·53 mole equivalent for A (mol. wt = 304) and 4·81 mole equivalent for B (mol. wt = 302). The results show that the number of double bonds in A is four and in B is five.

The samples were oxidized with either ozone or potassium permanganate and the carboxylic acids, which were formed by cleavage at the double bonds, were determined by gas chromatography. The authors were able to find caproic acid from A and propionic acid from B when the free carboxylic acids were separated through the use of a Porapak Q column; malonic and glutaric acid from both A and B were found through a determination of the methylated carboxylic acids using a DEGS column.

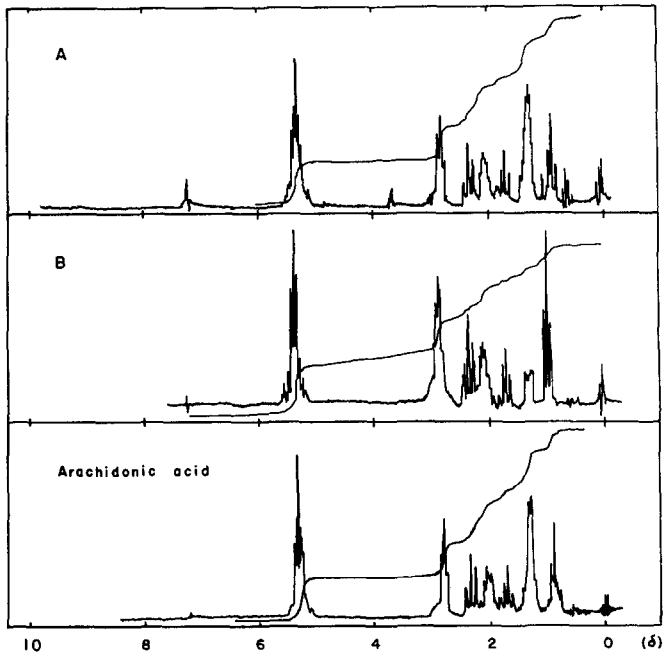

FIG. 5. Nuclear magnetic resonance spectra of A, B and arachidonic acid. Solvent, $CDCl_3$; apparatus. Varian HA-100.

The nuclear magnetic resonance spectra of A and B are compared with that of authentic arachidonic acid (Fig. 5), but no authentic sample of B ($C_{20:5}$) could be obtained. Each peak was assigned to the data in Table 1 according to the number of protons and the decoupling technique.

Infra-red spectra of A and B were compared with a spectrum of arachidonic acid which has four *cis* double bonds in its molecule (Fig. 6), and it was thought that all of the double bonds in both substances should be of the *cis*-type because there are *cis*-olefine absorptions between 700 and 710 cm^{-1}, but no *trans*-olefine absorptions between 960 and 970 cm^{-1} in their i.r. spectra.

From all of the results described above, the antipyretic substances A and B obtained from the Japanese earthworm were determined to be all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid) for A and all-*cis*-5,8,11,14,17-eicosapentaenoic acid for B.

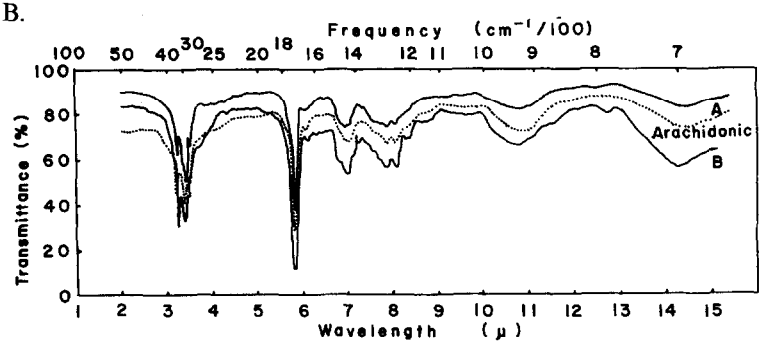

FIG. 6. Infra-red spectra of A and B. Apparatus, Hitachi EPI-2.

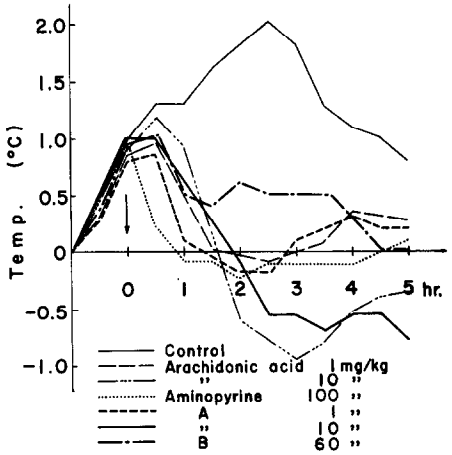

FIG. 7. Antipyretic effects of A and B against fever produced by intravenous injection of pyrogen (0·01 μg/kg). Pyrogen was administered at start (left side) and samples were added at zero hr (arrow).

Antipyretic activities. Antipyretic activities of A and B were compared with that of authentic arachidonic acid (Fig. 7).

Arachidonic acid and A caused a marked fall of pyrogen-induced fever at only 1 mg/kg and an unusual fall in temperature below normal occurred at 10 mg/kg; the temperature was found to be only a little lowered with B, even when 60 mg/kg was administered.

Antipyretic effects of A on normal (not-treated with the pyrogen) rabbits are shown in Fig. 8. Administration of 1 mg/kg of A did not cause an abnormal fall in temperature, but 10 mg/kg of A caused a marked fall in temperature.

Administration of A (1 and 10 mg/kg) 1 hr prior to pyrogen also suppressed the pyrogen-induced fever and the behavior of the temperature was almost the same as the results shown in Fig. 8.

No change was observed in the health of rabbits during these investigations.

DISCUSSION

Arachidonic acid, together with linoleic and linolenic acid, is known to be one of the essential fatty acids; it has also been known that these unsaturated fatty acids have medical action in relation to dermatitis.[13,14] Arachidonic acid is generally found in the organs of animals but not in plants.[13,14] The authors found a report[15]

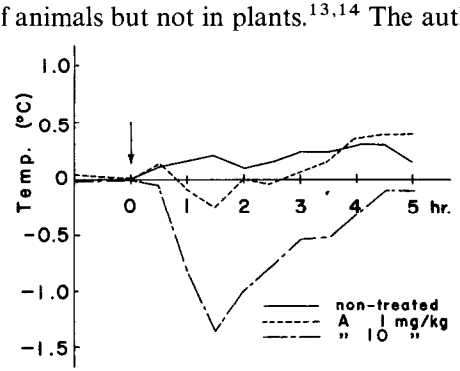

FIG. 8. Effects of A on temperature of normal rabbits. A was added at zero hr (arrow).

which investigated the unsaturated fatty acids in the earthworm, but no indication was given of individual substances and the fatty acids were not tested for antipyretic activity. Recently, arachidonic acid was found to be a precursor of prostaglandin E_2,[16–17] and several new metabolic pathways were reported.[18] No explanation can be given of the mechanism of antipyretic activity of arachidonic acid, but it is interesting to note that the unsaturated fatty acids showed antipyretic activities relating to the above metabolism of prostaglandins.

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