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Phlogistic Activity of Secalonic Acid A

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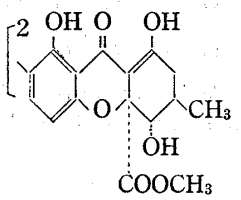
The phlogistic activity of secalonic acid A (S.A.A) was examined, isolation of which has recently been completed from *Aspergillus ochraceus*. S.A.A gave rise to a biphasic increase of vascular permeability in the abdominal cavity of mice by intraperitoneal application in the dye-leakage test. The peak of this biphasic increase was at about 1 hr and about 18 hr respectively. Exudation of leucocyte in the abdominal fluid was remarkable in the delayed phase. S.A.A also induced intensive sustained edema in a rat paw by a single local application, making the edema last more than 10 days. Pus-like thick fluid and a scab developed around the injected position 3—4 days after the injection of S.A.A and lasted as similarly as edema did. The increased vascular permeability and the rat paw edema were inhibited by aspirin, hydrocortisone and indomethacin in appropriate doses. The phlogistic activity of S.A.A considerably decreased when its chemical structure was changed. The effect of the abdominal fluid exuded by intraperitoneal application of S.A.A was examined on the isolated preparation of guinea pig ileum, rat duodenum and rat estrous uterus, respectively. The fluid contracted the ileum but this activity was supposed not to be due to S.A.A itself.

Secalonic acid A (S.A.A) has first been isolated from *Claviceps purpurea* by Frank, *et al.*[2] and recently been isolated by Yamazaki, *et al.*[3] from the culture of *Aspergillus ochraceus* on rice. Antibacterial activity of S.A.A on *Bacillus subtilis* and *Piricularia oryzae* has been recognized, but no other pharmacological properties have been reported so far. The fact having been observed recently by us that S.A.A gives rise to intensive sustained peritonitis in mice led us to further study and characteristic phlogistic activity of S.A.A was obtained in laboratory animals.

S.A.A



Experimental

Materials——S.A.A is insoluble in water and soluble in alkaline solution. Sodium hydroxide was used to prepare the test solution (pH 8.4—8.5). Saline was adjusted to pH 8.4 with sodium hydroxide and served as control. The test solution was prepared at use as it decomposes by being kept at room temperature for several weeks. Anti-inflammatory agents used include aspirin, hydrocortisone acetate, indomethacin and diphenhydramine hydrochloride. These were suspended in 0.5% carboxymethylcellulose solution if necessary. Doses are expressed as the above-named form of each drug.

Method——1. Increase of Vascular Permeability in the Mouse Abdominal Cavity: Experimental procedure was based upon Whittle's method.[4] Male mice of dd strain weighing about 20 g were used. S.A.A (12.5 and 50.0 mg/kg/10 ml respectively) was injected intraperitoneally (*i.p.*). Pontamine sky blue in 4% solution (0.1 ml/10 g body weight) was administered intravenously (*i.v.*) *via* the tail vein at each fixed time after the injection of S.A.A. Mice were killed after 20 min and abdominal fluid exuded was collected by washing the cavity in saline up to total amount of 10 ml. Solution of 0.1 ml of 1N sodium

1) Location: *a) No. 33, Yayoi-1-chome, Chiba; b) No. 9–1, Izumi-3-chome, Narashino, Chiba.*
2) B. Frank, E.M. Goottschalok, V. Ohnsorge, and F. Hüper, *Chem. Ber.*, **99**, 3842 (1966); B. Frank and G. Bauman, *ibid.*, **99**, 3863, 3875 (1966).
3) M. Yamazaki, Y. Maebayashi, and K. Miyaki, *Chem. Pharm. Bull.* (Tokyo), **19**, 199 (1971).
4) B.A. Whittle, *Brit. J. Pharmacol.*, **22**, 246 (1964).

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hydroxide was added to the dye-containing fluid. Amounts of dye of these samples were measured photometrically (590 mμ). Acetic acid in 0.7% solution was used as a referential phlogistic substance. Anti-inflammatory agents were administered orally 1 hr before the injection of S.A.A.

2. Induction of Edema in the Rat Paw: The experiment was carried out according to the method of Winter, *et al.* and Van Arman, *et al.*[5] Male rats of Wistar strain weighing about 150 g were used. S.A.A of 2% solution was injected subcutaneously (*s.c.*) into a hind paw (0.1 ml/rat). Edema induced was measured volumetrically hourly for 5 hr after application of S.A.A, and once daily thereafter. In order to examine the activity of various drugs on the edema two ways of administration were made as follows. 1) Preventive and Curative Administration: Drugs were administered orally 1 hr before S.A.A application and once a day for the succeeding 3 days. Tetracycline hydrochloride was administered orally (100 mg/kg/day) at the time different from drug administration. 2) Curative Administration: Drugs were given orally 5 hr after S.A.A application and for the succeeding 4 days (devided into 3 times in daytime). Mustard powder on the market served as a referential phlogistic substance.

3. Exudation of Leucocyte: After 1 or 18 hr of *i.p.* administration of S.A.A (12.5 mg/kg), mice were killed using chloroform and 1 ml of saline was injected into the abdominal cavity. One min later, the abdominal fluid was collected and total number of leucocyte in the exuded fluid was counted by means of staining with Türk solution.

4. Action of the Exuded Abdominal Fluid on Isolated Organs (Magnus method): After 1 and 18 hr of *i.p.* administration of S.A.A (50 mg/kg) and 1 hr of *i.p.* administration of AcOH (0.7%), mice were killed by chloroform and the abdomen was opened. The abdominal fluid was collected by washing with an appropriate volume of the bath solution up to 3 ml per mouse. Each solution of 3 mice was pooled as one sample. This solution was examined as soon as possible. Syringes and test tubes used were siliconized in advance. 1) Guinea Pig Ileum: Teated at 32° of bath temperature using Tyrode solution. 2) Rat Duodenum: Tested at 32° using De Jalon solution. 3) Estrous Rat Uterus Treated with *s.c.* Administration of Diethylstilbestrol (0.1 mg/kg) before 16—18 hr of Isolation: Tested at 28° using De Jalon solution.

Result

1. Increase of Vascular Permeability in the Mouse Abdominal Cavity

S.A.A increased vascular permeability of the abdominal cavity. The time course is shown in Fig. 1. S.A.A, especially in a higher dose, caused a biphasic change of permeability which displayed one peak at 1 hr and the other at 12—18 hr. The increased permeability returned to the basal level in 48—72 hr. Acetic acid showed a monophasic response with a peak at about 1 hr. Marked decrease of vascular permeable activity was observed when S.A.A solution was kept for 1 month at room temperature. No remarkable change in the symptoms of mice given S.A.A was observed until sacrifice. Writhing symptom was not induced either. Formalin (1%), formaldehyde (1%) and carrageenin (1%) were used as a referential agent in the early phase of enhanced permeability, but activity of these agents was very weak as compared with S.A.A (amount of dye exuded at 1 hr (μg/animal)——saline: 54 ± 8 ($n=8$), formalin (1%): 81 ± 21 ($n=12$), formaldehyde (1%): 93 ± 21 ($n=9$), carrageenin (1%): 92 ± 19 ($n=10$), S.A.A (50 mg/kg): 155 ± 22 ($n=12$) ($p<0.01$), AcOH (0.7%): 181 ± 22 ($n=11$) ($p<0.01$)).

Effect of anti-inflammatory agents is summarized in Table I. Aspirin, hydrocortisone and indomethacin inhibited the increased permeability. Diphenhydramine was inactive for the early phase even at 15 min, while aspirin lacked the inhibitory effect on the late phase if used in a dose smaller enough to show the effect on the early phase. As to 0.7% acetic acid, inhibitory action of diphenhydramine was shown at 15 min but not at 1 hr.

2. Induction of Edema in the Rat Paw

As shown in Fig. 2, S.A.A caused intensive sustained edema which reached more than edematous ratio of 50%. Development of edema was observed linear within 1 hr. This edema lasted for 15—20 days. Edema induced by mustard also lasted at increased level of 50—60% longer than 2 weeks. Effect of anti-inflammatory agents on the edema is summ-

5) C.A. Winter, E.A. Risley, and G.W. Nuss, *J. Pharmacol.*, **141**, 369 (1963); C.G. Van Arman, A.J. Begany, L.M. Miller, and H.H. Pless, *J. Pharmacol. Exptl. Therap.*, **150**, 328 (1965).

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arized in Table II and Fig. 3. Preventive and curative effect was obtained in aspirin 100 mg/kg, hydrocortisone 30 mg/kg and indomethacin 3 mg/kg. Aspirin 300 mg/kg, hydrocortisone 100 mg/kg and indomethacin 10 mg/kg showed the tendency of inhibition if given after development of edema was completed. When drug administration was discontinued, edema resumed the same level as control. The animals receiving 10 mg/kg of indomethacin began to die in 3 days. Effect of aspirin was reduced if high concentration of S.A.A (3%) was applied, while hydrocortisone inhibited the edema even in the same case. The inflammatory manifestation of S.A.A was as follows. The paw maintained red and swollen state for 3—4 days after injection. Then the injected position became white and yellow. Pus-like thick fluid extended around under the skin and formation of a scab took place. The scab continued for about 2 weeks and exfoliated thereafter. Further period was necessary to complete regeneration. S.A.A induced a similar phlogistic state in the mouse paw, too.

TABLE I. Inhibitory Effect of Anti-inflammatory Drugs on the Leakage of Dye into the Mouse Abdominal Cavity Induced by Secalonic Acid A and Acetic Acid

Inducer	Dose (*i.p.*) (mg/kg)	Drug	Dose (*p.o.*) (mg/kg)	15 min	1 hr	18 hr
S.A.A	12.5	——		100± 7(7)[a]	119± 6(12)	293±13(10)
S.A.A	12.5	diphenhydramine	50	115±10(11)	115±10(11)	
S.A.A	12.5	aspirin	100		82± 5(5)[b]	292±14(5)
S.A.A	12.5	aspirin	500		23± 2(6)[b]	126± 9(7)[b]
S.A.A	12.5	hydrocortisone	30		91±10(6)[c]	172±21(7)[b]
S.A.A	12.5	hydrocortisone	100		68± 5(6)[b]	118±11(7)[b]
S.A.A	12.5	indomethacin	10		80± 7(6)[b]	105±14(7)[b]
S.A.A	50.0	——		307±15(10)	267±32(6)	
S.A.A	50.0	diphenhydramine	50	257±21(10)	242±10(5)	
AcOH	0.7%	——		247±18(16)	274±19(10)	
AcOH	0.7%	diphenhydramine	50	131±17(16)[b]	254±25(8)	

a) amount of dye (μg/animal)±S.E. (number of animal)
b) $p<0.01$
c) $p<0.05$

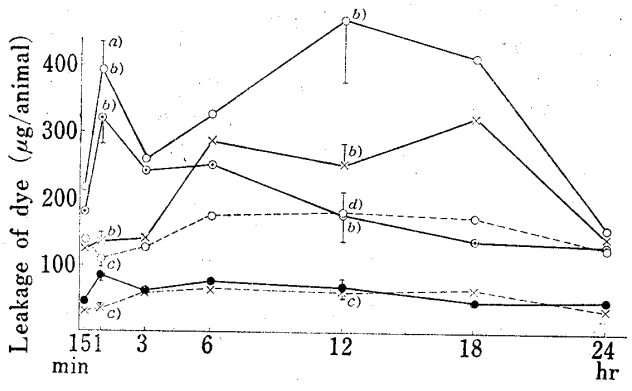

Fig. 1. Effect of Secalonic Acid A on the Leakage of Dye into the Abdominal Cavity in Mice

a) standard error *b*) $p<0.01$ for control
c) $p<0.01$ for fresh S.A.A *d*) $p<0.05$ for fresh S.A.A
●—●: control (n=6—12)
×—×: S.A.A (12.5 mg/kg) (n=5—6)
×---×: S.A.A (12.5 mg/kg, decomp) (n=6)
○—○: S.A.A (50.0 mg/kg) (n=5—10)
○---○: S.A.A (50.0 mg/kg, decomp) (n=5—6)
◉—◉: AcOH (0.7%) (n=5—6)

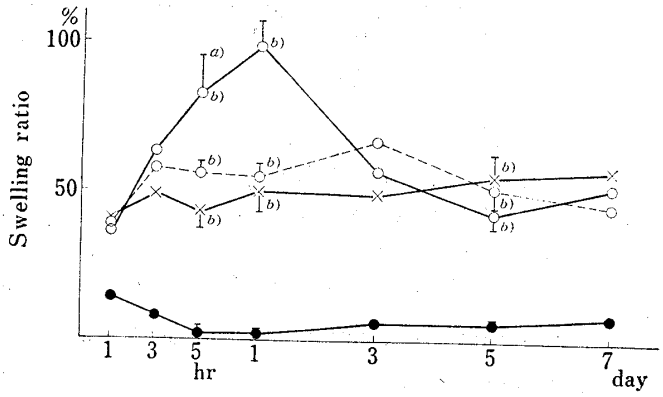

Fig. 2. Time Course of the Rat Paw Edema Induced by Secalonic Acid A and Mustard Powder

a) standard error *b*) $p<0.01$
●—●: control (n=13) ○---○: 2% S.A.A (n=5)
○—○: 3% S.A.A (n=5) ×—×: 5% mustard (n=6)

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TABLE II. Inhibtoiry Effect of Anti-inflammatory Drugs on the Rat Paw Edema Induced by Secalonic Acid A

Drug	Dose (mg/kg)	1 hr	3 hr	5 hr	1 day	3 day	5 day	7 day
Control	—	41.3±6.8[a]	56.8±5.9	61.4± 1.5	58.8±2.0	82.2± 4.5	67.0±3.9	48.4±1.7
Aspirin	100	22.8±2.9[b]	26.0±3.0[c]	26.6±10.0[b]	36.8±7.0[b]	55.8±10.0[b]	57.0±9.6	36.0±7.3
Aspirin	500	17.8±0.8[b]	12.5±3.3[c]	16.8± 4.7[c]	28.8±3.0[c]	50.5± 3.5[c]	60.0±4.1	54.8±3.9
Hydrocortisone	30	18.2±1.3[b]	15.0±2.6[c]	21.0± 2.7[c]	30.0±5.3[c]	50.4± 6.0[c]	48.4±4.7[b]	34.2±5.9
Hydrocortisone	100	22.8±4.3	23.0±1.0[c]	21.8± 0.9[c]	39.6±3.3[c]	45.0± 6.4[c]	48.4±4.1[b]	28.2±4.2[c]
Indomethacin	3	26.2±3.4	33.0±2.4[c]	31.8± 2.2[c]	44.2±4.9[b]	54.2± 5.9[c]	53.8±6.1	40.8±8.9
Indomethacin	10	26.0±2.5	22.0±2.5[c]	31.1± 4.4[c]	37.0±4.3[c]	26.3± 3.1[c]		
Control	—	42.3±5.0	65.5±4.9	47.0± 3.8	39.3±4.0	47.5± 4.6	41.8±9.1	36.0±4.1
Aspirin	100	31.2±3.6	38.4±3.1[c]	29.6± 1.9[c]	32.0±1.4	32.6± 4.9	27.2±2.9	37.2±3.1
Aspirin	500	11.6±3.0[c]	20.6±4.8[c]	11.6± 5.6[c]	15.0±3.9[c]	13.6± 1.7[c]	19.6±2.5	25.6±5.3
Hydrocortisone	30	28.6±1.6	34.0±2.5[c]	23.0± 2.5[c]	16.8±3.9[c]	14.6± 3.2[c]	16.0±2.6	33.2±3.0
Hydrocortisone	100	16.6±4.3[c]	30.8±3.9[c]	14.2± 3.9[c]	12.0±3.5[c]	15.6± 3.9[c]	24.0±5.4	44.0±6.3
Indomethacin	3	32.6±1.9	52.6±3.4	27.4± 4.6[b]	25.6±6.6	15.6± 2.4[c]	16.0±2.4	35.2±4.3
Indomethacin	10	16.0±4.7[c]	27.8±7.0[c]	15.8± 7.6[c]	16.4±7.8[c]	6.4± 1.9[c]		

Two sets of the experiment were carried out.
Five rats were used for each group.
Drugs were administered orally 1 hr before S.A.A and once a day for the succeeding 3 days.
a) swelling ratio (%) ± S.E. *b*) $p<0.05$ *c*) $p<0.01$

TABLE III. Total Number of Leucocytes Exuded into the Abdominal Cavity of Mice by Secalonic Acid A and Acetic Acid

		1 hr	18 hr
Control	—	25.6±3.8[a]	21.4± 3.7
S.A.A	12.5 mg/kg	27.3±4.0	171.8±30.8[b]
AcOH	0.7%	71.5±7.8	67.8±13.9[c]

Five mice were used for each trial.
a) number ± S.E. (per 1 mm² on Bürker–Türk plate)
b) $p<0.01$
c) $p<0.05$

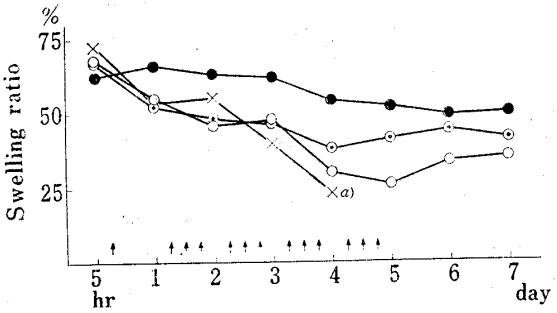

Fig. 3. Inhibitory Effect of Anti-inflammatory Drugs on the Rat Paw Edema Induced by 2% Secalonic Acid A

standard error at 1st and 4th day; control (5.8, 10.4), aspirin (7.0, 6.3), hydrocortisone (9.5, 3.3), indomethacin (7.2, 8.8)
↑ drug (*p.o.*)
a) two out of 4 animals died
●—●: control ($n=6$)
◉—◉: aspirin 300 mg/kg/day ($n=5$)
○—○: hydrocortisone 100 mg/kg/day ($n=4$)
×—×: indomethacin 10 mg/kg/day ($n=4$)

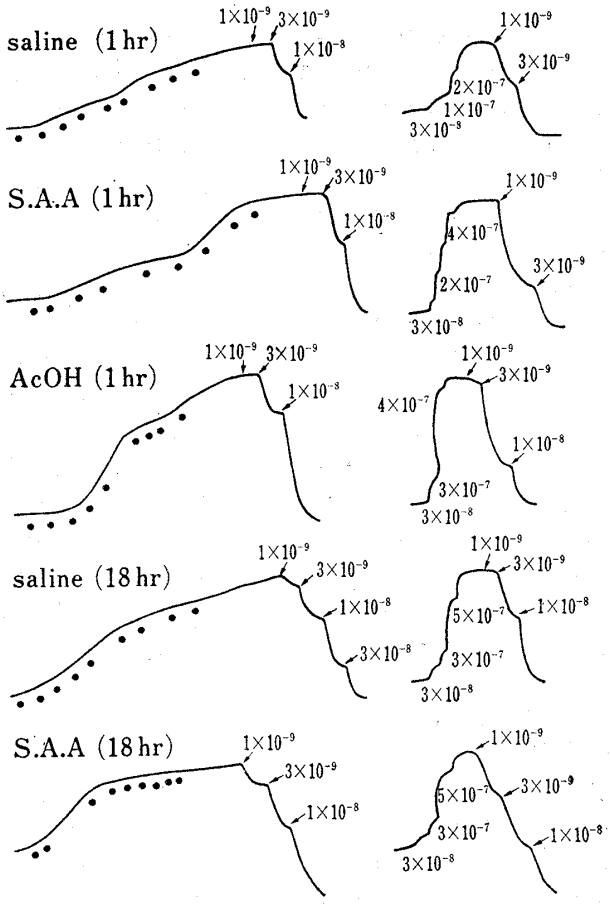

Fig. 4. Effect of the Mouse Abdominal Fluid Exuded by *i.p.* Administration of Secalonic Acid A and Acetic Acid on the Isolated Guinea Pig Ileum

●: abdominal fluid 1 ml (corresponding to 1/3 mouse)
relaxing agent: diphenhydramine HCl, concentration: g/ml

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3. Exudation of Leucocyte

Action of S.A.A on exudation of leucocyte is shown in Table III. Total number of leucocyte in the exuded abdominal fluid after 1 hr of S.A.A application was not different from that of control, whereas it remarkably increased after 18 hr as compared with the latter. In the case of acetic acid, leucocyte number increased in a small degree after 1 hr, maintaining the similar level even after 18 hr.

4. Action of the Exuded Abdominal Fluid on Isolated Organs

The result on guinea pig ileum is shown in Fig. 4. The number of ileum preparation was 8—14 respectively. All abdominal fluids which were exuded by either *i.p.* administration of S.A.A or AcOH contracted the ileum and, moreover, that of the corresponding saline-injected control group also gave a similar result. The difference of action between the phlogistic substance-injected groups and the saline group was hardly obtained. These contractions elicited by each fluid were relaxed by diphenhydramine in somewhat higher doses than those which were sufficient to relax the corresponding contraction induced by histamine. On the other hand, changes were observed neither in rat duodenum nor in rat uterus by these fluids.

Discussion

In order to study the phlogistic action of S.A.A, we examined the increased vascular permeability by S.A.A which is considered to characterize the 1st phase of inflammatory process. Action of S.A.A on the leakage of dye into the abdominal cavity of mice was of a biphasic change in which the late phase was especially remarkable. That of acetic acid, however, was of a monophasic change, displaying the peak at about 1 hr. It has already been known that phlogistic materials and factors such as heat (54° for 20 sec), UV light, streptococcal infection, turpentine oil *etc.* show a biphasic process of increased permeability. It would be of interest that such a chemically pure substance as S.A.A, the structure of which is known, is capable of producing a biphasic inflammatory process like this. Moreover, S.A.A has a feature that it does not exert any appreciable pharmacological and toxicological activity on the animal when given orally in a dose up to 1 g/kg. Wilhelm, *et al.*[6] reported various patterns of permeability process. This pattern varied depending on the intensity or quality of irritation and species difference. In addition, the pattern was inferred to depend especially upon the intensity more than the quality of a phlogistic substance. Therefore, S.A.A might give rise to a different pattern and intensity depending on doses and species used. S.A.A showed a little different pattern between two doses in the experiment.

All anti-inflammatory drugs except diphenhydramine inhibited both early and late phase. Diphenhydramine inhibited the early phase induced by acetic acid. This inhibition, however, was limited to the initial stage (at 15 min) in this early phase. It is commonly suggested that the early phase of the 1st phase of ordinary inflammation is related to histamine because of inhibition by antihistaminic agents and to other substances, and delayed phase is related to substance (s) which is not antagonized by diphenhydramine. According to our results, it was not recognized that histamine makes an important and primary role to the early phase of inflammation by S.A.A. Even in the case of acetic acid-induced inflammation, diphenhydramine inhibited only the earlier stage, suggesting the important participation of substance (s) other than histamine in the early phase.

The solution of S.A.A, kept for 1 month, lost the activity considerably. The study of thin-layer chromatography revealed the disappearance of S.A.A in this solution and its colour

6) D.L. Wilhelm, "Inflammation Biochemistry and Drug Interraction," ed. by A. Bertelli and J.C. Houck, Excerpta Media Foundation, Amsterdam, 1969, p. 136; D.L. Wilhelm, *Pharmacol. Rev.*, **14**, 251 (1962).

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reaction by $FeCl_3$ indicated the presence of phenolic radical in it. These facts suggest that the phlogistic effect of S.A.A is structure-specific and is not through presumable action of protein denaturation by phenolic radical.

S.A.A generated sustained edema in a rat paw lasting for 15—20 days. Garattini[7] examined duration of edema by various phlogistic substances and obtained a tendency for edema to decline from the peak within 6 hr. A number of reports often deal with the stage of edema within 5—6 hr and make no mention of the later process of edema. Stucki, *et al.*[8] reported that mustard produced sustained edema. The preparation of edema of a rat paw by mustard has been recognized as a model of sustained inflammation and has come to use in the study of anti-inflammatory drugs.[9] One of us[10] also used this preparation before and the duration of edema observed there was similar to that of present study. In addition to mustard, filipin, which is among antifungal agents and belongs to polyen derivatives, has been reported to produce sustained edema.[11] The mechanism is inferred to dissolve sterol which constitutes cell membrane and to destroy it. S.A.A is isolated from fungi and displays antibacterial activity, thus suggesting an interest in relevance to filipin. The development of edema was poorer in the solution kept for long period, as was the same in the test of dye leakage.

Anti-inflammatory agents inhibited edema in each of preventive and curative administration. The latter, however, did not give a significant inhibition. When daily administration was discontinued, the edema resumed the same level as control. Death which occurred in dose of 10 mg/kg of indomethacin seems to suggest toxicity of the drug. Tetracycline was used to prevent secondary infection but showed no influence on the process of swelling, body weight increase and condition of animals. Pus-like thick fluid and a scab developed 3—4 days after the injection of S.A.A and then lasted around the position of injection. This state was hardly inhibited by anti-inflammatory drugs and tetracycline.

The delayed phase of inflammation is considered to be characterized by exudation of leucocyte. The exact mechanism has not yet been elucidated. Polynuclear leucocyte first exudes in acute inflammation and monocyte follows it at the stage of the so-called delayed phase. Mediator (s) responsible for this exudation is thought to be different from histamine, serotonin, bradykinin. Yoshida, *et al.*[12] have recently isolated a substance, leukoegresin, as a mediator involved at this stage. Marked increase of leucocyte exudation took place 18 hr after application of S.A.A in this study, which suggests that this period corresponds to the delayed phase of acute inflammation. On the contrary, such increased exudation of leucocyte was not produced by acetic acid.

S.A.A showed a longer action on the increase of vascular permeability in the abdominal cavity of the mouse compared with that of acetic acid but did not give such sustained inflammatory state as obtained in rat paw edema. The difference of the duration between both the actions seems to be due to the site of application of S.A.A and/or to its amount in the affected area. In a model of the peritonitis, the inflamatory state induced by S.A.A is supposed to cease at the delayed phase of acute inflammation. In a model of the paw edema, however, the inflammation seems to proceed as far as the 3rd stage of acute inflammation and to remain in this state further. As this substance exerted an inflammatory state on the mouse paw similar to in the rat, species difference does not seem to exist in the paw edema model.

7) S. Garattini, "Non-steroidal Anti-inflammatory Drugs," ed. by S. Garattini, Excerpta Medica Foundation, Amsterdam, 1965, p. 151.
8) J.C. Stucki and C.R. Thompson, *Toxicol. Appl. Pharmacol.*, **4**, 362 (1962).
9) K. Tsurumi, M. Nozaki, M. Hayashi, A. Yamaguchi, Y. Hiramatsu, and H. Fujimura, *Nippon Yakurigaku Zasshi*, **68**, 662 (1972).
10) M. Harada, A. Yamashita, and M. Aburada, *Yakugaku Zasshi*, **92**, 750 (1972).
11) E.M. Glenn, B.J. Bowman, and T.C. Koslowske, "Chemical Biology of Inflammation," ed. by J.C. Houck and B.K. Forscher, Pergamon Press, Michigan, 1967, p. 27.
12) K. Yoshida, M. Yoshinaga, and H. Hayashi, *Nature*, **218**, 977 (1968).

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Both kinds of abdominal fluid at 1 and 18 hr after S.A.A application were examined using isolated organs. These samples exhibited contraction of guinea pig ileum but had no action on rat duodenum and rat uterus. Such contraction, however, was also shown in saline-treated control group in a similar degree. Consequently, at present, no knowledge could be obtained concerning the substances which are supposed to originate from S.A.A itself in the abdominal fluid exuded at the stage of the increase of vascular permeability by this substance.

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