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Evaluation of Analgesic Agents in Rats with Adjuvant Arthritis

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Rats with adjuvant arthritis were used for an analgesic test in which flection stimuli were applied to the arthritic joints.

TAI-284, indomethacin, phenylbutazone, mefenamic acid, ibufenac, aspirin and aminopyrine were effective at the relatively low doses in this method. Oral ED_{50}s of these compounds run parallel to their clinical doses. The method detects not only the analgesic action of morphine, pethidine and codeine, but also that of nalorphine, pentazocine and amphetamine. Prednisolone was mildly effective dose-independently. In spite of the high sensitivities, the method was specific in the sense that CNS-depressant, anti-depressant, anti-epileptic, naloxone, anti-serotonin and anti-histamine drugs were inactive or slightly active at toxic doses.

The arthritic flection pain test was concluded to be a sensitive and specific test.

Analgesic activities of nonsteroidal anti-inflammatory agents are currently considered to derive from a blockade of impulse generation at the peripheral chemonociceptors.[2] The assay for such weak analgesics and narcotic antagonist analgesics requires the chemically evoked pain or inflammatory sensitization procedures. Analgesic tests using intact animals[3] are not sensitive enough to detect their analgesic activities. At present, the phenylquinone writhing test in mice[4] and the Randall-Selitto's test in rats[5] are regarded to be relatively reliable for the experimental evaluation of the analgesic agents. Deffenu, *et al.*[6] and Abe, *et al.*[7] used the bradykinin-induced effects in rats as an assay for analgesic drugs including pentazocine. In our experiences, the phenylquinone writhing test was nonspecific for analgesic evaluation, the Randall-Selitto's test relatively gave a variance in the results, and the bradykinin-induced pain test was not sensitive enough for the analgesic evaluation. Adjuvant arthritis in rats resembles rheumatoid arthritis in man in the manifestation of inflammation and hyperalgesia. A gentle flection of the affected joints in rats with adjuvant arthritis evoked a clear-cut and reproducible sign of algesia, a squeaking response, the suppression of which by a variety of analgesics, particularly nonsteroidal anti-inflammatory drugs, presented successfully the analgesic activities.

Experimental

Test compounds used were 6-chloro-5-cyclohexylidan-1-carboxylic acid (TAI-284), indomethacin, phenylbutazone, mefenamic acid, ibufenac, aspirin, aminopyrine, prednisolone, morphine hydrochloride,

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pethidine hydrochloride, codeine phosphate, nalorphine hydrochloride, pentazocine, naloxone hydrochloride and amphetamine sulfate. Chlorpromazine hydrochloride, haloperidol, imipramine hydrochloride, diphenhydramine hydrochloride, diphenylhydantoin and cyproheptadine hydrochloride were also used.

Water-insoluble compounds were suspended with 4% gum acacia in water and water-soluble compounds were dissolved in water for oral administration. For subcutaneous injection, a saline solution was used instead of water. Dosages of compounds refer to the salt forms. The experiment was done following a double blind schedule.

SD-JCL male rats, 5 weeks old and weighing 130±10 g, and ICR-JCL male mice, 3.5 weeks old and weighing 20±2 g were used. Both animals were specific pathogen free and purchased from Japan CLEA Co.

Arthritic Flection Pain Test——Following the method of Newbould,[8] 0.05 ml of 0.5% Freund's complete adjuvant was injected intradermally at the plantar side of left hind paw in 70 rats. All of 70 rats injected with the adjuvant exhibited secondary lesions at non-injected hind foot and some other parts including ears and tail. The degree of secondary lesions at the right hind foot was scored in 0 (no abnormality), 1 (erythema), 2 (erythema and slight swelling), 3 (erythema and moderate swelling), 4 (severe swelling) and 5 (extremely severe arthritis showing hypersensitivity to noxious stimuli). As shown in Table I, the secondary lesions observed 10 days after the adjuvant injection almost corresponded to score 1. Then, the lesions rapidly progressed into score 2 or 3.

Pain response was examined by a gentle flection of the right tarso-tibial joints repeatedly 5 times at intervals of 4 to 5 sec with a finger of the operator. This is essentially similar to the procedures of LaBelle, *et al.*[9] or Margolin.[10] Although occasionally a struggling response was noted, only the squeaking response was regarded as an algesic sign. Animals with score 1 did not show any squeaking response. Animals with score 5 reacted hypersensitively to the stimuli. Eighteen days later, satisfactory pain responses were obtained, and animals with no pain response and with hypersensitivity were 8 of 70 rats.

Table I. Distribution of Numbers of Rats with Arthritis and/or Pain Response

Days after adjuvant injection		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Arthritic score	:0	60	35	10	7	—	1	0	0	0	0	—	—	0	0	0	0
	:1	9[a]	27	28	21	—	11	6	7	6	4	—	—	5	5	7	5
	:2	1	6	27	20	—	20	17	11	10	7	—	—	4	3	2	3
			(3)[b]	(9)	(13)		(10)	(7)	(6)	(9)	(6)			(3)	(3)	(2)	(3)
	:3	0	2	5	19	—	34	40	41	43	48	—	—	45	46	40	41
			(2)	(5)	(19)		(34)	(40)	(41)	(43)	(48)			(45)	(46)	(37)	(35)
	:4	0	0	0	3	—	4	7	11	10	10	—	—	14	14	19	18
					(3)		(4)	(7)	(11)	(10)	(10)			(14)	(14)	(19)	(18)
	:5	0	0	0	0	—	0	0	0	1	1	—	—	2	2	2	3
										(1)	(1)			(2)	(2)	(2)	(3)

a) No. of rats with arthritis
b) No. of rats with pain response

For the analgesic test, animals with no pain response or with hypersensitivity were excluded from the experiment 18 days after the adjuvant inoculation. Test compounds were administered orally(0.5 ml/100g B. W.) or subcutaneously (0.2 ml/100g B. W.). Thereafter, the series of 5 flection stimuli were applied hourly for 5 hr. For the evaluation of narcotics and narcotic antagonists, the stimuli were applied every 30 min for 3 hr. Animals showing no squeaking response when tested by 5 stimuli were defined as analgesic positive. ED_{50} was calculated by probit analysis from % analgesia: Sum of positive rats/ Total No. of rats tested for 5 hr or 3 hr.

Phenylquinone Writhing Test——Writhing and stretching responses were caused by an intraperitoneal injection of 0.1 ml/10g B. W. of 0.02% phenylquinone solution in mice.[4] The solubility of phenylquinone in water was increased by the inclusion of 5% ethanol. Test compounds were administered orally or subcutaneously 30 min before the phenylquinone injection. Then, the frequency of responses was counted in each animal for 20 min. Animals showing less than 50% of mean writhing response of control group were regarded as analgesic positive and ED_{50} was calculated by probit analysis.

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Result

Arthritic Flection Pain Test

Nonsteroidal Anti-inflammatory Drugs and Antipyretic Analgesic——The data in Table II are examples of analgesic effects of TAI-284 and aminopyrine in each animal and calculation of % analgesia. As shown in Table III, % analgesia in control group was extremely low. The oral doses of 0.25, 0.5 and 1.0 mg/kg of TAI-284 produced 16.7, 33.3 and 61.7% analgesia,

Table II. Effect of TAI-284 and Aminopyrine on Flection Pain in Rat Adjuvant Arthritis

Compound.	Oral dose mg/kg	Rat No.	Analgesia: Analgesic positive 1 hr	2 hr	3 hr	4 hr	5 hr	Analgesia: Rate[a)]	Analgesia: %
Control	—	1	−	−	−	−	−	0/30	0
		2	−	−	−	−	−		
		3	−	−	−	−	−		
		4	−	−	−	−	−		
		5	−	−	−	−	−		
		6	−	−	−	−	−		
TAI-284	0.25	1	−	+	+	+	+	8/30	26.7
		2	−	+	+	−	−		
		3	−	−	−	−	−		
		4	−	+	+	−	−		
		5	−	−	−	−	−		
		6	−	−	−	−	−		
	0.5	1	−	−	−	−	−	12/30	40.0
		2	+	+	+	+	+		
		3	−	−	−	−	−		
		4	−	+	+	+	+		
		5	−	−	+	+	+		
		6	−	−	−	−	−		
	1.0	1	−	−	+	+	+	18/30	60.0
		2	−	+	+	+	+		
		3	+	+	+	+	+		
		4	+	+	+	+	+		
		5	−	+	−	−	−		
		6	−	−	−	−	−		
Aminopyrine	50	1	+	−	−	−	−	9/30	30.0
		2	−	−	−	−	−		
		3	+	+	+	+	−		
		4	−	−	+	−	−		
		5	+	+	+	−	−		
		6	−	−	−	−	−		
	100	1	−	−	−	−	−	13/30	43.3
		2	+	+	+	+	+		
		3	−	+	−	−	−		
		4	+	+	−	−	−		
		5	+	+	−	−	−		
		6	−	+	−	+	+		
	200	1	+	+	+	−	+	24/30	80.0
		2	−	+	+	+	+		
		3	+	+	+	+	+		
		4	+	+	+	+	+		
		5	+	+	+	−	−		
		6	+	+	+	−	−		

a) sum of positive animals/total trials

Table III. Effect of Non-steroidal Anti-inflammatory Agents on Flection Pain in Rat Adjuvant Arthritis

Compound	Oral dose mg/kg	No. of rats	Analgesia: Positive animals 1 hr	2 hr	3 hr	4 hr	5 hr	Rate[a]	%
Control	—	42	0	2	1	0	0	3/210	1.4
TAI-284	0.25	6	0	2	1	1	1	5/30	16.7
	0.5	18	1	6	9	8	6	30/90	33.3
	1.0	12	4	8	9	8	8	37/60	61.7
Indomethacin	0.5	12	1	5	4	2	1	13/60	21.7
	1.0	12	4	4	7	8	4	27/60	45.0
	2.0	12	5	7	10	9	7	38/60	63.3
Phenylbutazone	2.5	6	0	2	0	0	1	3/30	10.0
	5.0	6	2	2	2	3	2	11/30	36.7
	10.0	12	6	6	8	7	7	34/60	56.7
	20.0	6	4	5	5	4	4	22/30	73.3
Mefenamic acid	2.5	6	2	0	0	0	1	3/30	10.0
	5.0	6	2	1	3	1	2	9/30	30.0
	10.0	6	2	2	3	3	2	12/30	40.0
	20.0	6	3	2	5	5	5	20/30	66.7
Ibufenac	10	6	2	2	2	1	0	7/30	23.3
	20	6	1	4	4	2	1	12/30	40.0
	40	6	3	5	5	4	1	18/30	60.0
Aspirin	50	6	1	1	2	1	2	7/30	23.3
	100	6	2	3	3	2	2	12/30	40.0
	200	6	2	5	5	4	5	21/30	70.0
Aminopyrine[b]	25	6	0	1	2	0	0	3/30	10.0
	50	6	3	2	3	1	0	9/30	30.0
	100	6	3	5	1	2	2	13/30	43.3
	200	6	5	6	6	3	4	24/30	80.0

a) sum of positive animals/total trials
b) antipyretic drug

respectively. Indomethacin (0.5—2.0 mg/kg), phenylbutazone (2.5—10 mg/kg), mefenamic acid (2.5—20 mg/kg), ibufenac (10—40 mg/kg) and aspirin (50—200 mg/kg) were also effective dose-dependently.

As shown in Fig. 1, the analgesic actions of these drugs reached the peak at 2 or 3 hr after administrations and decreased slowly except for ibufenac. Aminopyrine was dose-dependently effective in the doses of 25 to 100 mg/kg, and the onset and disappearance of the action seemed to be earlier than those of nonsteroidal anti-inflammatory drugs.

Narcotics and Narcotic Antagonists——As shown in Table IV, narcotics administered subcutaneously caused analgesia in the flection pain test in the decreasing order of morphine, codeine and pethidine. The relatively low doses of compounds were sufficient for their analgesic activities. Fig. 2 indicates that their analgesic actions promptly reached the peak and disappeared 3 hr after the administration.

Nalorphine (1.0—16 mg/kg) and pentazocine (0.75—6.0 mg/kg) also exhibited an analgesic action dose-dependently, but the slope of the dose response line for nalorphine was flatter than that of morphine. The analgesic pattern of nalorphine was similar to those of narcotics described above, but pentazocine had a somewhat longer action. Naloxone was almost ineffective even at 32 mg/kg.

Miscellaneous Compound——Amphetamine, a CNS-stimulant, was analgesic dose-dependently at 1 to 8 mg/kg *s.c.* in this test (Table IV). As shown in Table V, prednisolone in the doses of 5 to 20 mg/kg *p.o.* caused a mild analgesia, but no dose-response relationship was

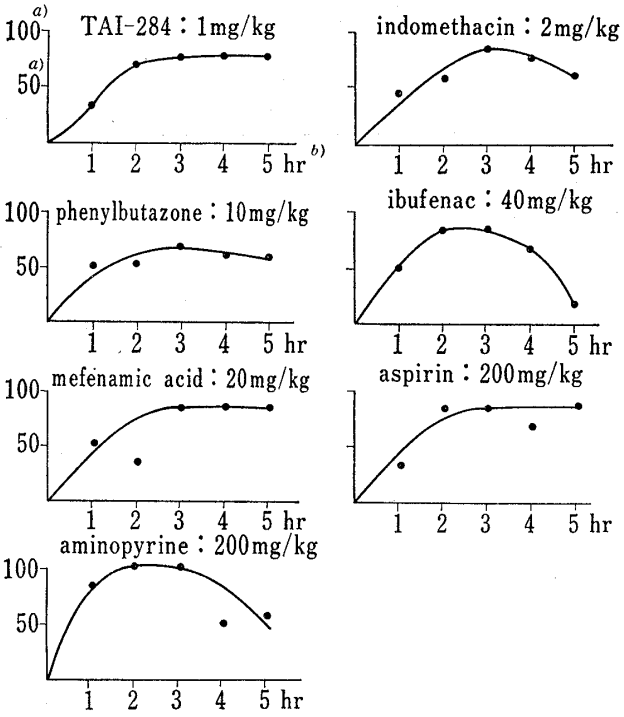

Fig. 1. Analgesic Pattern of TAI-284 and Other Anti-inflammatory Drugs in the Flection Pain Test

a) per cent analgesia *b*) time after administration

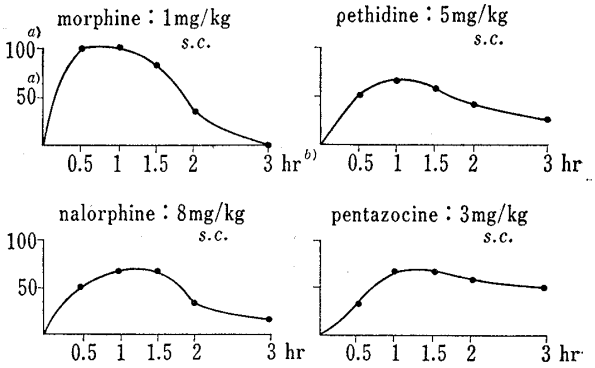

Fig. 2. Analgesic Pattern of Narcotics and Narcotic Antagonists in the Flection Pain Test

a) per cent analgesia
b) time after administration

TABLE IV. Analgesic Effect of Narcotics, Narcotic Antagonists and CNS-stimulant on Flection Pain in Rat Adjuvant Arthritis

Compound	Dose mg/kg *s.c.*	No. of rats	Analgesia: Positive animals 0.5 hr	1 hr	1.5 hr	2 hr	3 hr	Rate[a)]	%
Control	—	30	0	0	3	0	0	3/150	2.0
Morphine	0.25	12	2	6	4	1	0	13/60	21.7
	0.5	12	6	8	7	5	3	30/60	50.0
	1.0	6	6	6	5	2	0	19/30	63.3
Pethidine	2.5	12	1	5	3	1	1	11/60	18.3
	5.0	12	6	8	7	5	3	29/60	48.3
	10.0	6	5	6	6	4	2	23/30	76.7
Codeine	1.5	6	1	3	1	0	1	6/30	20.0
	3.0	6	3	4	4	1	0	12/30	40.0
	6.0	6	4	6	5	3	2	20/30	66.7
Naloxone	8	6	0	0	1	1	0	2/30	6.7
	16	6	0	1	1	0	0	2/30	6.7
	32	6	0	1	1	1	0	3/30	10.0
Nalorphine	1	6	2	2	1	0	0	5/30	16.7
	2	6	3	4	2	1	0	10/30	33.3
	4	6	3	5	4	1	0	13/30	43.3
	8	6	3	4	4	2	1	14/30	46.7
	16	6	4	5	6	3	3	21/30	70.0
Pentazocine	0.75	6	1	0	2	1	1	5/30	16.7
	1.5	12	1	3	4	4	2	14/60	23.3
	3.0	12	4	8	8	7	6	33/60	55.0
	6.0	6	3	6	6	6	3	24/30	80.0
Amphetamine	1	6	2	2	2	1	0	7/30	23.3
	2	6	2	3	4	2	0	11/30	36.7
	4	6	4	5	4	3	3	19/30	63.3
	8	6	6	6	6	5	2	25/30	83.3

a) sum of positive animals/total trials

Table V. Effect of Various Agents of Flection Pain in Rat Adjuvant Arthritis

Compound	Oral dose mg/kg	No. of rats	Analgesia: Positive animals 1 hr	2 hr	3 hr	4 hr	5 hr	Rate[a]	%
Control	—	18	1	0	0	0	0	1/90	1.1
Prednisolone	5	6	0	1	3	3	3	10/30	33.3
	10	12	2	4	5	3	3	17/60	28.3
	20	6	0	2	5	2	2	11/30	36.7
Chlorpromazine	5	6	2	2	2	0	0	6/30	20.0
	10	6	2	2	2	1	1	8/30	26.7
	20[b]	6	4	5	5	3	2	19/30	63.3
	50[b]	6	6	6	6	6	6	30/30	100.0
Haloperidol	2	6	2	2	1	2	0	7/30	23.3
	10	6	4	2	2	2	1	11/30	36.7
Imipramine	5	6	1	1	0	1	1	4/30	13.3
	50	6	1	1	1	0	0	3/30	10.0
Diphenylhydantoin	12.5	6	1	1	1	0	0	3/30	10.0
	25	6	0	1	0	0	0	1/30	3.3
	50	6	1	1	0	0	0	2/30	6.7
Diphenhydramine	50	6	2	0	2	0	0	4/30	13.3
Cyproheptadine	10	6	1	1	0	0	0	2/30	6.7
	50[b]	6	3	3	3	1	2	12/30	40.0

a) sum of positive animals/total trials
b) Behavioral changes were observed.

Table VI. Analgesic ED_{50}s of Various Drugs in Arthritic Flection Pain Test in Rats and Phenylquinone Writhing Test in Mice

Compound	Arthritic flection pain: Route	ED_{50} mg/kg	P.Q. writhing: Route	ED_{50} mg/kg
TAI-284	*p.o.*	0.66(0.56— 0.81)[a]	*p.o.*	14.8 (11.1 — 19.8)
Indomethacin	*p.o.*	1.26(0.98— 1.62)	*p.o.*	1.07(0.69— 1.66)
Phenylbutazone	*p.o.*	8.64(6.70— 11.41)	*p.o.*	51.4 (37.8 — 70.0)
Mefenamic acid	*p.o.*	12.2 (8.70— 22.70)	*p.o.*	32.4 (20.6 — 50.9)
Ibufenac	*p.o.*	28.2 (18.3 — 43.50)	*p.o.*	44.0 (31.2 — 54.3)
Aspirin	*p.o.*	118.9 (86.8 —183.40)	*p.o.*	41.5 (29.4 — 58.8)
Aminopyrine	*p.o.*	96.0 (73.9 —132.10)	*p.o.*	23.9 (20.1 — 28.6)
Morphine	*s.c.*	0.58(0.36— 0.74)	*s.c.*	0.26(0.2 — 0.33)
Pethidine	*s.c.*	5.17(4.00— 7.20)	*s.c.*	1.58(0.77— 3.90)
Codeine	*s.c.*	3.82(2.79— 5.21)	*s.c.*	5.66(3.82— 8.79)
Naloxone	*s.c.*	32 inactive	*s.c.*	>10
Nalorphine	*s.c.*	6.42(4.12— 12.55)	*s.c.*	0.84(0.47— 1.48)
Pentazocine	*s.c.*	2.70(2.20— 3.48)	*s.c.*	1.56(1.19— 2.05)
Amphetamine	*s.c.*	2.68(2.05— 3.62)	*s.c.*	0.17(0.07— 0.30)
Prednisolone	*p.o.*	>20	*p.o.*	20 inactive
Chlorpromazine	*p.o.*	>10	*p.o.*	1.22(0.75— 2.16)
Haloperidol	*p.o.*	>10	*p.o.*	0.18(0.09— 0.29)
Imipramine	*p.o.*	50 inactive	*p.o.*	18.6 (9.0 — 29.0)
Diphenylhydantoin	*p.o.*	50 inactive	*p.o.*	64.0 (37.1 —210.6)
Cyproheptadine	*p.o.*	10 inactive	*p.o.*	1.17(0.71— 1.87)
Diphenhydramine	*p.o.*	50 inactive	*p.o.*	3.66(1.34— 6.80)

a) ( ): 95% fiducial limits

obtained. Chlorpromazine in the doses of 5 to 50 mg/kg *p.o.* was apparently analgesic dose-dependently, but at 20 mg/kg a sedation and hypothermia were observed. Haloperidol was effective in a very high dosage. Imipramine, diphenhydramine, diphenylhydantoin and cyproheptadine were ineffective in the pharmacological doses.

Comparison of Flection Pain Test with Phenylquinone Writhing Test

Table VI indicates the ED_{50}s of various compounds obtained in the flection pain and phenylquinone writhing tests. In the writhing test, ED_{50}s were able to be calculated not only for analgesic drugs, but also for CNS-depressant, anti-depressant, anti-epileptic, anti-serotonin and anti-histamine drugs. In contrast to the writhing test, the flection pain test was very specific in the sense that miscellaneous drugs not known as analgesics are inactive, or slightly effective at toxic dosages, or did not have a dose response relationship.

Discussion

The phenylquinone writhing test is known to be a sensitive analgesic method providing reasonable parallelism to the clinical potency. However, CNS-depressant, anti-depressant, anti-epileptic, anti-serotonin and anti-histamine drugs exhibited a potent anti-writhing action. Such an unspecific property of this method has been reported by Pearl, *et al.*[11] and Emele, *et al.*[12] In this respect, the flection pain test was shown to have a definite advantage over the phenylquinone writhing test. Moreover, the ED_{50}s of nonsteroidal anti-inflammatory drugs were fairly low and well correlated with their clinical doses.[13]

As reported previously,[14] TAI-284 is presumed to possess a comparable analgesic activity to that of indomethacin in the Randall-Selitto's test in rats. In the flection pain test, TAI-284 was two times as potent as indomethacin, indicating the most potent of the nonsteroidal anti-inflammatory drugs tested. It was impressed that the flection pain test allowed a more precise evaluation of the analgesic potencies than did the Randall-Selitto's test. In contrast to the result in rats, the anti-writhing potency of TAI-284 in mice was about one tenth that of indomethacin. Kawai, *et al.*[15] reported that the oral acute toxicity of TAI-284 in mice was about one thirty that of indomethacin. These facts seems to be related to the high rate of its metabolism and excretion in this species.

Anti-inflammatory action of nonsteroidal anti-inflammatory drugs seems to play only a minor role in the anti-flection pain activity, because the established lesions are relatively tolerant to the anti-inflammatory action, and because their analgesic action was demonstrable at the very low doses. In addition, prednisolone which has a potent anti-inflammatory action was mild analgesic at the high doses.

Centrally acting analgesics were effective in the relatively low doses in this test, but the degree of correlation between the ED_{50}s and clinical doses was rather poor as compared with that observed with nonsteroidal anti-inflammatory drugs.[16] Naloxone was not effective.

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It is noteworthy that nalorphine and pentazocine are effective in this rather simple technique. The slope of the dose response line for nalorphine was flatter than that of morphine, as reported by Pearl, *et al.*[17] Amphetamine had a positive anti-flection pain activity. The analgesic action in man has been reported.[18] Prednisolone showed a dose-independent mild analgesic activity. Eichholtz, *et al.*[19] reported a centrally acting analgesic action of cortisone in rabbits. At present, the mechanisms for analgesic action of steroids remain unsolved.

Hirose, *et al.*[20] reported a new analgesic method in which the inflamed foot of adjuvant arthritis rats was stimulated by pinching, and efficiently demonstrated the analgesic action of several nonsteroidal anti-inflammatory drugs, morphine and hydrocortisone. In order to measure the degree of hyperalgesia in adjuvant arthritis, we have also been investigating various stimuli. For example, pressure stimulus was not favorable since the rats did not well respond to it. A gentle stimulus, flection of joint, was the best of stimuli tested, and squeaking responses thus produced were sensitive to many analgesics. In spite of the high sensitivity, the method showed the specificity, *i.e.*, ineffectiveness of chlorpromazine, haloperidol, imipramine, diphenhydramine, diphenylhydantoin and cyproheptadine. In addition, the onset and duration of analgesic action assayed without any tissue damage clearly suggests the pharmacodynamic aspect of compounds.

As for the shortcomings of the flection pain test, a considerable long period is required to establish arthritis. Moreover, 10 to 15% of adjuvant-injected rats did not show enough arthritis to respond to the flection stimuli or showed the extremely severe arthritis in which analgesics were not effective. Such animals had to be excluded from analgesic tests.

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