Br. J. Pharmac. (1974), **51**, 383-389

FORMATION OF A FACTOR INCREASING VASCULAR PERMEABILITY DURING ELECTRICAL STIMULATION OF THE SAPHENOUS NERVE IN RATS

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1 Increased vascular permeability following electric antidromic stimulation of the rat saphenous nerve was observed in the skin area supplied by the nerve, confirming previous results by other authors.

2 The phenomenon was not affected by pretreatment of the rats with diphenhydramine, burimamide or their combination; atropine, methysergide, methysergide plus diphenhydramine, carboxypeptidase B, acetylsalicylic acid, indomethacin or methiazinic acid. It was partially reduced by previous injection of cellulose-sulphate, a kininogen-depleting agent.

3 Perfusates from the subcutaneous tissue of the paw area supplied by the saphenous nerve contained permeability increasing activity as shown by intradermal tests in other rats. This activity was present in perfusates collected during nerve stimulation but not in those collected before stimulation. It was not destroyed by heating to 100° C, or by α-chymotrypsin or trypsin.

4 Bradykinin-like activity may appear later in the perfusates, depending on the intensity of the stimuli.

5 It is concluded that following electrical antidromic stimulation of the saphenous nerve a permeability increasing factor is released, possibly from nerves. It is dialysable and can be distinguished from acetylcholine, histamine, 5-hydroxytryptamine, plasma kinins, substance P, prostaglandins and high molecular weight proteins. The increased vascular permeability induced by this factor leads to plasma exudation and activation of the kinin system.

Introduction

Jancsó, Jancsó-Gábor & Szolcsányi (1967, 1968) provided evidence that inflammatory responses, characterized by arteriolar vasodilatation, enhancement of vascular permeability, protein exudation and fixation of injected colloidal silver on to the walls of venules, can be elicited by antidromic stimulation of sensory nerves. They suggested that a neurohumoral agent was released and was responsible for the observed response; it seemed to be different from acetylcholine, histamine, 5-hydroxytryptamine, adrenaline or noradrenaline.

Vasodilatation induced by antidromic stimulation of sensory nerves seems to depend on axon reflexes (Bayliss, 1901, 1923; Lewis, 1927; Dale & Gaddum, 1930) occurring at the terminals of superficial fibres mediating pain (Celander & Folkow, 1953a). It was suggested that the final vasodilator released was different from acetylcholine or histamine (Celander & Folkow, 1953b). The flare evoked by electrical stimulation of the human skin was not inhibited by antihistamines (Parrot, 1942), suggesting that, if histamine was released, it was not affecting the terminal mechanisms of the response. The production of a neurokinin at the onset of a flare reaction induced by noxious stimulation of the skin, was described by Chapman, Ramos, Goodell & Wolff (1961) and Chapman & Goodell (1964). However, such activity might be due to the activation of bradykinin-like substances from the leaked plasma, as pointed out by Keele & Armstrong (1964) and Jancsó *et al.* (1967).

These observations indicate that when sensory nerves are stimulated, vascular alterations occur which might depend on the presence of permeability factor(s) whose origin and nature are not fully identified.

In the present paper an attempt has been made to characterize further the release of a permeability factor following antidromic stimulation of sensory nerves. The results indicate that a factor is released from nerves, which can be

distinguished from acetylcholine, histamine, 5-hydroxytryptamine, plasma kinins, substance P, prostaglandins, or high molecular weight proteins.

Methods

Male adult Wistar rats (250-300 g) anaesthetized with pentobarbitone sodium (30-40 mg/kg) were used.

Electrical stimulation of the saphenous nerves

The saphenous nerves of both hind limbs were exposed, cut high in the thigh, and the incised areas kept under mineral oil as described by Jancsó *et al.* (1967). Rectangular pulses were applied to the peripheral end of the nerves by means of a bipolar stainless steel electrode and a stimulator (Grass, Model S4). The duration of the pulses was 20 ms, the frequencies 1, 10, 15 and 25 Hz and the intensities 1, 2 and 4 V.

Vascular permeability.

Evans blue dye (20 mg/kg) was injected intravenously as a 1% aqueous solution 5 min before the electrical stimulation of the saphenous nerve. The nerves of both paws were similarly exposed, but only one was stimulated. At the end of the 20 min stimulation period, the skin of both paws was resected up to the tibio-tarsal joint, the leaked dye was extracted with formamide (Reis, Okino & Rocha e Silva, 1971), and measured by spectrophotometry at 600 nm.

Coaxial perfusion of the subcutaneous tissue of the paw in the area supplied by the saphenous nerve

Polythene tubing (3 mm o.d. and 2 mm i.d.) was inserted through an incision high in the limbs, passed into the subcutaneous tissue and guided to the distal part of the paw as described by Rocha e Silva & Antonio (1960) and Garcia Leme, Hamamura & Rocha e Silva (1970). Narrower tubing, connected to a reservoir containing Tyrode solution kept at 5 to 10°C or at 37°C was introduced into the wider tube, in such a way that the perfusion fluid reached the subcutaneous space through the inner tube and could be collected through the outer tube. The perfusion (1 ml/10 min) started 10 min before the electrical stimulation of the nerve and continued during the 20 min period of stimulation. The first perfusate sample of 1 ml was collected before, and the second and third during the electrical stimulation of the nerve. These samples were submitted to pharmacological assays. During the collection of the first (control) sample, all handling of the nerve was avoided.

Drugs were given to the animals 30 min before the experimental procedures, unless indicated otherwise.

Assays of the perfusates

The three samples of perfusates collected were assayed as obtained (crude sample), after heating in boiling water for 30 min, or after incubation with chymotrypsin (200 μg/ml) or trypsin (200 μg/ml) at 37°C for 15 min followed by heating in boiling water for 30 minutes. In some cases corresponding samples from different animals were pooled for assays. Two types of assays were used:

(1) Intradermal injection of 0.5 ml of the samples in the abdominal wall of test rats previously given diphenhydramine 25 mg/kg and methysergide 2 mg/kg intraperitoneally (30 min before) and Evans blue dye 20 mg/kg in a 1% aqueous solution intravenously (5 min before). Twenty minutes after the injection of the samples the skin was resected, the areas around the injection site cut out, and the dye extracted and measured as described. The treatment with anti-histamine and anti-5-hydroxytryptamine drugs was sufficient to reduce by more than 90% the responses of the animals to doses as high as 10 μg histamine or 0.1 μg 5-hydroxytryptamine (in Tyrode solution). Similar reduction was observed in the effects of high doses of bradykinin (2 μg) after incubation with chymotrypsin 200 μg (in Tyrode solution; final volume 1 ml) followed by heating, treatment analogous to that used for the perfusates. This dose of bradykinin is larger than the amounts of bradykinin-like substances found in perfusions of inflamed areas (Rocha e Silva & Antonio, 1960; Garcia Leme *et al.*, 1970).

(2) The actions of the perfusates were examined on the rat isolated uterus (5 ml bath, de Jalón's solution, 28°C), the rat isolated duodenum (5 ml bath, de Jalón's solution, 37°C) and the guinea-pig isolated ileum (10 ml bath, Tyrode solution, 37°C).

Dialysis of the perfusates. Dialysis tubes from Union Carbide Co. were used and 1 ml samples dialysed for 12 h against 2 l of distilled water at 4°C, with stirring.

Plasma bradykininogen

Plasma bradykininogen was estimated in oxalated plasma according to Diniz, Carvalho, Ryan & Rocha e Silva (1961). The amount of kinin

released was assayed on the guinea-pig isolated ileum in the presence of atropine and diphenhydramine.

Kininase activity of carboxypeptidase B

To estimate the kininase activity of purified carboxypeptidase B, synthetic bradykinin 50 μg/ml was added to samples of heparinized rat blood diluted with Tris-NaCl buffer (Tris 50 mM; NaCl 100 mM; pH 7.5) in the proportion of 1 : 1 v/v (control-samples), and in the proportion of 1 : 9 v/v (test-samples). To test-samples 0.4 μg/ml of the enzyme was also added. Control and test-samples were incubated at 37° C and the rate of inactivation of bradykinin estimated on the guinea-pig isolated ileum in the presence of atropine. Complete destruction of the polypeptide was observed after 3 min in the test samples and after 9 min in control samples.

Drugs used

Acetylsalicylic acid (Aspirin), Bayer; 1-*p*-chlorobenzyl-2-methyl-5-methoxy-3-indolyacetic acid (Indomethacin), Merck, Sharp & Dohme; methyazinic acid (Soripal), Rhodia; soya-bean trypsin inhibitor (SBTI), Sigma; Evans blue dye, E. Merck A.G.; bradykinin (BRS-640), Sandoz; hexadimethrine bromide (Polybrene), Abbot; *N*-methyl*N*-(4-(5)-imidazolyl)butyl)thiourea (Burimamide), Smith Kline and French, kindly supplied by Prof. J.W. Black; purified carboxypeptidase B, Worthington Biochemical; acetylcholine hydrochloride, Roche; promethazine hydrochloride (Phenergan), Rhodia; diphenhydramine hydrochloride (Benadryl), Parke Davis; D-lysergic acid diethylamide (LDS-25), Sandoz; formamide, E. Merck A.G.; heparin (Liquemine), Roche; 5-hydroxytryptamine (Serotonin), Man Research; histamine diphosphate, Sigma; chlorpheniramine maleate (Polaramine), Schering; methysergide (Deseril), Sandoz; pentobarbitone sodium (Nembutal), Abbott; alpha-chymotrypsin, Man Research; atropine sulphate, Parke Davis; cellulose-sulphate, prepared according to Karrer, Köenig & Usteri (1943), kindly supplied by Prof. A.M. Rothschild; tris(hydroxymethyl)aminomethane (TRIS), E. Merck A.G.; trypsin, Worthington Biochemical; lidocaine (Xylocaine), Astra. For injections, drugs were dissolved in distilled water or saline. To dissolve indomethacin, NaOH was added to pH 9 and the solution neutralized afterwards by the addition of acid.

Results

Effect of electrical stimulation of the saphenous nerve on vascular permeability of the skin area supplied by the nerve.

Our results confirm previous data by Jancsó *et al.* (1967) showing that electrical stimulation of the saphenous nerve of the rat leads to an increased

Table 1 Evans blue content in the rat skin supplied by the saphenous nerve following electrical stimulation of the nerve for 20 min with pulses of 20 ms duration and different frequencies and intensities

Frequency (Hz)	Intensity (V)	Amount (μg) of dye leaked: Stimulated paw	Amount (μg) of dye leaked: Control paw	Number of animals
1	1	8.09 ± 2.02 ($P < 0.05$)*	2.48 ± 0.47	5
10	1	14.34 ± 1.37 ($P < 0.05$)	2.18 ± 0.21	6
15	1	15.27 ± 0.71 ($P < 0.05$)	2.09 ± 0.24	5
25	1	13.78 ± 1.12 ($P < 0.05$)	2.12 ± 0.30	5
10	1	18.21 ± 2.32 ($P < 0.01$)	2.44 ± 0.31	10
10	2	19.27 ± 2.08 ($P < 0.01$)	1.77 ± 0.33	5
10	4	25.55 ± 4.56 ($P < 0.01$)	3.68 ± 0.42	5

Results are means with s.e. mean.
* Student's *t* test; comparison with control values in parentheses.

vascular permeability in the skin area supplied by the nerve, as compared to the control, non-stimulated paws. Table 1 shows the amount of dye leaked when pulses of various frequencies and intensities, but constant duration (20 ms) and time of stimulation (20 min) were used.

In the following experiments, with a single exception, the parameters of stimulation were: frequency 10 Hz; intensity 1 V; duration 20 ms.

Effect of repeated electrical stimulation upon vascular permeability

The peripheral end of the nerve of one paw was stimulated for 40 minutes. This was followed by a 40 min rest period, and intravenous injection of Evans blue dye. The nerves of both paws were then stimulated for 20 minutes. The amount of dye leaked in the paw stimulated only once was 14.64 ± 1.04 μg and in the paw submitted to two periods of stimulation 4.93 ± 0.89 μg (mean with s.e. mean of 6 experiments; $P < 0.01$, Student's t test). Thus, previous stimulation of the nerve decreased the intensity of the blueing produced by a subsequent period of stimulation.

Influence of drugs on the increased vascular permeability following nerve stimulation

The following drugs had no significant effect when given before nerve stimulation: atropine (4 mg/kg, i.p.); diphenhydramine (25 mg/kg, i.p.); methysergide (2 mg/kg, i.p.); diphenhydramine (25 mg/kg, i.p.) plus methysergide (2 mg/kg, i.p.); burimamide (200 μg/kg, i.v.); burimamide (200 μg/kg, i.v.) plus diphenhydramine (25 mg/kg, i.p.); purified carboxypeptidase B (40-48 μg, i.v., 15 min before); acetylsalicylic acid (150 mg/kg, p.o., 30 and 60 min before); indomethacin (10 mg/kg, i.p., 30 and 120 min before); methyazinic acid (100 mg/kg, p.o.).

Cellulose-sulphate (3 mg/kg, i.v.), which reduces bradykininogen levels (Rothschild, 1967, 1968; Garcia Leme, Schapoval & Rocha e Silva, 1967; Eisen & Loveday, 1971), caused a partial reduction in the amount of dye leaked. The value obtained in stimulated paws of treated rats was 11.00 ± 2.13 and of untreated animals 17.04 ± 0.86 μg (mean with s.e. mean of 4 experiments; $P < 0.05$, Student's t test). Non-stimulated paws showed no significant differences. Bradykininogen determinations performed on the plasma of such animals, at the end of the electrical stimulations, showed that cellulose-sulphate reduced levels by 60-65% from 3.17 ± 0.19 μg/ml plasma to 1.19 ± 0.23 μg/ml (mean with s.e. mean of 4 experiments; $P < 0.05$, Student's t test).

As expected, when the exposed peripheral end of the saphenous nerve was immersed in lidocaine (10 ng-10 μg/ml), distally to the point of contact with the electrode, dye leakage following electrical stimulation was almost completely blocked.

Perfusion of paws and pharmacological analysis of perfusates collected before and during electrical stimulation of the saphenous nerve

Perfusions performed at 5-10° C. The subcutaneous tissue of the rat's paw was perfused and

Table 2 Amount (μg) of Evans blue dye leaked in abdominal skin areas of rats injected intradermally with 0.5 ml of perfusates collected from donor rat paws before (sample 1) and during (samples 2 and 3) electrical stimulation of the saphenous nerve (frequency 10 Hz; intensity 1 v; duration 20 ms)

Samples	*Crude* (n = 12)	*After heating* (n = 6)	*After incubation with chymotrypsin (200 μg/ml) + heating* (n = 6)	*After incubation with trypsin (200 μg/ml) + heating* (n = 6)
1	2.89 ± 0.25	3.19 ± 0.19	2.91 ± 0.32	2.69 ± 0.34
2	7.87 ± 1.22 ($P < 0.05$)*	8.60 ± 2.20 ($P < 0.05$)	8.74 ± 2.07 ($P < 0.05$)	7.88 ± 2.18 ($P < 0.05$)
3	8.65 ± 0.67 ($P < 0.05$)	7.34 ± 1.11 ($P < 0.05$)	7.67 ± 1.88 ($P < 0.05$)	8.39 ± 1.33 ($P < 0.05$)

Perfusions were performed at 5-10° C. Samples were collected in 10 min periods and tested either as obtained (crude-samples) or after heating in boiling water for 30 min or after incubation with chymotrypsin or trypsin at 37° C for 15 min followed by heating in boiling water for 30 minutes. Test rats were injected with benadryl (25 mg/kg) and methysergide (2 mg/kg i.p.) 30 min before, and with dye (20 mg/kg i.v.) 5 min before the injections of the perfusates. The skin areas of test rats were resected 20 min after the injections. n = number of experiments.

Results are mean with s.e. mean.

* Student's t test, comparison with effect of sample 1 in parentheses.

0.5 ml of three samples collected: (a) in the 10 min preceding the electrical stimulation of the nerve (sample 1); (b) in the first 10 min of stimulation (sample 2); and (c) in the second 10 min period of stimulation (sample 3). The samples were injected intradermally into the abdominal wall of test rats previously given Evans blue dye, anti-histamine and anti-5-hydroxytryptamine drugs. Dye leakage occurred in the areas injected with samples collected during stimulation of the nerve (samples 2 and 3); samples collected before stimulation (sample 1), caused practically no leakage.

The activity present in samples 2 and 3 was not affected by heating in boiling water for 30 min or by their incubation with chymotrypsin (200 μg/ml) or trypsin (200 μg/ml) at 37°C for 15 min followed by heating in boiling water for 30 min (Table 2).

The activity was reduced by dialysis. The amount of dye leaked in the abdominal skin of test rats injected with undialyzed samples 1, 2 or 3, was 3.46 ± 0.54, 12.35 ± 1.46, and 13.16 ± 1.54 μg respectively; after dialysis of these samples at 4°C for 12 h, it was 2.30 ± 0.13, 5.01 ± 0.46, and 4.12 ± 0.53 μg (means with s.e. mean of 6 experiments).

Storage below 0°C for 10 days partially reduced the activity in samples 2 and 3.

Samples 1, 2 and 3 produced no responses of the rat uterus, rat duodenum or guinea-pig ileum.

Perfusions performed at 37°C. When the perfusion fluid was heated to 37°C perfusates collected during stimulation of the nerve did not enhance permeability. However if the intensity of the electrical stimuli applied to the nerve was increased to 4 V, keeping constant the other parameters of stimulation, bradykinin-like activity appeared in samples 2 and 3. This factor contracted the rat isolated uterus and relaxed the rat isolated duodenum. These effects were not blocked by atropine or LSD in concentrations high enough to inhibit completely the responses of the preparations to the usual doses of acetylcholine or 5-hydroxytryptamine. Incubation of the samples with chymotrypsin 200 μg/ml for 15 min at 37°C abolished their activity (Figure 1). Addition to the perfusion fluid of soya-bean trypsin inhibitor or hexadimethrine bromide, which interfere with the activation of kinin-forming enzymes, prevented the appearance of the activity in the perfusates. No activity was found in perfusates from rats previously treated with cellulose sulphate intravenously. It should be noted that similar treatment with cellulose-sulphate only partially reduced dye-leakage in the paw following electrical stimulation of the nerve. Thus, the bradykinin-like activity detected with 4 V stimuli only partially contributes to the increased vascular permeability.

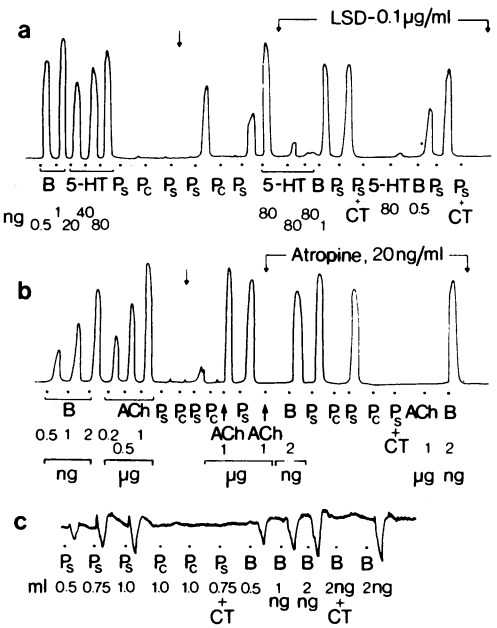

Fig. 1 Addition to the rat isolated uterus (a and b) and rat isolated duodenum (c) of perfusate samples collected from rats' paws submitted (P_s) or not (P_c) to electrical stimulation at 37°C (intensity 4 V; frequency 10 Hz; duration 20 ms) of the saphenous nerve. In a and b, P_s samples added to the left of the arrow were obtained before nerve stimulation, to the right, during nerve stimulation, and their volumes were 1 ml. In c, samples were collected during nerve stimulation. Note that no responses were observed by the addition of P_c samples during the whole assay or by the addition of P_s samples collected before stimulation. The addition of LSD or atropine to the bathing fluid completely abolished the responses to 5-hydroxytryptamine (5-HT) or acetylcholine (ACh), respectively, while it did not affect the responses to P_s or bradykinin (B). The activity in P_s disappeared after incubation (37°C) with 200 μg/ml α-chymotrypsin (P_s + CT).

On the other hand the bradykinin-like activity in perfusates collected during 4 V stimuli was not affected by previous treatment of the animals with atropine (4 mg/kg, i.p.); methysergide (2 mg/kg, i.p.); diphenhydramine (25 mg/kg, i.p.); acetylsalicylic acid (150 mg/kg, p.o.); indomethacin (10 mg/kg, i.p.); methyazinic acid (100 mg/kg, p.o.); promethazine (10 mg/kg, i.p.) or polaramine (2 mg/kg, i.p.).

Discussion

The results described in this paper confirm previous experiments by Jancsó *et al.* (1967) showing that electrical antidromic stimulation of the saphenous nerve of the rat induces increased vascular permeability in the skin area supplied by the nerve. The suggestion of these authors that a neurohumoral factor may be released in neurogenic inflammation, was examined in the present work.

Fluid perfusing the subcutaneous tissue of the rat's paw in the area supplied by the saphenous nerve contained permeability-increasing activity only during stimulation, and not before stimulation. This suggests that a factor is being released during stimulation of sensory nerves. The activity disappeared following dialysis of the perfusates, and decreased after their storage at -10°C for 10 days.

The intensity of the response observed in a paw rapidly decreased with repeated stimulation of the nerve, suggesting depletion of active material. Such rapid depletion would seem unlikely if the material originated in cells other than nerve fibres. Furthermore, responses following sensory nerve stimulation were seen only when the nerve terminals were intact (Pórszász & Jancsó, 1959; Jancsó *et al.*, 1967); desensitization of the animals by capsaicin was caused by lesions of pain receptors. They also observed that substances with a direct vascular action (histamine, 5-hydroxytryptamine) produced unchanged inflammatory effects after denervation, or in desensitized rats. In our experiments direct application of local anaesthetics to the nerve practically abolished the response.

The observed permeability factor appeared to be rapidly destroyed in the tissues, as it was only detected when perfusions were performed at 5-10°C and not at 37°C. Low temperatures may reduce the local inactivation of the factor.

It is unlikely that the permeability-increasing factor was histamine, acetylcholine or 5-hydroxytryptamine, as the response following stimulation of the nerve was not affected by previous treatment of the animals with antihistamines that block H_1 (diphenhydramine) or H_2 (burimamide; Black, Duncan, Durant, Ganellin & Parsons, 1972) receptors, singly or combined; with atropine, methysergide or methysergide plus diphenhydramine. Jancsó *et al.* (1967) also excluded the participation of these mediators in the phenomenon. Furthermore, we have always tested the perfusates in animals previously given effective doses of anti-histamine and anti-5-hydroxytryptamine drugs.

It is also unlikely that the factor is a prostaglandin as pretreatment of the animals with indomethacin or acetylsalicylic acid, which block the synthesis or release of prostaglandins (Ferreira, Moncada & Vane, 1971; Vane, 1971), did not interfere with the response to nerve stimulation.

Adenosine triphosphate (ATP) was reported by Holton (1959) to be released by antidromic stimulation of sensory nerve fibres in the skin of the rabbit's ear. However, the amounts of ATP necessary to match the vasodilator effect of stimulation were much greater than the maximum amount of ATP recovered from the perfusate after stimulation. In our experiments the permeability increasing activity could easily be recovered from the perfusates which argues against contamination with ATP.

Kinins (bradykinin, substance P) or kinin-like substances could be excluded as the activity present in the perfusates was not affected by their incubation with chymotrypsin or trypsin.

The factor was not destroyed by heating of the perfusates in boiling water for 30 minutes. It is therefore improbable that high molecular weight proteins could account for this activity.

It is possible that the vascular alterations following antidromic stimulation of sensory nerves are primarily due to the release of a new permeability factor from nerve fibres. Once initiated, the increased vascular permeability would lead to plasma exudation with activation of the kinin system in the subcutaneous tissue. The activated bradykinin-like substance(s), which was easily detected when stimuli of 4 V intensity were employed, might reinforce such vascular alterations and therefore contribute to the phenomenon. Under these circumstances kinins are mainly activated in the subcutaneous tissue, a situation similar to that occurring in 'thermic oedema' of the rat's paw (Garcia Leme *et al.*, 1970) and this may explain why cellulose-sulphate, which depletes the animals of their labile fraction of plasma bradykininogen, partially counteracts the increased vascular permeability following nerve stimulation, whereas carboxypeptidase B, intravenously injected, is ineffective. With electrical stimuli of intensity lower than 4 V, the amount of activated kinins in the subcutaneous tissue would not be enough to be detected by the usual biological tests employed.

The authors would like to thank Prof. M. Rocha e Silva for his criticism; Mr Rubens de Melo for excellent technical help; and the Conselho Nacional de Pesquisas, Brazil, for fellowships to one of us (L.H.) This work was supported in part by grants from the Fundação de Amparo à Pesquisa of the State of São Paulo (FAPESP).

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(Revised December 11, 1973)