where[1,2], rather than the internal or external laryngeal nerves.

These investigations now provide clear evidence that articular mechanoreceptor reflexes are involved in the normal co-ordination of activity in the laryngeal muscles, as we have suggested previously[1,3]. They also indicate that should abnormal situations arise in the larynx such that the pain-receptor system in the joint capsules (as well as, or apart from, that in the mucosa) be stimulated, then distorted reflex patterns of laryngeal muscular activity are provoked. Indeed, the stage has now been reached when laryngologists, phonologists and respiratory physiologists might well consider the possible role of laryngeal articular reflexes in the mechanisms of speech and respiration.

[1] Kirchner, J. A., and Wyke, B. D., *Nature*, **201**, 506 (1964).
[2] Kirchner, J. A., and Wyke, B. D., *J. Anat.* (Lond.) (in the press).
[3] Kirchner, J. A., and Wyke, B. D., *Nature*, **202**, 600 (1964).
[4] Kirchner, J. A., and Wyke, B. D., *J. Physiol.* (Lond.) (in the press).

KININASE INHIBITION BY A FLUORESCENT SUBSTANCE PREPARED FROM LIVER

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IT has been reported that a non-coagulable and fluorescent protein (polypeptide) fraction prepared from ox liver has several interesting biological properties. This fraction, which has been given the name 'urgocyton'[1], interferes with the growth of vaccinia virus *in vivo* and it gives protection in mice against infections with *Escherichia coli*[2,3]. Furthermore, it inhibits mitotic activity of human cells grown *in vitro*[4]. It also gives mice certain protection against cold stress[5]. A similar effect has also been reported for material prepared from ox blood[6]. As there is no explanation for these effects, it seemed desirable to search for significant actions of urgocyton in simple, controllable biological systems. We have thus investigated its possible relationship to the formation and inactivation of bradykinin.

In the discussions on the action of the plasma kinins and their possible biological significance, interest has been focused on the mechanisms for their formation and destruction[7]. An analysis of the conditions influencing or governing formation and destruction of these very active substances is probably essential for an evaluation of their physiological and pathological significance. Plasma kinin-forming and plasma kinin-destroying enzymatic activities have been identified in several biological fluids and tissues[7–11]. The great plasma kinin inactivating capacity of blood, of other biological fluids and of several tissue homogenates[10,11] is a striking and possibly important characteristic of such materials.

The investigations carried out with urgocyton revealed that this material caused a marked inhibition of the bradykinin-destroying activity of hæmolysed red blood cells and also, to some extent, of the bradykinin-destroying activity of plasma.

A ninhydrin-negative and fluorescent substance prepared by acid hydrolysis of urgocyton was shown to have kininase-inhibiting properties similar to those of urgocyton itself.

Methods for preparing urgocyton on a small scale. The method is a modification of one previously described[4]. One part of freshly minced ox liver was heated with 2 parts of distilled water at 90°–100° C for 30 min. When cool, the mixture was filtered and the residue washed with 1 part of water. The combined filtrates were freeze-dried and 2 parts of 70 per cent aqueous ethanol was afterwards added. The mixture was left at room temperature and filtered the next day. The *p*H of the filtrate (measured after a 4-fold dilution with water) was adjusted to 8·5 by addition of 2 N ammonia. Barium acetate dihydrate (1/200 part), dissolved in a small amount of water, was added and then ethanol up to a final concentration of 92 per cent. After storage at room temperature, the precipitate was removed by centrifugation the following day. The precipitate was dissolved in 0·1 part of water and the mixture passed through a suitable column of 'Amberlite *IR* 120 (NH_4)' to remove barium ions. The effluent was dialysed against water at +2° C for 48 h. On freeze-drying a white solid was obtained, the yield of which varied somewhat from batch to batch, but was usually about 0·05 per cent of the weight of the liver. The fluorescence of the material was measured in a Farrand fluoro-spectrophotometer. In 0·01 M phosphate buffer *p*H 7·0, it exhibited a fluorescence maximum at 440 mμ and an excitation maximum at 340–350 mμ.

Isolation of a fluorescent substance from urgocyton. 10 g of urgocyton was hydrolysed in a sealed tube at 110° C for 24 h with 6 N hydrochloric acid. The hydrolysate was concentrated to dryness in a vacuum under nitrogen and the residue dissolved in 200 ml. water (*p*H between 1–2). The solution was treated with 20 g of charcoal ('Norit') for a few hours and the charcoal filtered off and washed with water until free of chloride ions. The fluorescent substance was then eluted from the charcoal with 90 per cent aqueous phenol. The fluorescent eluant was added ether and water to displace the fluorescent material into water. After concentration of the aqueous phase in vacuum, the material was subjected to paper chromatography on Whatman 3 *MM* in butan–1–ol–ethanol (96 per cent)–aqueous ammonia (sp.gr. 0·91)–water (4 : 1 : 2 : 1, by volume). The strongly bluish fluorescent band was eluted with chloroform–methanol–aqueous ammonia (17 per cent w/v) (2 : 2 : 1, by volume) and the concentrated solution rechromatographed. The fluorescent band gave a negative ninhydrin reaction. The eluate of the band was concentrated to dryness, redissolved in water and treated with 0·6 g charcoal. The charcoal was washed with water until free of chloride ions and the fluorescent material again eluted with ethanol–water–aqueous ammonia (sp.gr. 0·91) (50 : 48 : 2, by volume). On concentration to dryness 5·5 mg of a nearly colourless substance was obtained. On Whatman No. 1 paper in butan-1-ol-ethanol–aqueous ammonia (sp.gr. 0·91)–water (4 : 1 : 2 : 1, by volume) and containing 0·2 per cent ammonium chloride, it had R_F 0·25. In thin-layer chromatography using cellulose '*MN* 300 *G*' (Macherey, Nagel and Co., Düren, Germany) as supporting medium and the above solvent system, it had an R_F of 0·49. In paper electrophoresis in a borate–potassium chloride buffer, *p*H 8·6 (500 ml. 0·2 M potassium chloride + 500 ml. 0·2 M orthoboric acid + 236 ml. 0·1 M sodium hydroxide in a total volume of 2,000 ml.), it moved 4·5 cm in 16 h towards the anode at 1·7 V/cm. In 0·01 M phosphate buffer it exhibited a

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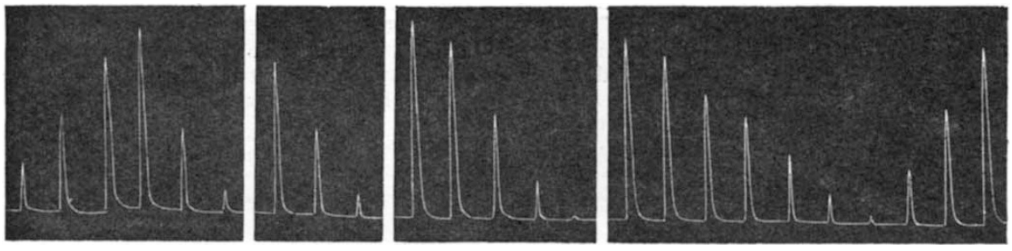

Fig. 1. Inhibition of plasma kininase by urgocyton. *A*, shows the inactivation of 500 ng of bradykinin in plasma diluted 1 : 10 with phosphate buffer. 0·9 ml. of diluted plasma was mixed with 0·1 ml. of saline containing 500 ng of synthetic bradykinin. The mixture was incubated at 37° C and aliquots of 0·1 ml. taken out after 1, 6 and 11 min and added to the rat uterus in the organ bath. Volume of organ bath 10 ml. Contact time 45 sec. In *B*, *C*, and *D* the same sort of incubation and testing was carried out, but with urgocyton present in the incubation mixtures. The final concentrations of urgocyton were: in *B*: 1 mg/ml., in *C*: 5 mg/ml. and in *D*: 10 mg/ml. Standard doses of synthetic bradykinin added before and after these tests

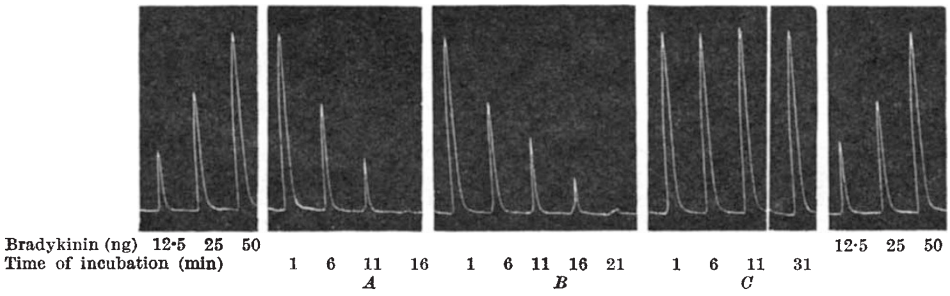

Fig. 2. Inhibition of hæmolysate kininase by urgocyton. Incubation at 37° C of 0·9 ml. of hæmolysate (see text) and 0·1 ml. of saline containing 500 ng of synthetic bradykinin. Aliquots taken out and tests carried out as described under Fig. 1. In *A* no urgocyton is present. In *B* 0·01 mg/ml. and in *C* 1 mg/ml. urgocyton in the incubation mixture

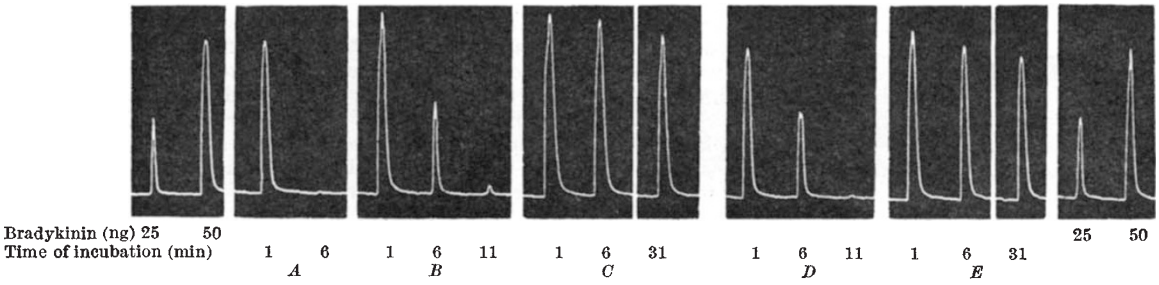

Fig. 3. Relationship between kininase strength of hæmolysate and inhibitory action of urgocyton. Incubation at 37° C of 0·9 ml. of hæmolysate with 0·1 ml. of saline containing 500 ng of synthetic bradykinin. Aliquots taken out and tests carried out as described under Fig. 1. In *A*, *B* and *C* the hæmolysate had its standard strength (see text); in *D* and *E* it was diluted 1 : 1 with phosphate buffer of *p*H 7·4. In *A* no urgocyton was present. In *B* and *E* 1 mg/ml. of urgocyton, in *C* 2 mg/ml. and in *D* 1/2 mg/ml. of urgocyton in incubation mixtures

fluorescence maximum at 430 mμ and an excitation maximum at 360 mμ.

Substrate plasma was prepared as previously described[12].

Saliva of human origin was centrifuged at 1,500 *g* for 15 min. In order to inactivate the kininase of the supernatant saliva, its *p*H was lowered to 2 for 10 min[13]. The preparation thus obtained was used undiluted.

Hæmolysate was prepared from human erythrocytes from citrated venous blood (1 vol. of 3·1 per cent aqueous solution of trisodium citrate dihydrate to 9 volumes of blood). The blood was centrifuged at 1,500 *g* for 20 min. Plasma was removed and used as a source for plasma kininase. The layer of white cells and thrombocytes on top of the erythrocytes was discarded. The remaining sediment was washed three times in saline after which it was suspended in a small volume of saline and exposed to ultrasonic treatment at 20,000 c/s for 4 min. It was then centrifuged at 1,500 *g* for 20 min. The sediment was discarded and the supernatant kept at −25° C as a stock solution for cellular kininase. This stock solution was diluted with 0·2 M phosphate buffer of *p*H 7·4 to the desired kininase activity. The standard hæmolysate dilution generally used contained about 4 mg of hæmoglobin per ml. 1 ml. of this dilution would inactivate 500 ng of bradykinin at 37° C in 6–10 min.

Plasma was prepared as already described here and kept at −25° C. Before being used, it was diluted 1 : 10 with 0·2 M phosphate buffer of *p*H 7·4. The kininase activity was then usually such that 1 ml. of the diluted plasma would break down 500 ng of bradykinin at 37° C in 10–16 min.

One g liver was homogenized in 9 ml. 0·25 M sucrose solution. The homogenate was centrifuged at 1,500 *g* for 15 min and the supernatant diluted 1 : 20 with 0·2 M phosphate buffer of *p*H 7·4. This dilution was used as a source of kininase.

Either (*a*) a rat uterus or (*b*) a guinea pig ileum preparation was used for estimating plasma kinin activity. (*a*) 0·1 ml. of the fluid to be tested was added to a virgin rat uterus suspended in aerated de Jalon solution in a 10 ml. organ bath. The contact time was 45 sec and the interval between each application was 5 min. The rats had been given intraperitoneal injections of 0·3 ml. of a 0·01 per cent aqueous solution of stilbœstrol 16–24 h before being killed.

(*b*) 0·1 ml. of the fluid to be tested was added to a piece of guinea pig ileum suspended in a 10-ml. organ bath. The contact time was 60 sec. and the interval between each application was 5 min.

The urgocyton was first tested for possible content of smooth muscle stimulating activity, of plasma kinin

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precursor, of kinin-forming enzymatic activity and of bradykinin-destroying activity.

Urgocyton, 0·4 mg/ml. in the organ bath, did not cause contractions of the rat uterus. Urgocyton itself did thus not contain any preformed plasma kinin.

A solution containing 10 mg/ml. of urgocyton and 500 ng/ml. of synthetic bradykinin in saline was prepared. Aliquots of 0·1 ml. of this solution gave the same degree of rat uterus contraction as did 0·1 ml. of a similar saline solution of bradykinin with no urgocyton added to it. This indicated that urgocyton did not interfere with the smooth muscle stimulating ability of bradykinin.

In order to test if urgocyton contained any substrate for plasma kinin formation, 0·8 ml. of a solution of urgocyton (20 mg/ml.) and 0·2 ml. undiluted human saliva was mixed and incubated at 37° C. Aliquots of the mixture were taken out at intervals up to 30 min and added to the rat uterus bath without causing any contraction of the organ. Thus urgocyton contained no substrate for plasma kinin formation.

To find out whether urgocyton possessed any plasma kinin-forming activity, 0·4 ml. of a solution of the substance (20 mg/ml.) was added to 0·6 ml. of the substrate plasma. Incubation was carried out at 37° C and aliquots of the mixture taken out and tested at intervals. No kinin formation was detected.

A mixture of urgocyton (20 mg/ml.) and synthetic bradykinin (500 ng/ml.) could be kept at 37° C for hours without any reduction in the bradykinin activity of the mixture. Thus urgocyton itself possessed no kininase activity.

Urgocyton did, however, interfere with the breakdown of synthetic bradykinin caused by the kininases of human plasma, of hæmolysed human red blood cells and of liver homogenate. The two former types of kininases were tested, because they are known to be different as regards the pattern of inhibition by other kininase inhibitors[14]. The kininase of plasma is efficiently inhibited by disodium EDTA, that of red blood cells is not. On the other hand, cobalt chloride or nickel sulphate, for example, inhibits the kininase activity of hæmolysate but not that of plasma[14].

When 500 ng of synthetic bradykinin was added to our plasma standard at 37° C, all kinin activity disappeared in about 16 min. Addition to this mixture of urgocyton to a concentration of 1 mg/ml. had no measurable effect on bradykinin inactivation. With 2·5 or 5 mg/ml. of urgocyton, however, the breakdown was retarded and with 10 mg/ml. markedly so (Fig. 1). 20 mg/ml. of urgocyton had no significantly greater effect, and the kinin breakdown in plasma could thus not be completely blocked by urgocyton.

In a freshly prepared hæmolysate standard the presence of as little as 0·01 mg/ml. of urgocyton retarded significantly the breakdown of bradykinin (500 ng/ml.) at 37° C, and with 0·1 mg per ml. of urgocyton the breakdown was markedly slowed down. One mg/ml. of urgocyton inhibited the kininase activity of the hæmolysate at 37° C completely (Fig. 2).

In order to decide whether there was a stoichiometric relationship between urgocyton and the kininase, the following experiment was carried out. The standard hæmolysate was incubated with bradykinin (final concentration 500 ng/ml.) and urgocyton (1 and 2 mg/ml. respectively) at 37° C. Urgocyton prolonged the inactivation of bradykinin markedly as shown in Fig. 3. When half as much urgocyton was present in mixtures containing half the above amount of kininase (standard hæmolysate diluted 1 : 1) the bradykinin breakdown at 37° C was inhibited to the same extent.

The low molecular fluorescent substance prepared from urgocyton by acid hydrolysis also inhibited the kininase activity of plasma as well as that of hæmolysate as shown in Figs. 4 and 5. Again the effect was most marked against the kininase of hæmolysate, where concentrations of this substance down to 100 ng/ml. had a detectable kininase inhibiting effect.

The kininase activity of a liver cell homogenate was inhibited by urgocyton as efficiently as was the kininase activity of hæmolysate (Fig. 6).

The kininase inhibiting activity of urgocyton and of the low molecular fluorescent substance prepared from it by acid hydrolysis was tested also with larger amounts of bradykinin present. Ten μg/ml. of bradykinin was incubated with the hæmolysate standard at 37° C with and without urgocyton or the low molecular substance present. After a period of 4 min aliquots of the mixture were taken out and mixed with ethanol at 80° C. The ethanol was evaporated and the sediments dissolved and tested for bradykinin activity either diluted 1 : 200 on the rat uterus or undiluted on the guinea pig ileum. Urgocyton and the low molecular fluorescent substance both retarded the inactivation of bradykinin significantly in concentrations of 0·1 mg/ml. With 1 mg urgocyton or low molecular fluorescent substance per ml. the inhibition of bradykinin breakdown was almost complete.

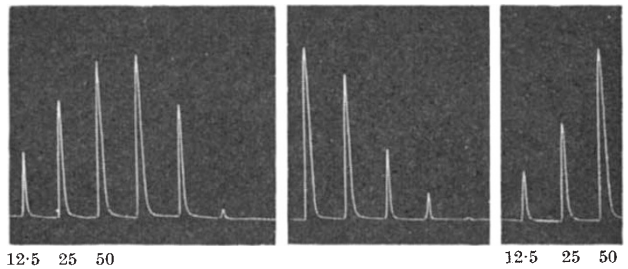

Fig. 4. Inhibition of plasma kininase by low molecular fluorescent substance. Incubation mixtures arranged and tests carried out as in the experiment of Fig. 1, the low molecular fluorescent substance being added instead of urgocyton. At *A* no such substance present in incubation mixture. At *B* 0·5 mg/ml. of the fluorescent low molecular substance in incubation mixture

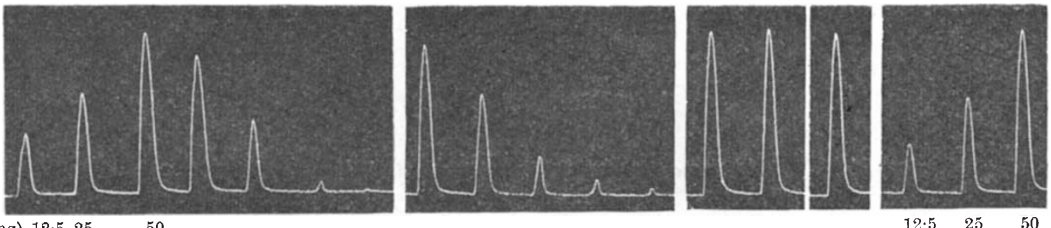

Fig. 5. Inhibition of hæmolysate kininase by low molecular fluorescent substance. Incubation mixtures arranged and tests carried out as described under Fig. 2, the low molecular fluorescent substance being added instead of urgocyton. At *A* no such substance present. At *B* 0·1 μg/ml. and at *C* 1 mg/ml. of the low molecular fluorescent substance in the incubation mixture

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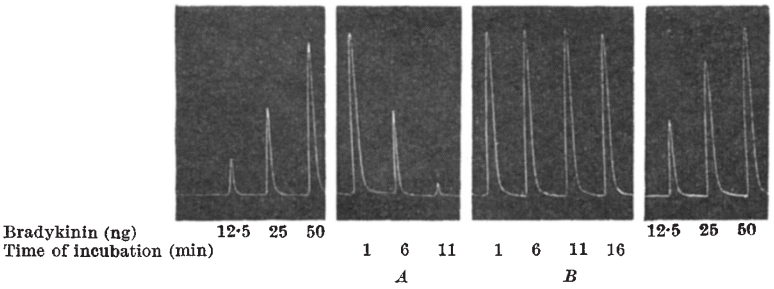

Fig. 6. Inhibition of kininase in liver cell homogenate by urgocyton. 0·9 ml. of a liver cell homogenate and 0·1 ml. of saline containing 500 ng of synthetic bradykinin incubated at 37° C. At *A* no urgocyton present. At *B* 2·5 mg/ml. of urgocyton in incubation mixture. Aliquots taken out and tests carried out as described under Fig. 1

It follows from its method of preparation that the ninhydrin-negative, fluorescent and low molecular substance is stable to acid. The experiments also suggest that it is linked covalently in urgocyton. The close similarity between the fluorescence characteristics of urgocyton and the low molecular substance suggests that urgocyton owes its fluorescence to this substance. This substance seems to be a novel one and its structure is under investigation.

The interference of urgocyton and of the fluorescent low molecular substance with kininase activity of plasma and hæmolysate makes these substances biologically interesting.

The small amount of urgocyton and of the fluorescent low molecular substance needed to influence bradykinin breakdown by hæmolysate, 10 μg/ml. and 100 ng/ml., respectively, seems to indicate that the inhibition is a specific one for the enzyme involved, although the amount of kininase present is not known. Furthermore, the experiments suggest that urgocyton combines with the kininase, thereby decreasing the rate of breakdown of bradykinin.

It is not surprising that the kininase-inhibiting ability of urgocyton is not equally marked against kininase of plasma and kininase of hæmolysed red blood cells. These two types of kininase are probably different ones, as they are known to be inhibited differently by inorganic inhibitors[14].

It has previously been suggested[3,4] that urgocyton, the fluorescent, non-coagulable protein (polypeptide) prepared from liver, may owe its interesting biological properties to a fluorescent low molecular substance being an integral part of the molecule. The fact that it has been possible to isolate, after hydrolysis of urgocyton, a substance with fluorescence characteristics similar to those of urgocyton, and which inhibits kininases as does urgocyton, lends some support to the above view.

The preparation of a fluorescent protein from ox blood[2,4], and a polypeptide from zymosan[15], with biological properties similar to those of urgocyton have previously been reported. It was suggested that the fluorescence of the liver fraction and of the materials isolated from blood and from zymosan was due to the same substance. This has partially been borne out since the low molecular fluorescent substance described in the present paper has now also been isolated after hydrolysis of polypeptides prepared from blood. In preliminary experiments the fluorescent materials prepared from ox blood or from zymosan both inhibit kininases in the same way as urgocyton.

With our present knowledge of plasma kinin-destroying enzymes in a variety of cells and tissues[10,11], the finding of a biological kininase inhibitor is very interesting. There is reason to believe that factors influencing formation and destruction of plasma kinins might change from time to time and from place to place in the organism. Balance between formation and destruction at any one time and place may determine whether a biologically active, local concentration of the kinins will develop or not. The possibility that kininase inhibiting principles might be present and active in the body must also be taken into consideration when one is analysing such a system of balance.

It is, however, too early to speculate about the possible biological role or importance of urgocyton. It remains to be shown whether the kininase inhibiting property of this substance has anything to do with its previously reported biological effects. Urgocyton should be tested, therefore, in various ways and situations, for ability to cause kininase inhibition *in vivo*.

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1 Eriksen, A., *Acta ophthal.*, Copenhagen (in the press).
2 Laland, P., Dedichen, J., Laland, S., and Thorsdalen, N., *Acta Med. Scand.*, **172**, 117 (1962).
3 Dedichen, J., Laland, P., Laland, S., and Voss, J., *Acta Med. Scand.*, **172**, 121 (1962).
4 Oftebro, R., Laland, P., Dedichen, J., Laland, S., and Thorsdalen, N., *Brit. J. Cancer*, **17**, 183 (1963).
5 Dedichen, J., Laland, P., and Laland, S. (unpublished results).
6 Dedichen, J., Laland, P., and Laland, S., *Acta Path. Microbiol. Scand.*, **58**, 219 (1963).
7 Lewis, G. P., *Physiol. Rev.*, **40**, 647 (1960).
8 Schachter, M., *Polypeptides which Affect Smooth Muscles and Blood Vessels*, 241 (London: Pergamon Press, 1960).
9 Erdös, E. G., and Sloane, E. M., *Biochem. Pharmacol.*, **11**, 585 (1962).
10 Amundsen, E., and Nustad, K., *Nature*, **201**, 1226 (1964).
11 Amundsen, E., and Nustad, K. (in preparation).
12 Amundsen, E., Nustad, K., and Waaler, B. A., *Brit. J. Pharmacol.*, **21**, 500 (1963).
13 Horton, E. W., *J. Physiol.* (Lond.), **148**, 267 (1959).
14 Erdös, E. G., Renfrew, A. G., Sloane, E. M., and Wohler, J. R., *Ann. N.Y. Acad. Sci.*, **104**, 222 (1963).
15 Laland, P., Dedichen, J., Laland, S., Oftebro, R., Thorsdalen, N., and Voss, J., *Nature*, **199**, 465 (1963).

EXCITATION OF PRIMARY MUSCLE SPINDLE ENDINGS BY β-AXON STIMULATION

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THE interest in slow muscle systems in the mammal has been heightened by the recent description of motor units in rat caudal muscles with a contraction time of 20–30 msec longer than the 15-msec contraction time of the fast units of the same muscle[1,2]. The axons innervating the slow extrafusal muscle fibres conduct action potentials with velocities between 35 and 20 m/sec, where 50–10 m/sec is the complete range for all the

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