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A DELAYED SLOW CONTRACTING EFFECT OF SERUM AND PLASMA DUE TO THE RELEASE OF A SUBSTANCE RESEMBLING KALLIDIN AND BRADYKININ

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During experiments on a delayed slow contracting effect of serum and plasma on the isolated guinea-pig ileum, it was found that this activity was not due to a substance originally present, but to one released or formed in the isolated organ bath during the test (Schachter, 1955). In the present experiments this phenomenon has been further analysed.

The incubation of Tyrode-diluted serum for 2–20 min. results in the release of a smooth-muscle stimulant; the onset and rate of contraction are therefore more rapid when the fluid bathing the test preparation is replaced by pre-diluted serum than when dilution of the serum occurs within the bath. The properties of the substance released resemble those of kallidin, which was originally called substance DK (Werle, Götze and Keppler, 1937; Werle and Grunz, 1939; Werle and Berek, 1950) and bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949; Prado, Beraldo and Rocha e Silva, 1950; Andrade, Diniz and Rocha e Silva, 1953). A provisional hypothesis is that dilution releases a smooth-muscle stimulant from serum through activation of serum kallikreinogen (see Frey, Kraut and Werle, 1950), resulting in the release of kallidin.

Most experiments were carried out with dialysed ox and guinea-pig serum, but a similar phenomenon, though quantitatively less striking, was observed with rat, dog, cat and human serum or plasma. Rabbit and hen serum either failed to exhibit this reaction or gave doubtful results.

Experiments were also carried out on the effectiveness of kallikrein (Padutin), saliva, urine, trypsin, renin, and fibrinolysin in releasing smooth-muscle stimulating agents from serum of different species. Their relative abilities to release muscle stimulating substances from different sera were not parallel. The histamine releasing agents, compound 48/80, egg white, and wasp venom, did not release kallidin or bradykinin on incubation with serum.

METHODS

Serum and Plasma.—Blood was collected in glass vessels and centrifuged at 1,400 *g* for 20 min. Serum or plasma was carefully removed from the cell layer without delay, and either tested or frozen at once in small volumes for future use. Samples were thawed at room temperature or with slight warming when required, and were still active after several months in the frozen state; thawed samples were never re-frozen.

Ox blood was obtained at slaughter and defibrinated; all other sera were obtained from clotted blood. Guinea-pigs were stunned by a blow on the head and blood collected by cutting a jugular vein. Rats, cats, dogs and rabbits were anaesthetized with intravenous or intraperitoneal pentobarbitone sodium (30 mg./kg.) and bled from a carotid artery. Human blood was obtained by venepuncture, and hen blood by cutting a vertebral artery.

When plasma was employed, blood was collected in 1% heparin-saline (1.0 ml./100 ml. blood). Serum or plasma was dialysed, when necessary, in cellophane sacs at 4° C. for 24 hr., against 10–20 volumes of saline, changed 3 times.

Isolated Smooth-muscle Test Preparations.—Isolated organ chambers (which varied in size with different preparations) were made with a constriction (2–3 mm. internal diameter) at the base, so that diffusion of added serum was restricted as much as possible to a definite volume. This precaution reduced variations in the delay and the degree of contraction following the addition of native serum to the chamber.

The stated volumes of the baths represent their fluid content in the presence of the particular test preparation, rather than the total volume; this distinction is important in determining the final concentration of serum in the muscle chamber. The fluid volumes of the chambers while containing the different preparations were approximately: 17 ml. for guinea-pig intestine or uterus, and for rat and cat uterus; 20 ml. for cat intestine; and 40 ml. for dog intestine.

Drugs and Other Agents.—Atropine and mepyramine were used as sulphate and maleate respectively; synthetic 5-hydroxytryptamine creatinine sulphate (weight ex-

pressed as base) was supplied by Abbott Laboratories and 48/80 by Burroughs Wellcome.

Renin, prepared from rabbit kidney, was kindly provided by Dr. W. S. Peart, and ox-serum bradykinin by Dr. M. Rocha e Silva. Ox-serum fibrinolysin, prepared by Dr. E. C. Loomis, was obtained through Dr. G. Ungar. Trypsin and chymotrypsin were crystalline preparations from ox pancreas (Armour). Soya bean and ovomucoid trypsin inhibitors were crystalline preparations (Worthington).

Padutin (Bayer) is a kallikrein preparation from hog pancreas preserved in 0.3% tricresol. It frequently produced a quick, rapidly relaxing contraction of the guinea-pig ileum, and the tricresol depressed the response of the ileum to various stimulants. These effects were eliminated by dialysis of Padutin (against 50–100 volumes of saline at 4° C. for 6 hr.) without affecting its ability to release kallidin. The residue, approximately doubled in volume, was made up to 4 kallikrein units/ml. with saline and used at once, or frozen in small volumes for future use.

Human mixed saliva was obtained after rinsing the mouth with saline. Cat chorda saliva was collected from the submaxillary duct during stimulation of the chorda tympani nerve under chloralose anaesthesia (80 mg./kg.).

Results

Release of a Smooth-muscle Stimulant from Serum by Dilution

Ox Serum.—The addition of 0.5–2.0 ml. dialysed ox serum to a 17 ml. bath (containing atropine and mepyramine) regularly caused a delayed, slow contraction of the guinea-pig ileum. The delay varied from 30 sec. to 3 min., and the contraction reached its peak in 2–6 min. The muscle then gradually relaxed in the presence of the diluted serum. The relaxation rate varied and was often more rapid in the presence of the greater quantities of serum, e.g., 2.0 ml. The largest contractions were obtained with the addition of 1.0–2.0 ml.; this volume of serum was optimal, since greater amounts produced smaller effects. The evidence presented below indicates that this delayed, slow contraction is due, not to a substance already present, but to its formation or release from serum when the latter is diluted in the test bath. (Dialysed ox serum also contains a pre-formed substance of the slow contracting type which becomes evident when 5.0 ml. or more of serum is tested in a 17 ml. bath.)

Diluted ox serum in concentrations of 1/35–1/10, incubated for 5–20 min. at 35° C., produced a marked contraction of the guinea-pig ileum when the bath fluid was replaced by the diluted serum. The total amount of serum in the 17 ml. bath was, as before, 0.5–2.0 ml. The contraction began in about 5 sec. and reached its maximum in about 60 sec.

The release of a smooth-muscle stimulant by dilution of ox serum with Tyrode solution was also demonstrated on isolated preparations of cat and dog intestine, and on guinea-pig, rat and cat

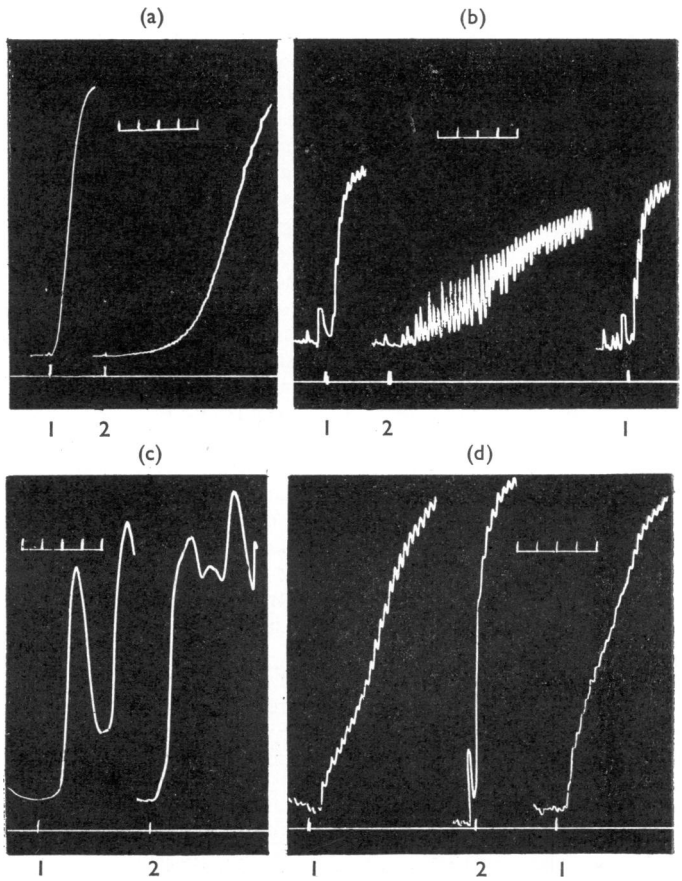

FIG. 1.—Delayed, slow contraction produced by dialysed ox serum; more rapid onset and rate of contraction if serum is diluted and incubated before testing. Bath temp. 35° C., except rat uterus, 28° C. Atropine (0.01 μg./ml.) and mepyramine (0.02 μg./ml.), present throughout. Serum was diluted with Mg-free Tyrode solution and incubated for 6 min. at 35° C. (for rat uterus at 28° C.). Time, 30 sec. (a) Guinea-pig ileum, 17 ml. bath. 1, 17 ml. diluted (0.5/17), incubated serum; 2, 0.5 ml. serum. (b) Dog small intestine, 40 ml. bath. 1, 40 ml. diluted (2/40), incubated serum; 2, 2 ml. serum. (c) Non-oestrous rat uterus, 17 ml. bath. 1, 0.5 ml. serum; 2, 17 ml. diluted (0.5/17), incubated ox serum. (d) Cat small intestine, 20 ml. bath. 1, 1 ml. serum; 2 20 ml. diluted (1/20), incubated serum.

uterus (Fig. 1). Rat uterus and cat intestine were particularly sensitive preparations.

Guinea-pig Serum.—A similar phenomenon, with quantitative differences, was observed with guinea-pig serum or plasma. Thus, guinea-pig serum produced a delayed, slow contraction of the guinea-pig ileum, but the delay of contraction was less than with ox serum, and the effect was usually optimal when only 0.2–0.25 ml. was added to the 17 ml. bath (Fig. 2). Also, as described later, the active substance in guinea-pig serum was released, and also inactivated more rapidly, than that of ox serum; diluted guinea-pig serum was therefore incubated for only 1–5 min. before testing. As with ox serum, this phenomenon was demonstrated with various smooth-muscle preparations.

Other Sera.—Rat, dog, cat, and human serum or plasma also released a smooth-muscle stimulant on dilution. These sera were usually dialysed, since the amounts tested often contained considerable quantities of 5-hydroxytryptamine or other quick contracting agents. The active agent was usually released when serum was incubated for 5–15 min. at the following concentrations: man 1/40–1/20, rat 1/10, dog and cat 1/50–1/15. Since only a limited number of observations was made with these sera, the concentrations found effective are not necessarily optimal. Human and cat serum did not produce effects as pronounced, or as regular after dilution, as did ox and guinea-pig serum; but an effect was usually detectable and was occasionally marked (cf., Fig. 4). With human and cat serum the release could usually be demonstrated by testing diluted, incubated (5–10 min.) serum, even if it was slight or undetectable when native serum was diluted in the test bath itself.

Rabbit and hen serum failed to show definite evidence of release of a smooth-muscle stimulant on dilution. Hen serum contained large amounts (approximately 1.0 μg./ml.) of a substance resembling 5-hydroxytryptamine (see Erspamer and Sala, 1954).

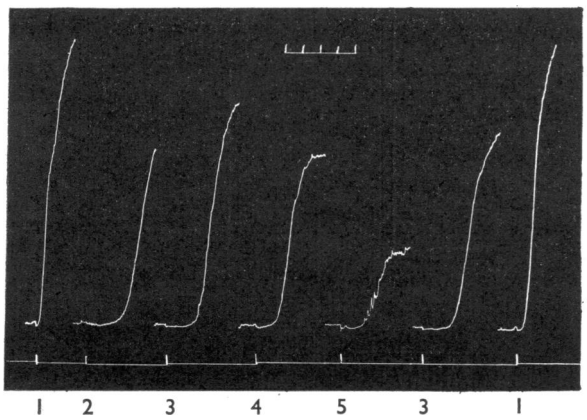

FIG. 2.—Delayed, slow contraction of the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by guinea-pig serum; more rapid onset and rate of contraction with serum diluted and incubated before testing. Time, 30 sec. 1, 17 ml. diluted (0.2/17) serum, incubated 2 min.; 2, 0.1 ml. serum; 3, 0.2 ml. serum; 4, 0.5 ml. serum; 5, 0.8 ml. serum.

Properties of the Smooth-muscle Stimulant Released by Dilution of Serum

The properties of the smooth-muscle stimulant released by dilution were mostly studied with ox and guinea-pig serum. The effect was not significantly reduced when the serum was additionally centrifuged at 17,000 *g* for 20 min.; the active substance must therefore be derived from serum and not from blood cells. It is readily distinguishable from acetylcholine, histamine, and 5-hydroxytryptamine both by a slower rate of contraction and subsequent relaxation of the ileum, and by the fact that the contraction is unaffected by atropine, by mepyramine, or by 5-hydroxytryptamine desensitization. It also differs from serum derivatives with smooth-muscle stimulating properties such as the kallikreins, which do not stimulate the guinea-pig intestine or uterus (see Werle, 1955), and from anaphylatoxin, which rapidly desensitizes the test preparation (Rothschild and Rocha e Silva, 1954). It does, however, share many properties with bradykinin and kallidin, both of which are derived from an α_2 serum globulin (van Arman, 1952, 1955; Werle, 1953, 1955).

In addition to producing a slow contraction of the guinea-pig ileum, like kallidin, it contracted the isolated intestine of the cat and dog, and the uterus of the guinea-pig, rat, and cat (Fig. 1). Also, its action on all these test objects paralleled that of a preparation of bradykinin both qualitatively and quantitatively. The slow contractor released by diluted (1/75) guinea-pig serum was usually inactivated in 15–30 min. at 35° C., although sometimes it took longer. A 1/35 dilution of ox serum became inactive in 60–90 min. It is of interest in this connexion that, of all mammalian sera studied, guinea-pig serum is the most effective in inactivating kallidin (Werle and Hambuechen, 1943). The addition of chymotrypsin to diluted serum markedly increased the rate of inactivation of the active substance (Fig. 5). Chymotrypsin and serum also quickly inactivate kallidin and bradykinin (Werle, Kehl, and Koebke, 1950; Rocha e Silva, 1951).

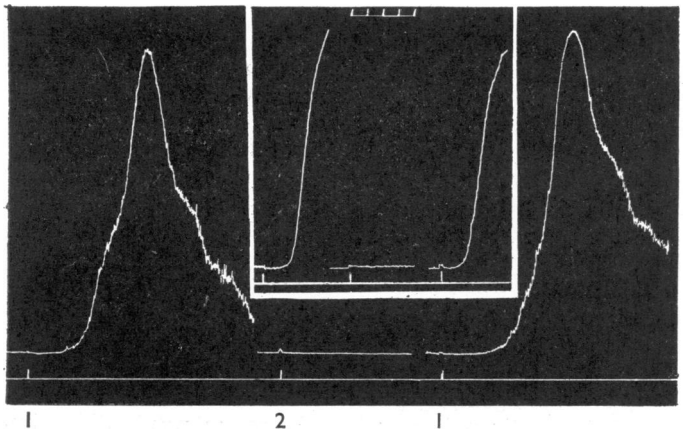

FIG. 3.—Delayed, slow contraction of isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by ox or guinea-pig serum, and inhibition of this effect by STI. Time, 30 sec. Main figure: 1, 2 ml. dialysed ox serum; 2, 2 ml. dialysed ox serum previously incubated (35° C.) for 1 min. with 0.4 mg. (0.4 ml.) STI. Inset figure: at first and last signals, 0.2 ml guinea-pig serum; at intervening signal, 0.2 ml. guinea-pig serum, but 0.4 mg. (0.4 ml.) STI present in bath.

Inhibition of Release of the Smooth-muscle Stimulant by Soya Bean Trypsin Inhibitor (STI)

Incubation of 0.5–2.0 ml. ox serum, or 0.20–0.25 ml. guinea-pig serum with 0.2–0.5 mg. STI (1 mg./ml.) for 1 min., regularly prevented the release of the smooth-muscle stimulant on subsequent dilution of the serum (Fig. 3). The inhibition of release was equally effective if the sera were diluted with 17 ml. Tyrode solution containing 0.2–0.5 mg. STI, i.e., 12–30 μg./ml. Double this concentration of ovomucoid inhibitor (OI) was ineffective. The addition of STI to diluted ox or guinea-pig serum which had already been incubated did not abolish the action of the released smooth-muscle stimulant.

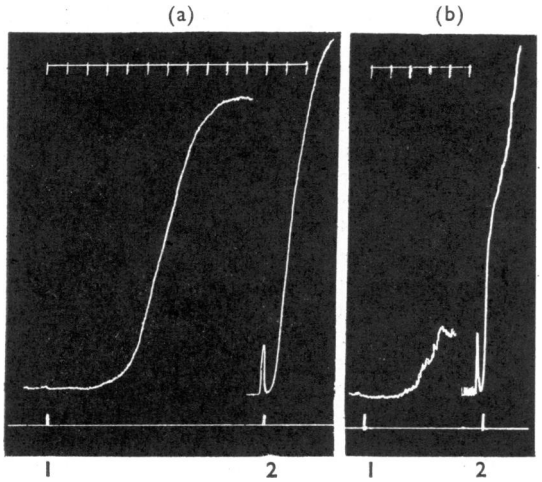

FIG. 4.—Delayed, slow contraction of isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by dialysed human and cat plasma; more rapid onset and rate of contraction with plasmas diluted with Tyrode solution and incubated (5 min., 35° C.) before testing. Time, 30 sec. (a) 1, 0.4 ml. human plasma; 2, 17 ml. diluted (0.4/17), incubated human plasma. (b) 1, 0.25 ml. cat plasma; 2, 17 ml. diluted (0.25/17), incubated cat plasma.

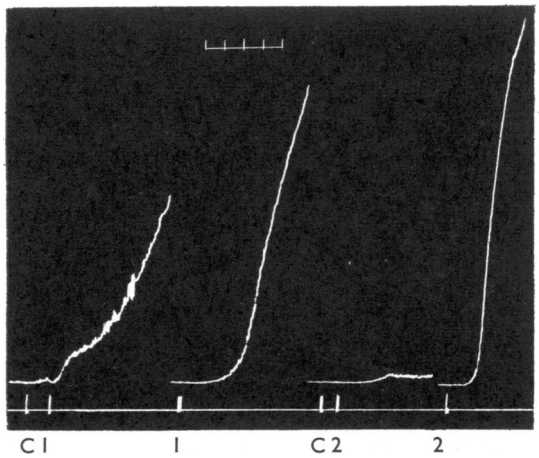

FIG. 5.—Reduced effect of ox and guinea-pig serum on the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) in the presence of chymotrypsin. Continuous tracing indicates bath not washed out. Time, 30 sec. C, 0.4 mg. (0.4 ml.) chymotrypsin. 1, 1 ml. dialysed ox serum. 2, 0.2 ml. guinea-pig serum.

However, the addition of STI to diluted guinea-pig serum, previously incubated for a few minutes, did substantially reduce the activity; with diluted ox serum the contraction was either unaffected or only slightly reduced. If, as suggested, dilution activates an agent (sensitive to STI) which releases a smooth-muscle stimulating polypeptide from a serum substrate, then the amount of active polypeptide present at any time would be the resultant of its rate of release and simultaneous inactivation by serum peptidase. Thus, if STI arrested the progressive release, but not the inactivation of the active substance, then a reduced effect, as observed, might be expected, particularly with guinea-pig serum where inactivation is more rapid. The above

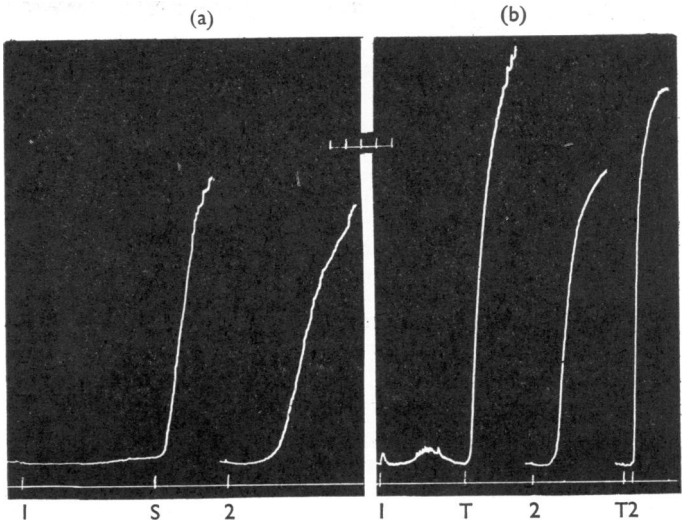

FIG. 6.—Ox or guinea-pig serum heated to 56° C. for 3 hr. fails to produce a delayed slow contraction of the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present); also, trypsin releases as much bradykinin from heated as from unheated guinea-pig serum. Continuous tracing indicates bath not washed out. Time 30 sec. Intestine desensitized to trypsin between (a) and (b). (a) 1, 0.5 ml. heated, dialysed ox serum; S, 0.2 ml. human saliva; 2, 0.5 ml. dialysed ox serum. (b) 1, 0.25 ml. heated guinea-pig serum; T, 0.3 mg. (0.3 ml.) trypsin; 2, 0.25 ml. guinea-pig serum.

facts, therefore, are not necessarily in disagreement with the hypothesis presented.

The concentration of STI which regularly prevented release of the muscle stimulant by dilution of ox or guinea-pig serum was only occasionally completely effective with human serum, although the effect was always reduced. STI only slightly reduced the release from cat serum. Thus, the relative effectiveness of STI in inhibiting the release of the active substance varies with different sera.

Failure to Release a Smooth-muscle Stimulant by Dilution of Ox and Guinea-pig Serum after Destruction of Kallikreinogen

Since kallikreinogen (kallikrein plus its inactivator) is destroyed by heating serum for 3 hr. at 56° C. (Werle *et al.*, 1937), it was of interest to see whether this treatment affected the release of the kallidin-like substance by dilution. It was indeed observed that ox and guinea-pig serum, heated in this way, released little or no smooth-muscle stimulant when subsequently diluted; such heated serum, none the less, released as much kallidin or bradykinin as unheated serum upon addition of saliva or trypsin (Fig. 6), indicating that heating in this way leaves kallidinogen and bradykininogen unaffected. That kallidinogen remains unaffected by this degree of heating was long ago noted by Werle *et al.* (1937).

It was also of interest to test whether serum exhausted of kallidinogen and bradykininogen would still release a kallidin-like substance on dilution. Several types of experiments were performed to test this possibility. Thus, the incubation of 0.25 ml. guinea-pig serum with 0.25–0.3 mg. trypsin (0.25–0.3 ml.) released large amounts of bradykinin which was inactivated, presumably by peptidase in serum, after about 60 min. at 35° C. This serum now released little or no kallidin on addition of Padutin (1 kallikrein unit in 0.25 ml.), or on dilution. The addition of 0.3 mg. renin (0.3 ml.), however, still released moderate amounts of a slow-contracting substance from the mixture, although the amount released was less than from untreated serum. Conversely, optimally diluted guinea-pig serum, after incubation for 10–60 min., released very little kallidin or bradykinin (by Padutin or trypsin), but the subsequent addition of renin was still moderately effective (Fig. 7).

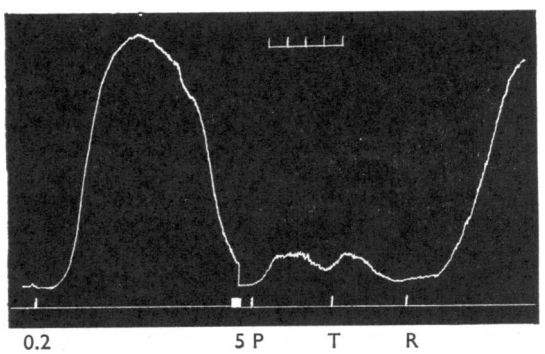

FIG. 7.—After the smooth-muscle stimulant is released by dilution, and inactivated in guinea-pig serum, the release of kallidin and bradykinin by Padutin and trypsin respectively is much reduced; but renin still releases a slow contractor. The longer delay required for renin to release a slow contractor is characteristic. Isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present). Continuous tracing indicates bath not washed out. Time, 30 sec. At 0.2, 0.2 ml. dialysed guinea-pig serum; at 5, kymograph stopped for 5 min. P, Padutin (0.25 ml., 1 kallikrein unit); T, 0.3 mg. trypsin (0.3 ml.); R, 0.3 mg. renin (0.3 ml.).

Incubation of 0.5 ml. diluted ox serum for long periods, unlike guinea-pig serum, still released large amounts of kallidin on subsequent addition of saliva. Similarly, saliva still released large amounts of kallidin from diluted human serum. This is perhaps because the kallidinogen and bradykininogen of guinea-pig serum are largely exhausted by optimal dilution, whereas those of ox and human serum are only reduced.

Relative Effectiveness of Padutin, Trypsin, Fibrinolysin, Renin, Saliva and Urine, in Releasing Smooth-muscle Stimulants from Different Mammalian Sera

During the above experiments it was observed that various agents releasing kallidin or bradykinin possessed this ability to a strikingly different degree with different sera as substrates (Fig. 8). For example, Padutin was a potent releasing agent from guinea-pig serum, but completely ineffective, under the same conditions, with human, cat, or rabbit serum; on the other hand, human mixed saliva, and particularly cat chorda saliva, were potent releasing agents with human, cat, and rabbit serum, but ineffective with guinea-pig serum. These results indicate a degree of species specificity of kallikrein and renin for their substrates—kallidinogen and hypertensinogen (Table I). Relatively high concentrations of a purified bovine fibrinolysin preparation (5 mg./ml.) were required to release significants amounts of a smooth-muscle stimulant from ox, human, and guinea-pig serum.

STI and OI were also tested for their ability to prevent release of the smooth-muscle stimulants from serum by these various agents. The releasing abilities of human saliva and of urine were unaffected by incubation with STI or OI (1.0 mg./ml.) for 1–2 min. The active releasing agents in these instances are therefore, in all probability, the kallikreins of saliva and urine, respectively (for literature on kallikrein in saliva, see Werle and von Roden, 1936; Ungar and Parrot, 1936; Guimarais and Tavares, 1942; Werle and Maier, 1952). The releasing ability of fibrinolysin was completely inhibited by STI (0.5 mg./ml.), but unaffected by the same amount of OI. The sensitivity of fibrinolysin to inhibitors is therefore similar to that of the serum kallikrein prepared by the acetone method (Werle and Maier, 1952).

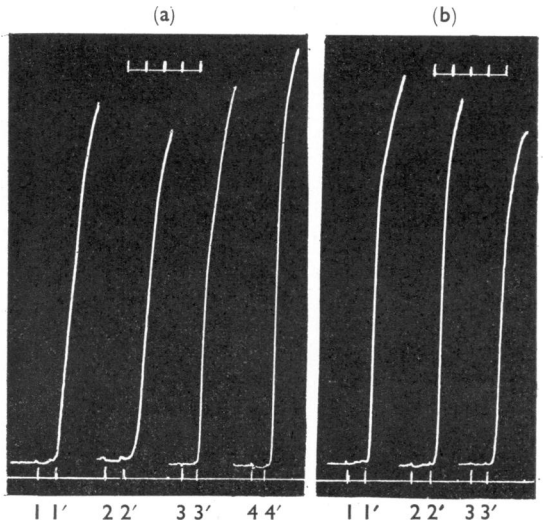

FIG. 8.—Difference in relative ability of Padutin, saliva and urine in releasing a kallidin- or bradykinin-like substance from serum of different species; also, failure of wasp venom, egg white, and 48/80 to release a smooth-muscle stimulant from human serum. Isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present). Dialysed serum or plasma (0.7 ml.) incubated with various agents at 35° C. for 2 min. Continuous tracing indicates bath not washed out. Time, 30 sec. (a) 1, guinea-pig serum+0.5 ml. human urine; 1′, Padutin (1 kallikrein unit); 2, ox serum+0.5 ml. human urine; 2′, 0.2 ml. human saliva; 3, guinea-pig serum+0.5 ml. guinea-pig urine; 3′, Padutin (1 unit); 4, cat plasma+Padutin (1 unit); 4′, 0.1 ml. cat chorda saliva. (b) 1, human serum+0.2 mg. (0.3 ml.) dialysed wasp venom; 1′, 0.2 ml. human saliva; 2, human serum+10% hen egg white (0.3 ml.); 2′, 0.1 ml. cat chorda saliva; 3, human plasma+0.03 mg. 48/80 (0.3 ml.); 3′, 0.5 ml. human urine.

TABLE I

RELATIVE ABILITY OF VARIOUS AGENTS TO RELEASE A SMOOTH-MUSCLE STIMULANT FROM DIFFERENT MAMMALIAN SERA AFTER 2 MIN. INCUBATION AT 35° C.

Releasing Agent and Its Source	Serum (0·7 ml. Dialysed)				
	Ox	Guinea-pig	Man	Cat	Rabbit
Padutin—hog pancreas (1.0 kallikrein unit; 0.25 ml.)	+	++++	−	−	−
Trypsin—ox pancreas (0.3 mg., 0.3 ml.)	+	++++	++	n.t.	n.t.
Renin—rabbit kidney (0.3 mg., 0.3 ml.)	++++	++++	−	+++	++++
Saliva—man (0.2 ml.)	++++	−	++++	++	++
Saliva—cat, chorda (0.1 ml.)	++++	−	++++	++++	++++
Urine—man (0.5 ml.)	−	−	++++	−	n.t.
Urine—guinea-pig (0.5 ml.)	−	−	−	−	−

+ to ++++, relative amount of smooth-muscle stimulant released; −, no release detected; n.t., not tested.

The observation that the administration of histamine liberators to animals results in the appearance of a slow-contracting substance in plasma (Paton, 1951; Jaques and Schachter, 1954) suggested the possibility that these agents might release such a substance from serum. Compound 48/80 (Paton, 1951), egg white (Schachter and

Talesnik, 1952) and wasp venom (Jaques and Schachter, 1954), however, all failed to release kallidin or bradykinin on incubation with human serum (Fig. 8).

Discussion

The present results are of interest from a practical standpoint, since they indicate the possibility of a potent smooth-muscle stimulant being formed under conditions which exist in many biological assays. The possibility that errors of interpretation may occur for this reason during assay of different body fluids, as well as with plasma or serum, must be considered.

The process whereby dilution releases a smooth-muscle stimulant from serum is of considerable interest from a theoretical point of view. It is not surprising that new properties of serum result from dilution, since dilution is known to dissociate large molecular complexes such as trypsin-antitrypsin (Hussey and Northrop, 1923) and toxin-antitoxin (Glenny and Barr, 1932). Dilution of serum also activates profibrinolysin (MacFarlane and Pilling, 1946); results in the appearance of substances in guinea-pig serum which increase capillary permeability, and "bind" small amounts of histamine (MacKay, Miles, Schachter and Wilhelm, 1953; Miles and Wilhelm, 1955); and produces an anaphylatoxin in rat serum (Rothschild and Rocha e Silva, 1954). The observation that human serum releases a substance resembling bradykinin on first contact with glass (Armstrong, Keele, Jepson and Stewart, 1954) is of particular interest. Again, contact with glass has been reported to activate (independently of platelet disruption) many of the large molecular serum clotting factors, e.g., a thromboplastin precursor, prothrombinogen, and proconvertin (see Rapaport, Aas and Owren, 1955). Further work is desirable to determine whether the similar smooth-muscle stimulants released from serum on first contact with glass, and by dilution, are end-results of the same or different mechanisms.

How does dilution of serum or plasma result in the release of a substance resembling kallidin and bradykinin? The present observations do not justify more than a speculative interpretation based on the observations described and on the properties of known substances and their precursors in serum. Of the known agents derived from serum, and capable of releasing a smooth-muscle stimulant similar to the one released by dilution, the serum kallikreins stand out as likely possibilities, particularly kallikrein, prepared by the acetone method, whose kallidin releasing action is also sensitive to STI but not to OI (Werle and Maier, 1952). Furthermore, the release of a kallidin-like substance by dilution failed to occur when ox or guinea-pig serum was first heated for 3 hr. at 56° C.; kallikreinogen, the inactive precursor of kallikrein, is likewise destroyed by this treatment (Werle *et al.*, 1937). Also, guinea-pig serum exhausted of kallidinogen by Padutin (or of bradykininogen by trypsin) failed to release a smooth-muscle stimulant on subsequent dilution. The properties of the latter agent correspond to those of kallidin and bradykinin in its instability in serum, susceptibility to chymotrypsin, and action on various test preparations. All the above observations are therefore consistent with the hypothesis that dilution of serum releases kallidin through the activation of kallikreinogen. Purified kallikrein preparations release detectable amounts of kallidin when only 0.003 μg. is incubated with serum (Werle *et al.*, 1937). This order of activity is about a thousandfold greater than observed in the present experiments for the bradykinin-releasing activity of crystalline trypsin, and far greater still than for a purified preparation of fibrinolysin. Also, in the present experiments, as little as 0.01 ml. cat chorda saliva, most probably through the action of salivary kallikrein, released detectable amounts of a kallidin-like substance from cat serum.

Thus, the dilution of serum might simply dissociate the kallikrein-inactivator complex, or it might activate serum proteases (like fibrinolysin), which in turn might release kallikrein. It has, in fact, been shown recently that trypsin releases kallikrein from its inactive complex in serum (Werle, Forell, and Maier, 1955). The possibility also arises, therefore, that the release of bradykinin from serum by trypsin is entirely due to such an activation. This is not so, however, since in the present experiments trypsin released as much bradykinin from heated serum (which destroyed kallikreinogen) as it did from unheated serum.

The fact that exhaustion of kallidinogen of guinea-pig serum by Padutin (or of bradykininogen by trypsin) abolished the subsequent release of a kallidin-like substance by dilution of the same serum suggests that the smooth-muscle stimulants are released from a common substrate in these instances; however, since renin released moderate amounts of a slow contracting substance from serum previously exposed to all the above procedures, it would appear that hypertensinogen is either distinct from kallidinogen and bradykininogen, or the same protein, but split at a different site. These conclusions regarding the specificity of substrates are, however, made with reservation, since the possibility cannot be excluded that the activities of fibrinolysin

and renin are to some degree due to contamination with kallikrein, or those of Padutin to contamination with trypsin. A definite answer to this question can only be obtained by further experiments with highly purified releasing agents and their serum substrates.

Summary

1. Dilution of ox, guinea-pig, rat, dog, cat and human serum or plasma releases a smooth-muscle stimulating agent resembling kallidin and bradykinin. The optimal dilution, the amount of muscle stimulant released, and its rate of inactivation vary with the sera of different species. The release of this substance is demonstrable when dilution occurs in the test bath, but the effect is more striking if diluted serum is incubated for a definite period and then tested.

2. The release of the kallidin-like substance by dilution of ox or guinea-pig serum is greatly reduced or abolished: (a) in the presence of soya bean trypsin inhibitor, (b) if serum is heated before dilution at 56° C. for 3 hr. to destroy kallikreinogen.

3. The smooth-muscle stimulant released by dilution resembles kallidin and bradykinin in that: (a) it contracts, in a characteristic way, the guinea-pig, cat, and dog intestine, and the guinea-pig, rat, and cat uterus ; (b) it is inactivated by serum and by chymotrypsin.

4. A comparison of the relative ability of various agents which release smooth-muscle stimulants from serum showed that this property was not parallel with sera of different species. The results indicate a degree of species specificity of kallikrein and renin for their serum substrates.

5. Rabbit and hen serum failed to release a smooth-muscle stimulant on dilution. Compound 48/80, egg white, and wasp venom did not release kallidin or bradykinin from serum.

6. The hypothesis is suggested that dilution of serum releases kallidin through activation of kallikreinogen.

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