Blood-Borne Factors Affecting Vascular Tone

Arterial vessels normally display a state of tone, by which is meant they exhibit a state of partial contraction intermediate between the fully relaxed and the maximally contracted artery. Vascular tone can also be characterized by viscoelastic properties of the partially contracted artery; these values are intermediate between those of the fully relaxed and the maximally contracted artery[1].

It has long been postulated that vascular tone is maintained in part by vasoactive substances dissolved in circulating plasma. Battelli[2] introduced the term 'vasoconstrictine' for what he believed to be the contracting principle of plasma. It is now generally agreed that plasma contains several substances that can contract arterial smooth muscle. Some of these are 'effective vasoconstrictors'[3], that is under physiological conditions they exist in blood at a concentration sufficient to elicit a contraction, and therefore, may contribute to the overall effect of vascular tone. Wurzel and Zweifach (1964) found serotonin to be the prinicipal effective contractor substance in the blood of rabbits. Histamine was present in concentrations which had only a modest contracting effect and was reinforced by a reciprocal potentiating action of serotonin. It is commonly assumed that plasma catecholamines – norepinephrine (NOR) and epinephrine (EPI) – are 'effective' vasotonins. In rabbit plasma, however, they were never found in concentrations sufficient to contract vascular smooth muscle in vitro.

A number of observations made since 1962 have led us to believe that along with serotonin, blood contains a so-far unidentified dialysable substance, temporarily referred to as SVPx[4]. Thus, concentrates of a protein-free dialysate of plasma uniformly induced maximal contraction of the aortic strip, whereas serotonis had only a partial effect. An intracutaneous injection of SVPx, but not of synthetic serotonin, produced a necrotic area in the rabbit skin. Furthermore, topical application of SVPx on the mesentery of the rabbit and of the rat resulted in a powerful and prolonged vasoconstriction, while serotonin produced only a limited narrowing of venules.

In the present paper additional supportive evidence is given for the occurrence of SVPx, (Substance for Vasoconstriction from Plasma) by use of DBMC, a selective antagonist of serotonin (Dombro and Woolley[5]), and of mepyramine (MEP), an antihistaminic. We have subsequently succeeded in separating SVPx from serotonin by paperchromatography.

Methods and materials. Rabbits were used because their plasma contains fairly large measurable amounts of contracting substances. The isolated rabbit aortic strip, a representative of large arteries, was used both as an index of the sensitivity of a large artery, and for assay of contractor substances.

The rabbit aortic strip was prepared according to Furchgott and Bhadrakom with a modification to permit convenient application of single doses (see [3]). The excised spirally cut aortic strip was suspended in Krebs-HCO_3-Ringer solution at 37 °C, and aerated with a gas mixture of 5% CO_2 and 95% O_2. Isotonic contractions were obtained to single doses of norepinephrine (NOR), serotonin, or plasma dialysate applied for 3 min. The tissue was preincubated with antagonists for 4 min, and then the agonists were added for 3 min[6].

In experiments in which direct visualization of the microcirculation was carried out (see introduction), the tissues were exteriorized and kept at body temperature and moist by a well-established technique[7]. The effects of SVPx preparations on the permeability of skin vessels (see introduction) were studied by the technique of Miles and Wilhelm[8]. Each of the experiments was repeated 4–5 times.

Results. (1) *Contracting potency of 1:1 plasma dialysate.* Rabbit blood was rapidly cooled after collection by way of a polyethylene cannula in the carotid artery into plastic

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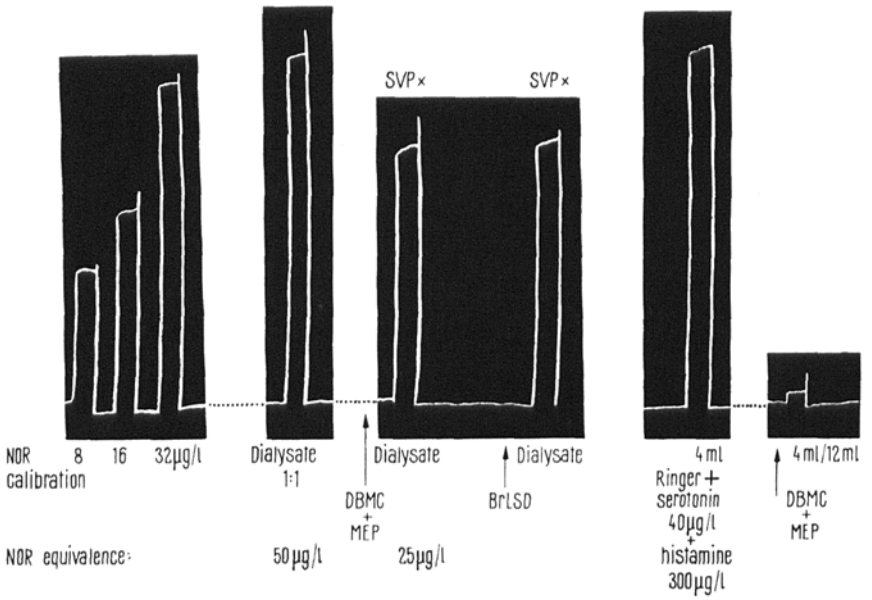

Fig. 1. Evidence for the occurrence in plasma of SVPx, an unidentified contracting substance. On the left: plasma dialysate, 1:1, has a contracting potency equivalent with that induced by 50 µg/l norepinephrine bitartrate monohydrate (NOR), and therefore, undiluted plasma should be equivalent to 50 µg/l NOR-base. In the middle: a contraction induced by plasma dialysate can only partially be antagonized by 2 selective antiserotonin compounds. The remaining contracting potency is equivalent to that of 25 µg/l NOR-base. On the right: a contraction induced by Ringer supplemented with the physiological concentration of serotonin and histamine (and NOR-EPI as well) is completely antagonized by a mixture of DBMCand mepyramine.

bags (Transpak from Abbott Labs.) containing heparin 20 U/ml final concentration, agitated in a mixture of ice and water. Plasma obtained by centrifugation at 10,000 *g* was dialyzed against an equal volume of Krebs-HCO_3-Ringer to allow diffusible vasoconstrictors in the plasma to equilibrate with the dialysate – thus, each of the 2 solutions contained at equilibrium one half the total diffusible materials. Aliquots of the dialysate preheated to 37 °C were used undiluted on an isolated aortic strip. As seen in Figure 1 the aortic strip contracted. The extent of this contraction exceeded 50% of the maximal contraction. In this type of experiment the equivalent concentration of NOR-EPI needed to produce a contraction of the same extent is 50 µg/l.

(2) *Fluorimetric determination of catecholamines in rabbit plasma.* In a previous study using the method of VON EULER and FLODING we found less than 1 µg/l (see [3]). In the present study we preferred an adaptation of the fluorimetric method of ANTON and SAYRE[9] using a large volume of plasma dialysate as the starting material and treating several 30 ml portions of dialysate with the same sample of alumina. The values obtained were 2.5–3 µg/l. As indicated in Figure 1 it is obvious that NOR-EPI are not present in sufficient concentration to account for the contracting activity of plasma which is equivalent to as much as 50 µg/l.

(3) *Fluorimetric determination of rabbit plasma serotonin.* Similar measurements for serotonin were also performed with 100 ml of starting material, which had been reduced to a small volume by flash evaporation and was then subjected to the procedure of UDENFRIEND et al.[10]. The findings are in fairly good agreement with JOHANSSON's data (1960) (see also [3]), since we found plasma to contain approximately 40 µg/l.

(4) *Evidence for a vasoconstrictor substance in rabbit plasma in addition to serotonin and histamine.* Krebs-HCO_3-Ringer was supplemented with serotonin, histamine, and catecholamines at concentrations which we believe to exist in plasma (results 2, 3, and [3]). An antiserotonin agent synthesized by DOMBRO and WOOLLEY[5], DBMC, and an antihistaminic, mepyramine (MEP), were applied on the aortic strip at an appropriate concentration to antagonize each of these principles successfully[6]. When plasma dialysate was the stimulus, only part of the contraction was antagonized (Figure 1). The remaining activity was equivalent to 25 µg/l (Figure 1). The above data indicate clearly that plasma dialysate contains an unidentified substance (SVPx) exclusive of NOR-EPI, serotonin, and histamine.

(5) *SVPx, unlike serotonin, is not stored and released from blood cells.* The unidentified constrictor principle does not appear in substantial amounts when the cellular elements of blood are disrupted by freezing-and-thawing (see Figure 2). Heparinized blood, 0.8 U/ml, was incubated at 37 °C, and aliquots were taken at 10 min intervals for assay in the absence and in the presence of DBMC and MEP antagonists. It can be seen that while serotonin was gradually released with time in large amount (see

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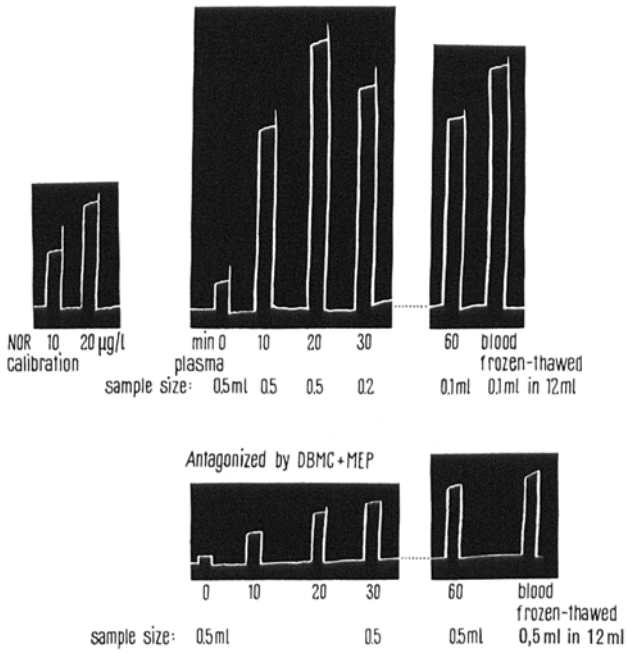

Fig. 2. Release of contractor substances into plasma upon incubation of 0.8 U/ml heparinized blood at 37 °C. Upper half: aliquots of blood taken at 10 min intervals showed gradually higher potency. At the extreme right, plasma of non-incubated but frozen-and-thawed blood induced a contraction only slightly higher than plasma of blood incubated for 60 min. Lower half: contractions induced by all samples were substantially antagonized by DBMC with MEP indicating that the concentration of the unidentified substance, in contrast to that of serotonin, has not changed substantially during incubation or upon freezing-and-thawing the blood.

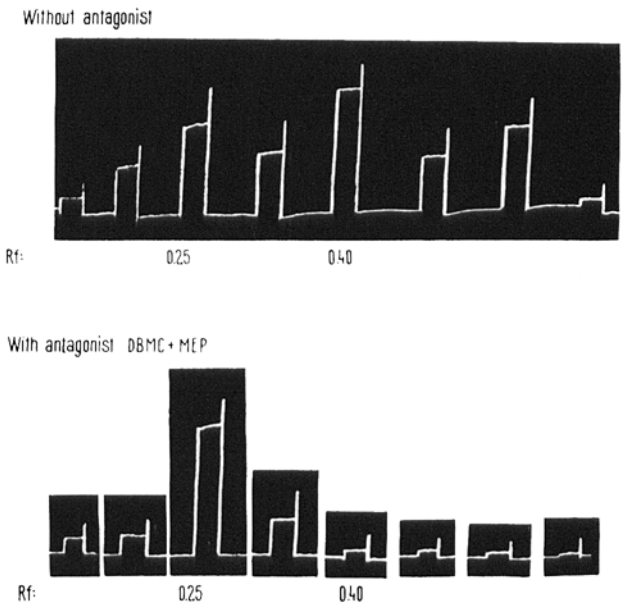

Fig. 3. Separation of the unidentified contracting substance of plasma from serotonin on a paperchromatogram. 400 ml plasma dialysate, 1:1, was concentrated to 2 ml on a rotary evaporator, and layered on 2 sheets of Whatman 4 paper along a line of origin. The solvent system was *n*-butanol:pyridine:water (1:1:1), and its front ran for 14 inches (12 h). Contractor substances contained in strips of paper 1 inch wide were eluted in Krebs-HCO_3-Ringer solution, and assayed on a rabbit aortic strip. The effect of the unidentified substance, at Rf 0.25, was not diminished by antagonists, but that of the eluate around Rf 0.4 was practically abolished.

also [3]), the concentration of SVPx does not change significantly after 10 min of incubation.

(6) *Separation of SVPx by paperchromatography.* When a chromatogram was run with *n*-butanol:acetic acid:water (12:3:5), the solvent mixture used earlier[3], the active principle eluted at Rf 0.5 consisted of a mixture of 2 types of substances. As in the case of the unpurified plasma dialysate (Figure 1), one of the factors is DBMC + MEP sensitive, and the other DBMC + MEP insensitive. By use of *n*-butanol:pyridine:water, Figure 3, 2 active substances were separated. One, which peaks around Rf 0.25, is not antagonized by DBMC + MEP. The other, which peaks around Rf 0.4, is blocked by DBMC + MEP. Presumably the latter fraction contains serotonin (see [3]).

Discussion. For historical reasons we felt it might be appropriate to continue to designate the total of the contracting substances of plasma as 'vasoconstrictine', the term introduced in 1905 by BATELLI[2]. In the rabbit it consists of a mixture of serotonin, SVPx, histamine, and barely detectable amounts of NOR-EPI. There then remains an unknown factor (SVPx) which exists in concentrations equivalent in constrictor activity to the concentration of serotonin in plasma. At the present time we can state unequivocally that SVPx exists and can be separated. Its nature, whether a known or a so-far unknown substance, remains to be investigated.

Note. Separation of serotonin from the unidentified 'effective' vasoconstrictor was also achieved by differential dialysis[11], and by countercurrent distribution technique (to be reported in detail)[12].

Résumé. La «vasoconstrictine» (BATTELLI 1905), c'est-à-dire l'ensemble des vasoconstricteurs «effectifs» dissous dans le plasma du lapin, consiste en un mélange de sérotonine, SVPx (substance non-identifiée) et d'histamine. Par l'usage d'une antisérotonine sélective, DBMC, et d'une mépyramine antihistaminique, il a été possible de reconnaître et d'isoler SVPx. La concentration des catécholamines dans le plasma n'est pas suffisante pour produire une contraction de la paroi d'une artère.

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[12] This study was supported, in part, by grants from the Ontario Heart Foundation grant No. 5-5, and from the Medical Research Council of Canada, grant No. MA-2046.

Secondary Antibody Responses of Mice to Bacterial Somatic Antigens

The physical form of the antigen, the interval between multiple injections, and the techniques used to assess the immune response, are known to influence the results and the interpretation of immunizing procedures. We have used the haemolytic plaque technique, indirect haemagglutination and immunoelectrophoresis to study the secondary responses of mice to bacterial somatic antigens.

Female mice, 8 weeks old, were injected i.p. with a mixture of $2.5 \cdot 10^7$ boiled *Salmonella newport* cells and 12.5 μg of purified *S. typhi* lipopolysaccharide (LPS), a Difco product. The antigens were used together so that the responses induced could be compared in individual animals. One group received no further injections; the animals were bled at weekly intervals to determine the course of the primary immune response. 6 other groups were given a second dose of the same mixture by the same route, at intervals of 2–7 weeks after the first injection; 6 days after the second injection, the spleens and blood sera were examined. Spleen cells forming antibodies (IgM or 19S type) to *S. newport*, *S. typhi* or sheep erythrocytes were counted in duplicate by the plaque method described previously[1], using sheep erythrocytes either sensitized with the appropriate bacterial antigens or non-sensitized as required. A correction was applied for the small number of plaques caused by antibody to sheep erythrocytes. Circulating antibodies were titrated in duplicate by haemagglutination of sheep erythrocytes sensitized as above. Susceptibility of the antibodies to reduction was determined by diluting sera 1 in 10 with phosphate buffer of pH 7.4, containing 0.1 *M* 2-mercaptoethanol (ME), and titrating after 1 h at 37 °C. Alkylation of a number of reduced sera by dialysis against iodoacetamide was found not to alter their titres.

Selected sera were subjected to agar gel immunoelectrophoresis, the precipitin arcs being developed with goat antiserum to whole mouse serum or to mouse γ-globulin. Those arcs which contained antibacterial antibody were detected in the gel by treatment with ^{14}C-labelled soluble antigen and autoradiography on X-ray film.

Both spleen plaque numbers and serum antibody titres were highly variable from one mouse to another, as exemplified in Table I, which shows the detailed results obtained from one of the groups after secondary stimulation. In terms of numbers of spleen cells producing haemolytic antibody, the whole bacterial cells were the more potent immunogen and also gave rise to noticeably larger plaques. There was, however, no correlation with serum antibody levels; individual mice sometimes responded well, in terms of serum antibody, to an antigen inducing only minimal reaction in the spleen. On the average, about 50% of the *S. typhi* antibody was reducible but only about 25% of the *S. newport* antibody.

Table II shows the data from the groups of mice which received antigen mixtures at different intervals. There was no consistent change in the numbers of spleen plaques as the primary-secondary interval increased, in contrast to serum antibody which reached a maximum at the 4-week interval then remained approximately constant. That the latter result was a true secondary response (rather than the slow development of antibody

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