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THE CARDIO-ACTIVE PRINCIPLE IN SPLEEN

BY

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A substance has been demonstrated in acetone extracts of freeze-dried spleen which had cardiotonic properties on isolated papillary muscles from the cat and perfused hearts of the cat and guinea-pig. This activity of the extract could not be explained by the presence of choline, acetylcholine, histamine, 5-hydroxytryptamine, adrenaline or noradrenaline.

The progressive deterioration of a heart-lung preparation has been shown to be abolished by the introduction of the liver into the circuit (Bassani, 1933; Pinotti, 1942; Rein, 1942). These authors demonstrated that glucose, bile ingredients or sympathomimetic amines could not be responsible for the tonic effect of the liver, and Rein (1942) postulated that, since the tonic effect resembled that of the cardiac glycoside strophanthin, the liver might add a steroid to the blood.

Subsequently, Rein, Mertens, and Bücherl (1949) demonstrated a spleen-liver regulatory system which protected the organism as a whole, and the heart specifically against oxygen lack by regulating oxidative metabolism. Under conditions of cardiac hypoxia, Bücherl and Rein (1949) found that the spleen released a substance (*hypoxie-l'enen*) which was transported by the blood stream to the liver, where it stimulated the release of an active material resembling strophanthin since it caused greater economy of cardiac work. Rein (1951) found that transhepatic administration of splenic venous blood from a donor to a recipient animal with acute cardiac hypoxia overcame progressive cardiac failure.

Extracts of liver were shown by Green (1952) to have a pronounced stimulant effect upon isolated papillary muscle of the cat. Green and Nahum (1957) examined commercial liver extracts for cardiotonic activity and found some contained tyramine, although the presence of this substance was thought to be due to bacterial contamination.

Stimulant actions of splenic extracts upon the heart have been attributed by Rothlin (1920) to histamine which was subsequently isolated from spleen (Dale and Dudley, 1929). We have previously shown that extracts of spleen can stimulate isolated cat papillary muscle (Cobbin and Thorp, 1957). The purpose of the present paper is to show that this activity cannot be due to histamine, adrenaline, noradrenaline, acetylcholine, choline, or 5-hydroxytryptamine, all of which occur in splenic tissue.

METHODS

Preparation of the Splenic Extracts

Three separate extractions of spleen were performed.

Extraction (1).—Ox spleen (3.37 kg.) from the abattoir was decapsulated; passed through a hand mincer and freeze-dried. A sample was extracted in a Soxhlet apparatus with the following purified solvents, first petroleum ether, then ether and finally acetone. The acetone fraction was evaporated to dryness and yielded a dark brown tarry residue (2.37 mg./1.0 g. of fresh spleen).

Extraction (2).—A freeze-dried preparation from 17 kg. of ox spleen by *Extraction* (1) was powdered in a mortar, transferred to a Soxhlet apparatus and extracted with anhydrous ether. A cold extraction with anhydrous acetone followed, the acetone was removed under reduced pressure and the residue was taken up in distilled water. Impurities were extracted first with cold ether and then with light petroleum ether (40 to 80°). The aqueous solution was then evaporated to dryness under reduced pressure and stored below 3°. The yield was 0.33 mg. from 1 g. fresh spleen. Although this procedure gave a less potent product than *Extraction* (1), the extract was readily water soluble, there being less contamination by fat.

Extraction (3).—Ox spleen (22.5 kg.) was freeze-dried as in *Extraction* (1) and extracted in a Soxhlet apparatus first with ether and then with acetone. The acetone extract was evaporated to dryness under reduced pressure. The residue was dissolved in petroleum ether and extracted with water until no more coloured material appeared in the water layer, which was then evaporated to dryness under reduced pressure. A yield of 1.5 mg. of water soluble material was obtained from 1 g. of fresh spleen.

Biological Test Preparations

The Blood Pressure of the Cat.—Cats were anaesthetized with pentobarbitone sodium (50 mg./kg., intra-

peritoneally). The blood pressure was recorded with a mercury manometer from the left carotid artery. Intravenous injections were made into the right saphenous vein. The splenic extract [*Extraction* (2)] was dissolved in 0.9% NaCl solution in a concentration of 5 mg./ml.

Isolated Papillary Muscle Preparation.—Cats were anaesthetized with ether. Papillary muscles were dissected from the right ventricles and mounted in a 20 ml. bath of 0.9% NaCl solution at 37°. Two other solutions were used, being the modifications (a) and (c) (Creese, 1949) of the Krebs-Hensleit formula.

The muscle was stimulated by rectangular pulses of about 1 to 5 msec. duration at a rate of 75 to 90/min. through a fine silver wire (0.005 in. diameter) lightly wound round the muscle. An indifferent electrode was placed in the fluid bathing the preparation.

The force of contraction was recorded by means of a variable-inductance-transducer attached to the spring of an isometric lever to which the upper end of the muscle was tied. The transducer was coupled to an ink recorder and the relationship between tension and pen deflection was linear. At first, the muscle developed a tension of 0.5 to 2 g. according to size with a resting tension of approximately 3 g. With the usual sensitivity setting of the apparatus a tension of 1 g. corresponded to a deflection of the myograph spring of about 0.05 mm. at the point of attachment of the muscle.

When a muscle was set up first in solution (a) it contracted quite strongly and might continue to do so for many hours. In solution (c), which differed from solution (a) in the absence of $NaHCO_3$ and a reduction of Ca, the contractions of the muscle declined quite quickly. Usually this was allowed to continue until the force of contraction fell to 20 to 30% of the value when first bathed in solution (a).

The splenic extracts were amorphous pastes and were weighed on a spatula and dissolved directly in the solution (c).

Since papillary muscles set up in this way were very sensitive to changes of temperature and *p*H, all test solutions were adjusted to the same *p*H, kept at the same temperature and aerated with O_2 in the same way as the solution in the muscle bath.

The Isolated Langendorff Heart Preparation.—The isolated hearts of kittens were arranged for perfusion by the method of Langendorff at a pressure of 30 to 50 cm. of water. Ringer-Locke solution at 37° was perfused through the hearts from a reservoir containing 30 ml. of fluid maintained at a constant level by a small non-metallic centrifugal pump. Solutions of the extract were added to the reservoir or injected through a side-arm of the cannula supporting the heart. The amplitude of contraction was recorded on a kymograph by a light lever arranged to make an electrical contact at each beat which operated an impulse timer (Thorp, 1948), thus giving a record of the heart rate. Coronary flow was measured by the outflow recorder of Andrews (1952) which was calibrated after each experiment.

Isolated Guinea-pig Intestine.—A segment of guinea-pig intestine (20 to 25 cm. from the pylorus) was removed from animals of either sex weighing between 200 to 250 g., washed and mounted in a 4 ml. bath containing Tyrode solution at 35° bubbled with 5% CO_2 and 95% O_2.

Isolated Rabbit Intestine.—Rabbit intestine (20 to 25 cm. from the pylorus) was taken from animals of either sex, washed and mounted in a 10 ml. bath containing Ringer-Locke solution at 35° gassed with 5% CO_2 and 95% O_2.

Isolated Rat Uterus.—On the day preceding the experiments, virgin female rats weighing between 150 to 180 g. were given 50 μg. of oestradiol monobenzoate intramuscularly. One horn of the uterus was suspended in a 4 ml. bath containing the solution of Gaddum, Peart, and Vogt (1949) at 29°.

Results

The Action of Splenic Extract on Papillary Muscles

Splenic extract exerted a positive inotropic action on isolated cat papillary muscle confirming the findings of Cobbin and Thorp (1957). The results of ten experiments performed with the sample prepared by *Extraction* (1) are given in Table I. The threshold dose required to produce an inotropic action was greater than the equivalent of 50 mg. of fresh tissue/ml. of solution in the bath. At a dose corresponding to 100 mg. of fresh tissue/ml., a mean increase in tension to 237% of the control tension occurred, and at higher doses a graded response was found which gave an approximately linear log dose/response relationship; only single observations were made with the higher doses. The time taken to reach the peak response varied considerably, although in general the larger the dose employed the later was the peak response and the longer the duration of the effects. Frequently experiments were ended before the control amplitude was regained.

The sample prepared by *Extraction* (2) was much less potent than that prepared by *Extraction* (1). This is probably because cold acetone gives incomplete extraction. However, positive inotropic activity was found with this batch (Table I). *Extractions* (1) and (3) gave samples of approximately the same strength (see Table I).

In order to estimate the molecular size of the substance causing the positive inotropic effect, a concentrated solution was prepared in modification (c) of Krebs-Hensleit solution and passed through a cellophane ultrafilter under pressure overnight at 2°. Tested on papillary muscle, similar doses of ultrafiltrate and of crude extract gave comparable increases in amplitude of beat. Since oxytocin could just pass through the cellophane membrane, the material responsible for cardio-activity in spleen probably had a molecular weight of less than 1,000.

TABLE I

THE EFFECTS OF THE EXTRACTS OF SPLENIC TISSUE UPON THE ISOLATED CAT PAPILLARY MUSCLE

Where more than one experiment was performed the mean values are given. See text for details of *Extractions* (1), (2), and (3).

Extraction	Dose of Extract (mg. Fresh Tissue/ml.)	No. of Experiments	Increase in Amplitude (Control = 100)	Time to Maximum Effect (min.)	Duration of Response (min.)
(1)	50	3	No change	—	—
	100	4	237	14	>60
	160	1	260	50	>70
	200	1	330	8	>90
	400	1	465	90	>120
(2)	1,000	2	138	14	60–80
	2,000	1	180	15	100–120
	4,000	5	386	19	Not recorded
(3)	33	1	208	3	28–30
	67	2	350	15	>90
	100	1	230	20	>60
	300	1	420	20	>90

The Effect of Splenic Extract on the Isolated Cat Heart

When doses of 5 mg of splenic extract were injected into the side arm of the cannula attached to the isolated cat heart, the usual response was an initial depression of the amplitude of contraction lasting approximately 30 sec., and then a slight increase in amplitude together with a moderate rise in the heart rate and decrease in coronary flow. Subsequently the flow of perfusion fluid rose slightly above the control level (Fig. 1*a*). These effects were all transient, since the volumes injected were small and would have passed rapidly through the heart

When 10 mg. of extract was added to the upper reservoir essentially the same effects were observed, although they were more prolonged (Fig. 1*b*). First the heart rate returned to its control level, then the amplitude and finally the coronary flow. With very small doses, it was sometimes possible to elicit changes in amplitude without accompanying alterations in heart rate or coronary flow.

The Action of Splenic Extract in the Anaesthetized Cat

Since splenic tissue is known to contain considerable quantities of noradrenaline and lesser amounts of adrenaline (von Euler, 1950; von Euler and Purkhold, 1951a; von Euler, 1954), doses of *Extraction* (2) were injected into anaesthetized cats to see if there was a pressor response. With constant intravenous doses, it was first shown that tachyphylaxis to repeated injections did not occur. The extract caused a considerable fall in blood pressure (Fig. 2). A diminished fall in blood pressure was found 12 to 15 min. after the administration of 0.5 mg./kg. of body weight of atropine

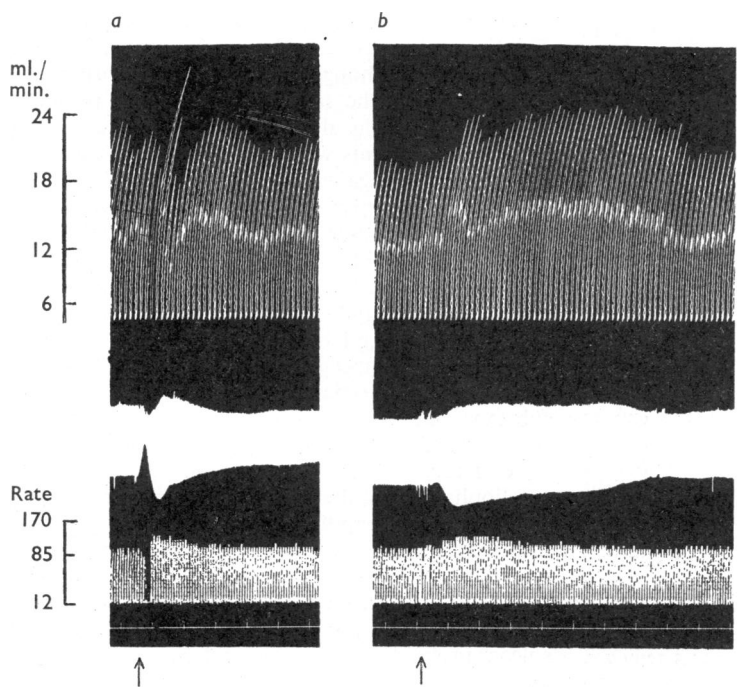

FIG. 1.—The effects of splenic extract [*Extraction* (3)] on the isolated heart of the cat. (*a*) Effect of 5 mg. crude extract injected (at arrow) into the side arm of the cannula near the entrance to the aorta. (*b*) The effect of 10 mg. crude extract added (at arrow) to the upper reservoir of fluid (vol. 30 ml.). Records from above down: coronary flow; amplitude of contraction; heart rate/min.; time, min.

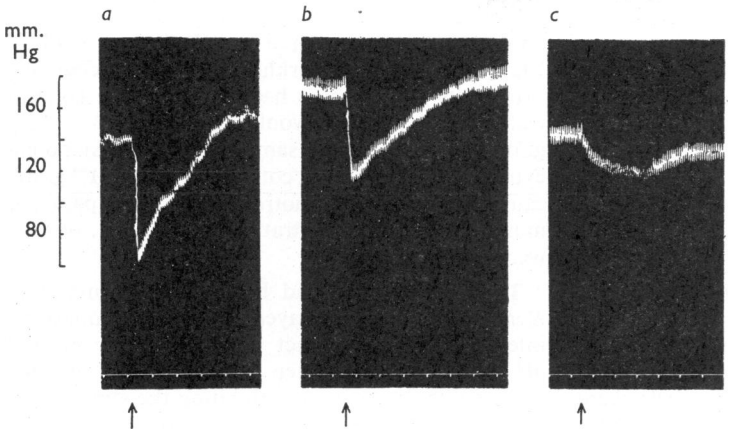

FIG. 2.—The effects of splenic extract [*Extraction* (2), 5 mg./ml. 0.9% NaCl] on the blood pressure of the anaesthetized cat. (*a*) 1 mg./kg. crude extract. (*b*) 1 mg./kg. after atropine sulphate 0.5 mg./kg. (*c*) 1 mg./kg. after promethazine hydrochloride 1.5 mg./kg. Between (*a*) and (*b*) an interval of 12 min.; between (*b*) and (*c*) an interval of 16 min. Time, min.

sulphate. Promethazine hydrochloride (1.5 mg./kg. given 15 min. previously) reduced the depressor response still further, and indeed altered the timing of the response, since the maximum fall was delayed.

Thus most of the depressor effect appeared to be due to substances resembling choline (or its esters) or histamine. The lack of a pressor response after injection of atropine and promethazine suggested the absence of sympathomimetic amines.

Exclusion of Catecholamines as the Cause of Activity in Splenic Extracts

Solutions of splenic extract in modified Krebs solution were brought to *p*H 11±0.1 with 4% NaOH and incubated at 37° for 1 hr. with MnO_2 and O_2 bubbled through the solution (von Euler and Purkhold, 1951b). After cooling to room temperature, the solutions were restored to *p*H 7.5 with N-HCl. Any precipitate in the alkaline solution was redissolved by the acid. The solutions were filtered to remove the MnO_2, warmed to 37° and tested on hypodynamic papillary muscles. Solutions of splenic extract not subjected to the alkaline treatment were used as controls on the same muscles.

Fig. 3 shows an experiment using doses from *Extraction* (3). It will be seen that the treated extract still shows positive inotropic activity of the same order as the untreated material. Experiments performed with *Extraction* (2) showed a similar magnitude of response for both treated and untreated extract, but the response of the incubated extract was sometimes delayed by up to 50%.

Control experiments were done with the maximum quantities of noradrenaline which might have been extracted from the spleen according to the estimates of von Euler (1954). Thus for the weakly active samples [*Extraction* (2)] the usual dose used on papillary muscles was equivalent to 3 g. fresh tissue/ml. of fluid in the 20 ml. bath. If all the noradrenaline in this quantity of spleen had appeared in the acetone extract, a total of 240 μg. would be present. After 240 μg. noradrenaline had been added to 20 ml. of modification (c) of Krebs-Hensleit solution and incubated according to the conditions described above, it had practically no action on the hypodynamic papillary muscle. This quantity of noradrenaline added without prior incubation to a papillary muscle always produced about a 300% increase in amplitude, appearing within 5 to 10 min. and thereafter decaying in an approximately linear manner. Adrenaline in similar doses was also destroyed by alkali.

To be certain that the splenic extract did not contain a substance which protected catecholamines from destruction by this procedure, 240 μg. noradrenaline was added to splenic extract and incubated as described above. When tested on papillary muscle, there was no response typical of noradrenaline, but the response to splenic extract appeared as before, reaching a maximum in 15 to 20 min. The same dose of noradrenaline and splenic extract without alkaline treatment had a strong positive inotropic action which reached a maximum in 5 to 15 min. In each experiment the

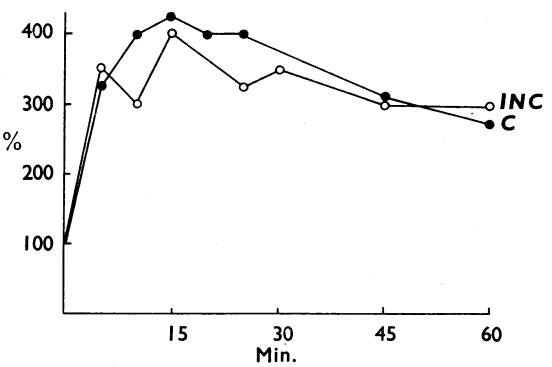

FIG. 3.—Comparison of the effects on papillary muscle of splenic extract [*Extraction* (3)]. *C*, without treatment; *INC*, after incubation at 37° at *p*H 11.0 in the presence of MnO_2 and O_2. In each instance, the dose was equivalent to 67 mg. of fresh spleen/ml. of fluid. Both experiments were performed on the same muscle, the incubated sample being tested second.

response was far greater than a simple addition of the two independent effects.

Exclusion of Acetylcholine, Choline, and Histamine as Agents Causing Activity in Splenic Extracts

The reduction by atropine of the depressor response in the anaesthetized cat suggested the presence of choline or of its esters in the splenic extracts. Fresh spleen contains acetylcholine and choline in amounts of 5 to 30 μg./g. and 2 mg./g. respectively (Dale and Dudley, 1929; Chang and Gaddum, 1933).

Acetylcholine is well known to have a negative inotropic action on cardiac tissue, and when tested on papillary muscle only depressant effects were observed. Rather high concentrations, about 10^{-7} g./ml., were required to depress the contractile force by 1/3. This effect took about 1 min. to appear, and wore off in about 5 min. Fresh spleen can yield 9×10^{-5} g. acetylcholine from 3 g. of tissue, and a 9×10^{-5} g./ml. solution of acetylcholine added to a papillary muscle resulted in a decline in the contractile force to about half the control level.

It was unlikely that acetylcholine was present in the extracts, since the tissue was minced in air, which Dale and Dudley (1929) have shown to result in rapid enzymatic hydrolysis of acetylcholine. Thus only choline should be present in these extracts. *Extraction* (2) would yield a concentration of choline of 6×10^{-3} g./ml., assuming all the choline was extracted from 3 g. fresh tissue/ml. This dose of choline depressed papillary muscle.

Similarly, the histamine content of spleen is about 8 μg./g. of fresh tissue (Dale and Dudley, 1929; von Euler and Purkhold, 1951b). If complete extraction of histamine had occurred, the concentration of histamine would have been 2.4×10^{-5} g./ml. in the strongest samples of the least potent extract. Histamine in a concentration of 10^{-4} g./ml. caused only negative inotropic effects on papillary muscle. Lower concentrations, 10^{-5} g./ml. or less, were without effect.

The acetylcholine and histamine in samples of *Extraction* (2) were assayed on isolated guinea-pig intestine. Some extract was incubated at 37°, *p*H 11 for 1 hr. and then tested to see if catechol amines were present and inhibiting the stimulation. Doses of untreated and treated extract were equipotent on the guinea-pig gut. Constant submaximal responses of similar size were obtained with acetylcholine, histamine, and the splenic extract (Fig. 4). With small doses of atropine sulphate (5×10^{-10} to 1.5×10^{-9} g./ml. in the bath) there was progressive abolition of the response to acetylcholine, but the response to histamine was only slightly affected by higher doses. The response to splenic extract, whether incubated or not, was partially abolished, suggesting that only part of its stimulation was due to choline-like materials (Fig. 4). If all the action of splenic extract had been due to acetylcholine the concentration would have been 0.001 μg./g. of fresh tissue, or for choline, 1 μg./g. fresh tissue, allowing an activity ratio of acetylcholine to choline of 1,000 (Dale and Dudley, 1929).

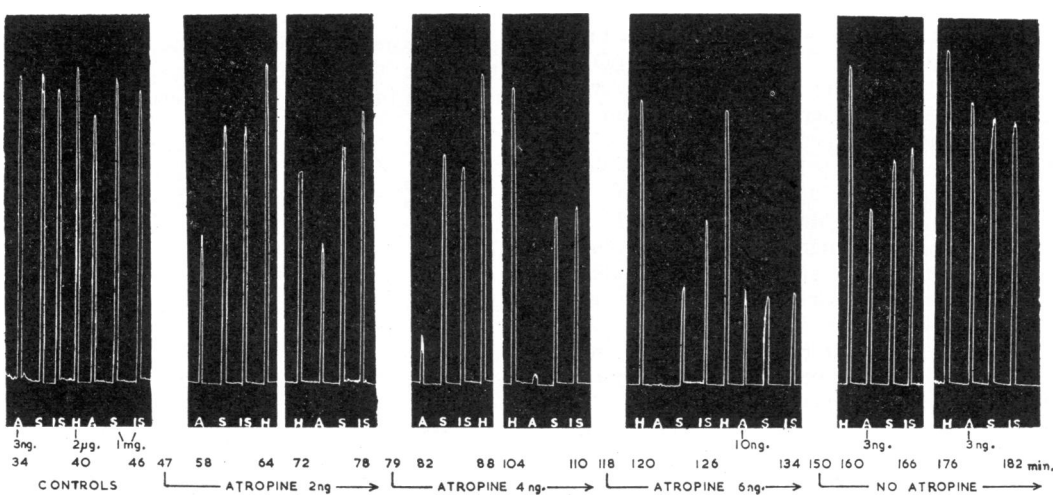

FIG. 4.—The effects of splenic extract [*Extraction* (2)], histamine and acetylcholine on the isolated guinea-pig intestine in a 4 ml. bath. *A*, acetylcholine; *H*, histamine; *S*, splenic extract; *IS*, splenic extract after incubation at *p*H 11.0 for 60 min. at 37° in the presence of O_2 and MnO_2. Atropine was present where indicated. All doses refer to the quantities added to the bath indicated in the left-hand panel, and are constant except where otherwise stated.

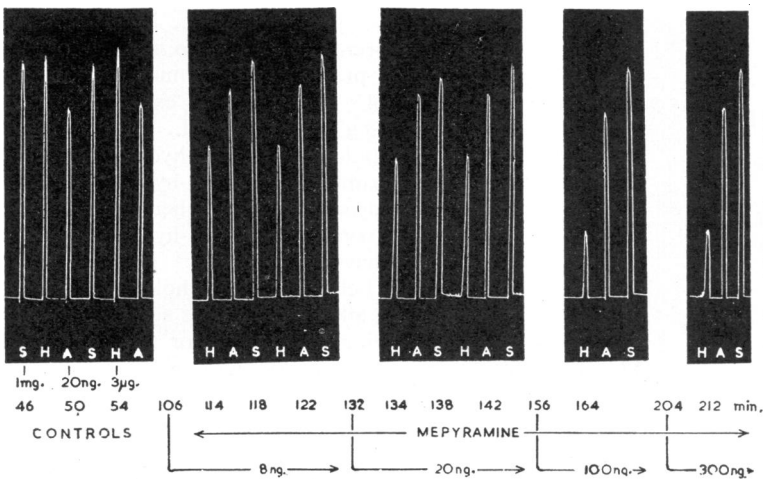

FIG. 5.—The action of splenic extract [*Extraction* (2)] upon isolated guinea-pig intestine in the presence of mepyramine maleate. All doses refer to quantities added to the bath. Abbreviations as in Fig. 4.

Similar experiments were performed using mepyramine maleate (2×10^{-9} g./ml. to 7.5×10^{-8} in the bath) as an antagonist. The response to histamine was gradually diminished, but the actions of acetylcholine and the splenic extract were not affected (Fig. 5). Thus very little of the smooth muscle stimulation by splenic extract could be attributed to histamine. However, if all the contraction following a dose of splenic extract had been due to histamine, the maximum amount present would have corresponded to 1 μg./g. of fresh spleen. Computation of the maximum histamine content from Fig. 4 gives a similar result. (An estimate from Fig. 5 of the maximum acetylcholine content of fresh spleen yields a seven-fold higher result, and since this was the highest value obtained in this series of experiments, it has been used in the work reported below.)

In experiments with *Extraction* (2) on papillary muscles, using a dose equivalent to 3 g. of fresh tissue/ml., the concentrations of histamine and choline would have been less than 3×10^{-6} g./ml. and 2.1×10^{-5} g./ml. respectively. As reported above, these concentrations were without effect on papillary muscle. It was concluded, therefore, that neither histamine, acetylcholine, nor choline were responsible for the inotropic activity of the splenic extract.

Figs. 4 and 5 show that about one-half of the stimulation of smooth muscle by the splenic extract could have been due to cholinergic materials of which atropine is an antagonist, and that very little could have been due to histamine.

Exclusion of 5-Hydroxytryptamine as an Agent Causing Activity in Splenic Extracts

5-Hydroxytryptamine is another smooth muscle stimulant known to occur in spleen which might have caused the residual activity. Since 5-hydroxytryptamine is soluble in acetone, it seemed probable that the residual activity on guinea-pig gut could be due to this substance.

Ox spleen contains up to 8 μg. of 5-hydroxytryptamine/g. of tissue (Erspamer, 1954). The 5-hydroxytryptamine in the extract was assayed on the isolated uterus of the virgin rat, a tissue insensitive to histamine, and about 1,000 times less sensitive to acetylcholine than to 5-hydroxytryptamine. Unlike guinea-pig intestine, the uterus of the virgin rat does not show tachyphylaxis to 5-hydroxytryptamine.

Constant submaximal responses to acetylcholine, 5-hydroxytryptamine, and the splenic extract were obtained (Fig. 6). In the presence of atropine (5×10^{-8} g./ml.) the responses to acetylcholine were blocked, but those to 5-hydroxytryptamine and splenic extract were unaffected. Lysergic acid diethylamide (3×10^{-9} g./ml.) antagonized the actions of 5-hydroxytryptamine and the splenic extract to similar extents, while the response to acetylcholine was hardly affected.

The doses (1 mg.) of *Extraction* (2) used in the rat uterus experiments would have contained 0.02 mg. of acetylcholine/mg. of extract, if this drug had been responsible for their activity. However this dose of acetylcholine is sub-threshold on the rat uterus. In view of the similarity in behaviour of the splenic extract and 5-hydroxytryptamine, it is not unreasonable to assume that all of the oxytocic activity of the extract is due to 5-hydroxytryptamine; the maximum quantity of 5-hydroxytryptamine in the extract being 0.02 μg./g. of fresh tissue.

When tested on papillary muscle, 5-hydroxytryptamine had a positive inotropic action. No action was seen at concentrations of 10^{-6} g./ml. or less. At a concentration of 5×10^{-5} g./ml., the force of contraction was increased by about 50% and reached a maximum in 5 to 6 min. Higher concentrations (10^{-5} and 10^{-4} g./ml.) doubled and quadrupled respectively the force of contraction, and

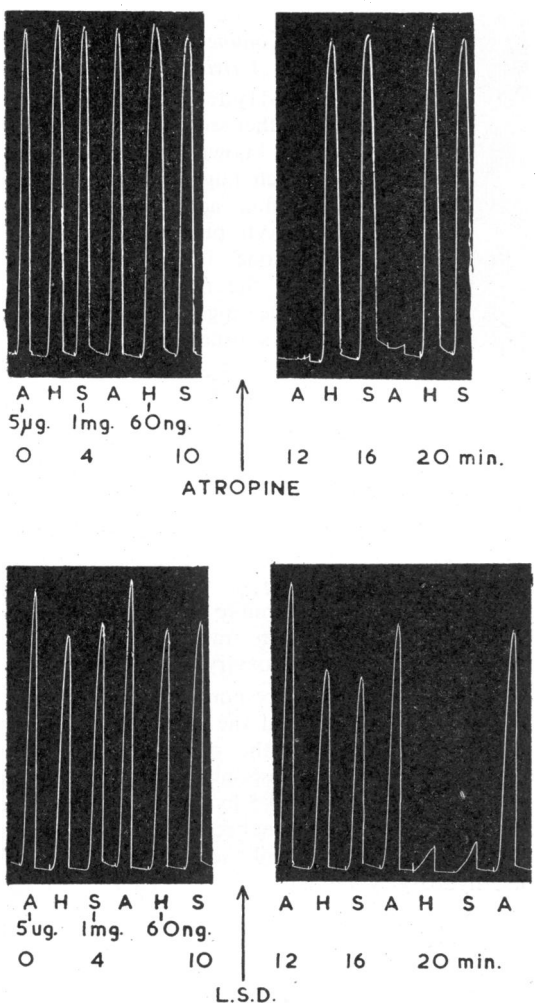

FIG. 6.—The action of splenic extract [*Extraction* (2)], 5-hydroxytryptamine and acetylcholine upon the isolated uterus of the virgin rat at oestrus. The upper panels show the action of atropine in a concentration of 5×10^{-8} g./ml. affecting the response to acetylcholine only. The lower panels are similar except that the antagonist was lysergic acid diethylamide (L.S.D.), 3×10^{-9} g./ml. Abbreviations as in Fig. 4 except that H refers to 5-hydroxytryptamine.

with both strengths the response reached a maximum in about 5 min. The action lasted well over 1 hr. when the concentration was 10^{-5} g./ml.

When 5-hydroxytryptamine was dissolved in modification (c) of Krebs-Hensleit solution at a concentration of 10^{-5} g./ml. and subjected to the alkaline incubation in the presence of MnO_2 and O_2 at *p*H 11, 37° for 1 hr., all the positive inotropic action disappeared. As reported earlier the splenic extract retained its activity under these conditions.

If all the 5-hydroxytryptamine in the sample of spleen had been extracted into the acetone, the concentration in the papillary muscle bath would have been 2.4×10^{-5} g./ml. in experiments using extracts from 3 g. fresh tissue/ml. This is very close to the threshold dose of 5-hydroxytryptamine. Using the maximum value for 5-hydroxytryptamine actually found in the extract when assayed on the rat uterus, the concentration of 5-hydroxytryptamine would only have been 6×10^{-8} g./ml., which is considerably below the threshold for papillary muscle. Thus all our evidence is against 5-hydroxytryptamine being responsible for the positive inotropic action of splenic extract on isolated papillary muscle of the cat.

The maximum quantities of catecholamines, histamine, choline, and 5-hydroxytryptamine found are given in Table II. In fact, the actual quantities of most of these substances were considerably less than the values quoted. Table II also contains the quantities of these materials which would have been present in a papillary muscle experiment using the equivalent of 3 g. of fresh spleen/ml. For comparison, the mean quantities reported elsewhere of these substances are included.

TABLE II

MAXIMUM QUANTITIES OF SOME KNOWN MATERIALS FOUND IN *EXTRACTION* (2)

The quantities (in µg.) which would have been present in the 20 ml. bath in a papillary muscle experiment using the equivalent of 3 g. of fresh tissue/ml. are also given in col. (3). For comparison, the mean values obtained from the literature are quoted with the respective source. References in col. (5): 1, von Euler and Purkhold (1951a); von Euler (1950); von Euler (1954); 2, Dale and Dudley (1929); 3, von Euler and Purkhold (1951b); and 4, Erspamer (1954).

Substance	Acetone Extract of Spleen		Values Reported by Other Workers	
	(µg./g. Fresh Tissue)	Quantity Bath	µg./g. Fresh Tissue	References
(1)	(2)	(3)	(4)	(5)
Adrenaline ..	None found	None	0·07	1
Noradrenaline	,,	,,	4·0	1
Choline ..	"7·0"	420	2,000	2
Histamine ..	1·0	60	8	2, 3
5-Hydroxy-tryptamine	0·02	1·2	8	4

To be quite certain that these known materials did not interact to cause a positive inotropic action on papillary muscle, the maximum quantities which could have been present were combined together in a " cocktail " and assayed. No inotropic activity was observed, and it was concluded that the cardiotonic factor in spleen was neither adrenaline, noradrenaline, choline, acetylcholine, histamine, 5-hydroxytryptamine, nor a combination of these.

Discussion

From Table II it is apparent that the spleen contains a number of biologically active materials of considerable potency. The neurohormones acetylcholine, adrenaline, and noradrenaline are usually considered to be present in an amount proportional to the degree of innervation of the tissue. Since the spleen is innervated both by vagal nerve fibres and by sympathetic postganglionic nerve fibres from the coeliac ganglion, these neurotransmitters should be present in reasonably large quantities. The presence of histamine in this tissue is not so easily explained; there is about the same quantity as of acetylcholine. Finally, Erspamer (1954) has explained the relatively large concentrations of 5-hydroxytryptamine as originating from the serum and in particular from degenerating platelets.

To obtain these materials from the spleen various authors have used extraction methods designed to yield the maximum quantity of each of the substances. For acetylcholine, choline, and histamine the solvent employed was cold alcohol and, to prevent rapid destruction of acetylcholine, the tissue had to be minced in the solvent. To extract the total sympathomimetic neurohormones from spleen either acidified ethanol or trichloracetic acid was used (von Euler, 1950). 5-Hydroxytryptamine was prepared from splenic tissue by the use of an acetone extract (Erspamer, 1940a, 1940b). In the extracts used in this study it is not surprising that lesser quantities of known materials than those reported in the literature were found. Since the spleen extract was prepared without the special precautions necessary to preserve acetylcholine, the only cholinergic activity present in the acetone fraction was most probably due to choline. This supposition is supported by the fact that incubation of the extract under alkaline conditions did not diminish the stimulant action on the isolated guinea-pig ileum.

The stimulation of smooth muscle by the extracts could have been due to choline or its esters since the action was partially blocked by atropine. The residual stimulation was not due to histamine since there was no diminution of response in the presence of mepyramine. When tested for 5-hydroxytryptamine on the isolated rat uterus the splenic extract was found to contain a small quantity which would account for the residual activity on guinea-pig ileum in the presence of atropine. Thus it is most likely that the smooth muscle stimulation by the splenic extract can be explained in terms of its content of choline and 5-hydroxytryptamine. It is strange that the yield of 5-hydroxytryptamine in the extract should be so low compared with the concentrations found by others in ox spleen. The only explanations which can be offered are either that the extraction of 5-hydroxytryptamine was incomplete, or that it could have been eliminated during the removal of fat soluble materials from the crude acetone fraction.

The fact remains that a cardiotonic principle is present in an acetone extract of spleen. This principle cannot be histamine, choline, or choline esters since these were found to have only a depressant action on the force of contraction of isolated papillary muscle, even when given in the maximum concentrations at which they might occur (as shown by the smooth muscle experiments). The active material was neither adrenaline nor noradrenaline since it was not destroyed on alkaline incubation in the presence of MnO_2 and O_2, whereas all the noradrenaline in an equivalent quantity of fresh spleen was destroyed by this procedure. Further evidence that noradrenaline was not responsible is that added noradrenaline was destroyed by incubation at *p*H 11, showing that the splenic extract did not contain a substance protecting noradrenaline from destruction.

5-Hydroxytryptamine is interesting since concentrations of 5×10^{-6} g./ml. are sufficient to cause a regular increase of about 50% in the force of contraction of isolated papillary muscle. Green and Nahum (1957) found the same order of activity although Loubatières, Sassine, and Mauche (1955) reported that concentrations up to 10^{-4} g./ml. had no inotropic effect. When 5-hydroxytryptamine (10^{-5} g./ml.) was incubated with MnO_2 and O_2 at *p*H 11.0, the inotropic action was destroyed. Quantities of 5-hydroxytryptamine as small as those in our extracts were without effect on papillary muscle and consequently the cardiotropic principle cannot be due to 5-hydroxytryptamine. Erspamer (1942) has shown that 5-hydroxytryptamine is partly inactive when first extracted, but that it may be fully activated either by heating for 10 min. at 100° at *p*H 6 to 8 or by incubating in buffer solution at 40° and *p*H 9 to 11 for 1 hr. or longer. A similar procedure did not increase the activity of the splenic extract but destroyed the action of pure 5-hydroxytryptamine on the heart. It therefore seems safe to conclude that low apparent concentrations of 5-hydroxytryptamine in the extracts (assayed on the rat uterus) were not increased by alkaline incubation, and even if activation had occurred initially it would have been abolished by continuing the incubation for 1 hr.

The final proof that histamine, acetylcholine, choline, and 5-hydroxytryptamine were not responsible for the activity of the acetone extract was

that, when the maximum concentrations at which the drugs could occur in *Extraction* (2) were combined and tested on papillary muscle, no inotropic effect was observed.

As to the nature of the cardiotonic principle in spleen we have the following information: it is soluble in acetone and water, is not destroyed by incubation at alkaline *p*H in the presence of MnO_2 and O_2 and it is ultrafiltrable. A material soluble in acetone has been extracted from various bodily tissues and identified as palmitoyl lysolecithin (Titus, Weiss, and Hajdu, 1956; Hajdu, Weiss, and Titus, 1957).

These authors did not include the spleen among the organs surveyed for palmitoyl lysolecithin. Their techniques for estimation of cardio-activity included measuring the abolition of the staircase phenomenon in the isolated frog heart and determining the increase in systolic tension of isolated right ventricular muscle of the squab. It is doubtful whether information derived from these tests correlates with effects upon the mammalian heart since, in experiments performed in this laboratory, synthetic palmitoyl lysolecithin (in concentrations up to 50 μg./ml.) caused no increase in the tension developed by isolated papillary muscle.

It remains to be determined whether the substance in the spleen is related to the "*hypoxielienen*" of Bücherl and Rein (1949). Rein (1942) suggested that a digitalis-like material was present in the body and it is possible that a steroid might appear in an acetone extract. Whether or not a steroid would be stable on alkaline incubation would depend on the peripheral chemical groupings surrounding the nucleus.

The spleen-liver reaction of Rein *et al.* (1949) involves more economical and efficient function in a heart failing from hypoxia. Noradrenaline (and adrenaline) cannot account for the spleen-liver reaction since the effects of noradrenaline are too transient (Meesmann and Schmier, 1955). Meesmann and Schmier (1956) have shown that the spleen-liver reaction could not be due to a variety of known substances including 5-hydroxytryptamine, adenyl compounds, and commercial spleen and liver extracts.

Further work is in progress to identify the cardiotonic material in these extracts of freeze dried spleen.

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References

Andrews, W. H. H. (1952). *J. Physiol. (Lond.)*, **117**, 45P.
Bassani, B. (1933). *Arch. Fisiol.*, **32**, 223.
Bücherl, E., and Rein, H. (1949). *Naturwissenschaften*, **36**, 260.
Chang, H. C., and Gaddum, J. H. (1933). *J. Physiol. (Lond.)*, **79**, 225.
Cobbin, L. B., and Thorp, R. H. (1957). *Nature (Lond.)*, **180**, 242.
Creese, R. (1949). *J. Physiol. (Lond.)*, **110**, 450.
Dale, H. H., and Dudley, H. W. (1929). Ibid., **68**, 97.
Erspamer, V. (1940a). *Arch. exp. Path. Pharmak.*, **196**, 343.
—— (1940b). Ibid., **196**, 391.
—— (1942). Ibid., **200**, 60.
—— (1954). *Pharmacol. Rev.*, **6**, 425.
Euler, U. S. von (1950). *Methods in Medical Research*, Vol. 3, p. 131. Chicago: Year Book Publishers.
—— (1954). *Pharmacol. Rev.*, **6**, 15.
—— and Purkhold, A. (1951a). *Acta physiol. scand.*, **24**, 212.
—— —— (1951b). Ibid., **24**, 218.
Gaddum, J. H., Peart, W. S., and Vogt, M. (1949). *J. Physiol. (Lond.)*, **108**, 467.
Green, J. P. (1952). *Amer. J. Physiol.*, **170**, 330.
—— and Nahum, L. H. (1957). *Circulation Res.*, **5**, 634.
Hajdu, S., Weiss, H., and Titus, E. (1957). *J. Pharmacol. exp. Ther.*, **120**, 99.
Loubatières, A., Sassine, A., and Mauche, J. (1955). *C.R. Soc. Biol. (Paris)*, **149**, 1634.
Meesmann, W., and Schmier, J. (1955). *Arch. exp. Path. Pharmak.*, **227**, 265.
—— —— (1956). *Z. Kreisl-Forsch.*, **45**, 335.
Pinotti, O. (1942). *Arch. Fisiol.*, **42**, 170.
Rein, H. (1942). *Klin. Wschr.*, **21**, 873.
—— (1951). *Pflüg. Arch. ges. Physiol.*, **253**, 435.
—— Mertens, O., and Bücherl, E. (1949). *Naturwissenschaften*, **36**, 233.
Rothlin, E. (1920). *Pflüg. Arch. ges. Physiol.*, **185**, 111.
Thorp, R. H. (1948). *Brit. J. Pharmacol.*, **3**, 271.
Titus, E., Weiss, H., and Hajdu, S. (1956). *Science*, **124**, 1205.