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PHARMACOLOGICALLY ACTIVE SUBSTANCES FORMED IN EGG YOLK BY COBRA VENOM

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This paper deals with the pharmacological activities acquired by egg yolk when incubated with cobra venom, and is concerned with the nature of the substances responsible for these activities. It has been known since the work of Lüdecke (1905) and Delezenne & Ledebt (1912) that lysolecithin is formed from the lecithin present in egg yolk by enzymic cleavage of one fatty acid. The enzyme responsible for this cleavage is phospholipase A. In 1938 Feldberg, Holden & Kellaway examined the effect of envenomed egg yolk on smooth muscle preparations. They found that it caused on the isolated guinea-pig ileum preparation a contraction, which was followed at first by an increase in sensitivity of the preparation to histamine and to acetylcholine and later by a decrease. The contractions produced by envenomed egg yolk diminished on repeated administration. Since the contractions occurred after a latency and developed slowly, the active principle responsible for it was referred to as 'slow reacting substance', or, abbreviated, as 'SRS'. Two active fractions were separated from the envenomed egg yolk: one fraction had the properties of lysolecithin and caused the inhibitory effect, the decrease in histamine sensitivity; and the other produced the slow contraction. The authors concluded that the inhibiting effect of envenomed egg yolk on the guinea-pig ileum preparation was due to the lysolecithin. Whereas Rocha e Silva & Beraldo (1948), as well as Habermann & Neumann (1954), accepted and confirmed this conclusion, Boquet, Dworetzky & Essex (1950) were unable to obtain with lysolecithin a decreased sensitivity of the guinea-pig ileum to histamine and attributed the inhibiting effect of envenomed egg yolk to a different substance, which they called in memory of Delezenne and Ledebt 'D-L substance'. It has therefore been necessary to re-investigate this problem: and it is now shown that the inhibiting effect is due to lysolecithin.

No detailed experiments have hitherto been performed concerning the nature of SRS. Nor do we know whether SRS is responsible also for the

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increased sensitivity which follows the contraction. Several polypeptides are known to have slow contracting smooth muscle effects, and some snake venoms are able to form such polypeptides from suitable substrates. However, SRS was found by Feldberg *et al.* (1938) to be soluble in acetone and in ether, which excludes a polypeptide structure. The present experiments show that SRS consists of one or more unsaturated fatty acids, but not of oleic acid, which was also excluded by Feldberg *et al.* Further, unsaturated fatty acids, either the same as those responsible for the slow contraction or others, are responsible for the increased sensitivity as well.

The term SRS refers in the present paper only to the slow contracting substance or substances formed in egg yolk by cobra venom and does not apply to other slow reacting smooth-muscle-stimulating substances found under different conditions; some of these are certainly different from SRS.

MATERIALS AND METHODS

Incubation of egg yolk with cobra venom. Egg yolks were suspended in an equal volume of saline or Tyrode solution, to which 0·2–5 mg of cobra venom, dissolved in a small amount of saline, was added per egg yolk. After mixing, the suspension was incubated at 37° C for varying times up to 16 hr ('envenomed egg yolk'). The pH of the incubate decreased gradually, but, except in one experiment in which the pH was brought to pH 7 from time to time, no adjustment of pH was made. At the end of the incubation four volumes of alcohol were added and the precipitate formed was centrifuged off after standing in an ice box during the night. The precipitate was washed several times with 95% alcohol and finally with absolute alcohol. The combined supernatant and washings were used for further investigation. The precipitate, which was inactive on the isolated guinea-pig ileum preparation, was discarded.

Enzymic cleavage of lecithin. The method of Hanahan, Rodbell & Turner (1954) was used. Lecithin was dissolved in peroxide-free ether to give a final concentration of 1–5%. For the paper chromatography experiments, samples of 2 ml. of this ethereal lecithin solution were mixed with 0·02 ml. of a 0·5% aqueous cobra venom solution, shaken, and then kept at room temperature (20–22° C) for varying times. The enzymic reaction was then stopped by adding 2 ml. of methanol, which at the same time redissolved the precipitated lysolecithin. Measured parts of this solution were then applied to paper for chromatography.

For preparing lysolecithin and liberated fatty acids larger amounts of lecithin were similarly dissolved in ether, mixed with cobra venom, and incubated for 14–16 hr. The precipitated lysolecithin was then collected by centrifugation, washed with ether, and dried. The combined ether solution and washings, which contained the fatty acids, were evaporated in a stream of N_2, and redissolved in petrol ether; this solution was then washed with water and afterwards dried over anhydrous Na_2SO_4.

Preparation of pure lecithin. Lecithin was extracted from egg yolk and purified by chromatography on aluminium oxide according to the method of Hanahan, Turner & Jayko (1951). The alcoholic solution of pure lecithin obtained by this procedure was evaporated nearly to dryness under reduced pressure and in the absence of oxygen. The residue was taken up in a small volume of petrol ether and precipitated with four volumes of acetone. A colourless lecithin preparation was obtained, which, after drying in a desiccator, turned faintly yellow.

Chemical hydrolysis of lecithin. 70 mg of lecithin was emulsified in 1·4 ml. of N-NaOH in a sealed tube and left at 37° C for 15 hr. From the reaction mixture fatty acids were extracted with ether after diluting and acidifying with HCl. The ethereal solution was evaporated in a stream of N_2 at room temperature. The residue was dissolved in water to which an adequate amount of

NaOH had been added, and was then treated with 10% Pb acetate. This precipitated the lead salts of the fatty acids, which were collected by centrifugation, washed with water, and dried. The dry material was suspended in 4 ml. of ether; this suspension was centrifuged and the clear supernatant was treated with H_2S to precipitate PbS, which was then removed by centrifugation. The ethereal solution now contained the unsaturated fatty acids of the lecithin.

Paper chromatography. Whatman no. 1 paper was used and the chromatograms were run in ascending direction in a tightly closed chamber in a N_2-atmosphere. Samples of lecithin-cobra venom incubates corresponding to 120–150 μg of lecithin or equivalent amounts of split-products were applied to the paper as single spots. The solvent was butanol, saturated with water (Huennekens, Hanahan & Uziel, 1954). Equilibration of the liquid and gaseous phases was allowed for 2–4 hr before dipping the chromatogram into the solvent.

Lecithin and lysolecithin were located by spraying with the phosphate reagent of Hanes & Isherwood (1949), heating the paper to about 80° C for 7–10 min and then irradiating with ultraviolet light for 10 min (Bandurski & Axelrod, 1951). Furthermore, unsaturated groups were detected by exposure of the papers to osmium tetroxide vapours, thus locating lecithin and liberated unsaturated fatty acids. Choline was found by staining with Reinecke salt solution (Hack, 1953) or by the molybdenum blue method of Bevan, Gregory, Malkin & Poole (1951). For biological assay, several unstained chromatograms were divided into transverse sections. Corresponding sections were combined and extracted with methanol; the methanol solution was evaporated on a water-bath in a stream of nitrogen, directed at its surface, and the residue was taken up in saline for the measurement of haemolytic activity and for biological assay on the guinea-pig ileum preparation.

Estimation of haemolytic activity. Washing of red cells and measuring of haemolysis was done as advised by Ponder (1948). Thrice washed red cells from guinea-pig blood were used as a suspension in saline, corresponding to 5% of the original blood volume. To 0·2–4 ml. of this suspension were added 0·02–0·2 ml. of the solution to be tested made up in saline. The time needed for complete haemolysis was determined at constant temperature (21 or 22° C). Occasionally, the percentage haemolysis was measured. For this purpose samples were incubated for 10 min in centrifuge tubes and were then centrifuged for 5 min. Measured portions of the supernatants were taken for colorimetric estimation, after converting the liberated haemoglobin to acid haematin by the addition of an equal volume of 0·33 N-HCl.

Biological assay. Isolated pieces of guinea-pig terminal ileum were suspended in a 15 ml. bath at 34° C in Tyrode solution, aerated with oxygen; 0·2 μg atropine was added to the bath throughout the experiment. Test doses of histamine were administered every 1·5 min. Each dose was allowed to act for 15 sec followed by a 15 sec wash and a further 60 sec rest. The samples of envenomed egg yolk fractions on test were left in the organ bath for varying times. If the envenomed yolk fractions had no direct effect, histamine was administered at the end of the exposure time before washing out. Otherwise the sample was washed out and the next dose of histamine was applied after 1 min rest.

Materials. The histamine used for the biological assay was the acid phosphate. All doses refer to the base. Cobra venom was a sample supplied by the late Dr C. H. Kellaway. Linoleic acid was a sample analysed and found to be pure by Dr A. T. James, who kindly supplied it. Oleic acid and stearic acid were commercial preparations, not subjected to further purification.

RESULTS

As found by Feldberg *et al.* (1938) alcoholic extracts of envenomed egg yolk produce a slowly developing contraction of the isolated guinea-pig's ileum followed by periods of decreased and increased sensitivity of the preparation to histamine. The predominant change was a decrease in sensitivity, and sometimes this effect was so strong and occurred so early as to interfere with and

even to prevent the slow contraction itself. In that case the decreased sensitivity of the preparation was the sole effect of the extract which could be observed.

When the alcoholic extracts of envenomed egg yolk were dried and extracted with ether, the material contained in the ether filtrate had a stimulating effect on the ileum and the contractions were followed by a period of increased sensitivity of the preparation to histamine. A period of decreased histamine sensitivity of the preparation was usually not observed after application of the ether filtrate, or, if present, the effect was weak. On the other hand, the ether-insoluble residue, taken up in saline solution and tested on the ileum preparation, rendered it less sensitive to histamine. Thus it was possible by extraction with ether to separate the principle in envenomed yolk

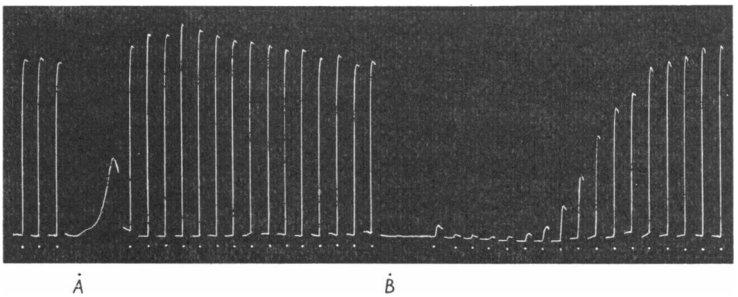

Fig. 1. Guinea-pig ileum, suspended in 15 ml. Tyrode solution. Effect of ether-soluble (*A*) and ether-insoluble (*B*) fractions of alcoholic extract of envenomed egg yolk, left in the bath for 80 sec. At the white dots 0·04 μg of histamine, left in the bath for 15 sec and repeated at intervals of 90 sec (for details see text).

extracts which produced the inhibitory effect from that which caused the slow contraction (SRS) as well as the increase in sensitivity. The effects of the ether-soluble and ether-insoluble fractions on the guinea-pig's ileum are illustrated in Fig. 1. The fractions were obtained from egg yolk incubated with 230 μg of cobra venom per ml. of egg yolk for 16 hr at 37° C. At *A* 415 μg of the ether-soluble material were given and at *B* 1 mg of the ether-insoluble fraction, each dose corresponding to 0·015 ml. of egg yolk.

The action of SRS on the guinea-pig ileum

Not only did the contraction produced by SRS develop rather slowly, but when the bath was replaced with fresh Tyrode solution, relaxation was also slow. In fact, when histamine was given $1\frac{1}{2}$ min later, that is after the usual time interval between applications of histamine, the preparation was often not fully relaxed.

The amounts of extracts necessary to produce a slow contraction depended upon the time of incubation with the venom. In the experiment of Fig. 1 the

contraction at *A* was produced by extract corresponding to 0·015 ml. of egg yolk incubated with cobra venom for 16 hr. Some SRS, however, was formed in much shorter periods of incubation. Thus under similar conditions pharmacological activity was produced after 3 min incubation with an amount of extract corresponding to 0·2 ml. egg yolk. In another experiment, a sample incubated for 4 hr had a stronger effect than a corresponding sample incubated for ½ hr, and a sample incubated for 10 hr had again a stronger effect than the 4 hr sample. Thus the formation of SRS increased with the time of incubation. In this respect, SRS differs from the muscle-stimulating polypeptide bradykinin since the bradykinin which is formed by the action of *Bothrops jararaca* venom is destroyed when incubation is continued for more than a few minutes.

Decreased responses to SRS after repeated application. Feldberg *et al.* (1938) showed that the slow contraction produced by extract of envenomed egg yolk decreased with repeated application and finally disappeared, although the preparation remained sensitive to histamine. This has been confirmed with the ether-soluble fraction which contained SRS.

The diminution in the response to SRS could be obtained with amounts which did not produce maximal contractions. The diminished response was obtained even when the SRS was applied at intervals as long as 15–20 min. The reduction in the response to SRS when given repeatedly varied with different ileum preparations. In some experiments the second dose of SRS was already ineffective, whereas in others it produced a slight diminution only, which increased on further application of SRS. In some preparations a small contraction persisted, however often the application of SRS was repeated, suggesting that there were two different contracting effects with the SRS, only one being abolished by repeated administration. The same impression was gained from experiments in which SRS was kept in the bath after a maximal response had been reached. In these experiments the ileum started to relax gradually after a short time, but in some experiments it did not relax fully but remained slightly contracted. The small contraction which was not abolished by repeated SRS administration also persisted as long as the SRS was kept in the bath.

Another effect was sometimes seen with large doses of SRS (100 μg/ml. or more). When they were given to a preparation made insensitive to smaller doses by their repeated administration, a large dose sometimes produced a transient quick small contraction. At the same time the fluid became turbid probably as a result of precipitation of Ca^{2+} ions and the contraction may therefore have been brought about by the resultant increase in the K/Ca ratio.

The histamine-sensitizing effect of SRS extracts. The sensitization of the histamine response varied in different preparations. In some it was only slight but in others there was a fourfold increase in the histamine contractions. The sensitization was independent of the direct contraction produced by SRS,

since it occurred also when, after repeated administration, SRS no longer contracted the ileum. In fact, in some experiments the sensitizing effect increased as the direct contracting effect diminished.

Chemical properties of SRS

SRS was found to be soluble not only in ether, but also in acetone, benzene and petrol ether. Extracts made with these solvents produced the slow contraction as well as the increase in sensitivity of the preparation but lacked the inhibitory effect.

The solubility of SRS in organic solvents such as petrol ether made it unlikely that it was a polypeptide, even on the assumption that lipids present in the extracts could have acted as solubilizing agents. On the other hand, the solubility in lipid solvents suggested that SRS might be an acidic substance since all known lipid-soluble smooth muscle stimulating substances, such as Prostaglandin (von Euler, 1936; Bergström, 1949), Darmstoff, fraction 'X' (Vogt, 1955) and Irin (Ambache, 1956), are acidic in nature. Evidence of the acidic nature of SRS was obtained as follows.

The ether-soluble filtrate obtained by extraction of dried alcoholic extract of envenomed egg yolk was shaken with an equal volume of water, to which a few drops of N-NaOH had been added to produce a definitely alkaline pH. The two phases of the system were separated, neutralized, evaporated to dryness and redissolved in saline solution. By estimating the contracting effect of the two fractions on the guinea-pig's ileum it was found that most of the SRS had migrated into the aqueous phase; the activity of the ether phase was comparatively weak. If, however, a similar distribution of SRS was made at acidic pH, i.e. after the addition of a few drops of N-HCl instead of N-NaOH to the ether-water mixture, the slow contraction was elicited by the ether phase, the water phase now being ineffective. In both partitions the fraction which contained the SRS also produced the increase in histamine sensitivity of the ileum. These results indicate not only that SRS is an acidic substance but also that it or a similar substance is responsible for the sensitizing effect on the guinea-pig ileum preparation.

The finding that SRS is acidic in nature and lipid-soluble suggested that it might be a fatty acid. Cobra venom contains a phospholipase A, which splits off the unsaturated fatty acid from egg lecithin. Since egg lecithin is not a uniform substance, different unsaturated fatty acids are liberated by the venom and SRS might be one or several of these unsaturated fatty acids. To prove the unsaturated nature of SRS, use was made of the solubility of the lead salts of unsaturated fatty acids in ether since it is known that the corresponding lead salts of the saturated fatty acids are insoluble in ether.

An aqueous solution of SRS was treated with lead acetate until no further precipitation occurred. Precipitate and supernatant were separated by centri-

fugation; the precipitate was then dried and extracted with ether and the ether-soluble material separated by filtration from the ether-insoluble residue. From all three fractions lead was eliminated with H_2S, the solvents were evaporated and the residues taken up in saline solution. The filtered ether-soluble fraction produced a slow contraction followed by increased sensitivity to histamine, whereas the other fractions, i.e. the aqueous supernatant and the ether-insoluble precipitate, did not contract the ileum, nor did they alter the histamine response. Thus it was clear that SRS had formed a lead salt, insoluble in water but soluble in ether, which indicated that it was indeed an unsaturated fatty acid.

Properties of the substance responsible for the decreased sensitivity of the guinea-pig ileum to histamine

The ether-insoluble residue of the alcoholic extracts of envenomed egg yolk, if given in sufficient amounts, rendered the guinea-pig ileum insensitive to doses of histamine which formerly gave a strong contraction, and recovery occurred gradually after about 10 min (Fig. 1). With smaller amounts of the ether-insoluble residue the histamine responses were not abolished but only reduced, and recovery was quicker. The substance responsible for this effect was insoluble in dry acetone but when extracted with 90% acetone a little activity was sometimes found in the acetone filtrate. The substance was also insoluble in petrol ether, but readily soluble in methyl- or in ethyl alcohol and in water.

The extracts containing the inhibitory substance were strongly haemolytic, indicating the presence of lysolecithin. Attempts to separate the inhibiting from the haemolytic effect by various procedures such as extraction, liquid-liquid distribution, and paper chromatography, were unsuccessful. This supports the assumption made by Feldberg *et al.* (1938) that the inhibitory effect was due to lysolecithin itself. Moreover, the areas of paper chromatograms, which showed the gut-inhibiting and haemolytic effect (see page 139), gave a positive reaction for phosphate and choline, and their R_F value after running with butanol agreed with that given for lysolecithin by Huennekens *et al.* (1954).

Enzymic formation of SRS and inhibitory substance from pure lecithin

The experiments so far described indicated, but did not prove, that SRS originated from the lecithin in the egg yolk, and that the inhibitory substance was lysolecithin. It seemed necessary, therefore, to repeat the experiments by incubating cobra venom with pure lecithin, thus ensuring that no other substrate was available to the cobra venom, and further, to incubate under conditions in which no other reaction could occur but the cleavage of the unsaturated fatty acid and the formation of lysolecithin.

Lecithin was prepared from egg yolk, purified chromatographically and incubated with cobra venom in ether solution. It is known that in ethereal solution no other reaction but cleavage of the unsaturated fatty acid takes place (Hanahan, 1952). Samples were incubated at room temperature for 0, 25 min, 4 and 14 hr, after which times the reaction was stopped by addition of methanol. Measured portions of the incubated samples, corresponding to 120–500 μg of lecithin or of its split products, were placed as single spots on

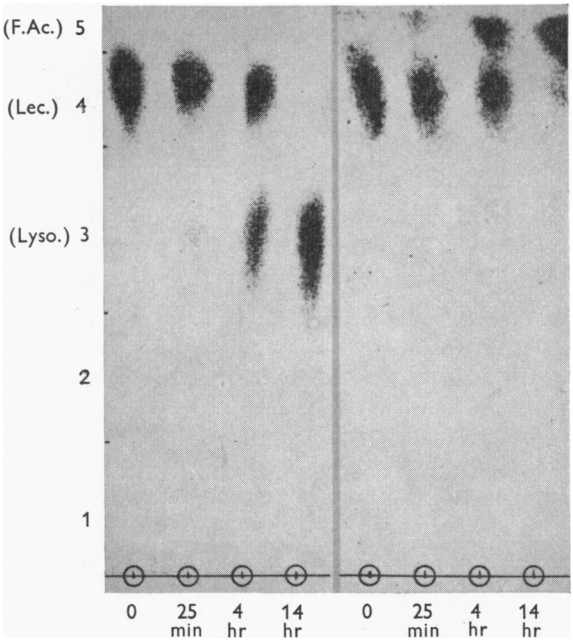

Fig. 2. Chromatographic identification of the split products of lecithin after incubation with cobra venom in ether solution for varying times as indicated below the origin. Each spot applied at the origin corresponded to 250 μg of lecithin, or of split products, respectively. The chromatogram on the left was stained for phosphate with the reagent of Hanes & Isherwood, showing (originally blue) spots in the position of the lecithin (Lec.) and the lysolecithin (Lyso.) regions. The chromatogram on the right, exposed to OsO_4 vapour, showed (originally grey) spots in the positions of lecithin and of unsaturated fatty acids (F.Ac.). The numbers 1–5 on the left margin refer to corresponding transverse slices from parallel chromatograms which were used for biological assay (for details see text).

Whatman No. 1 paper and were then chromatographed with water-saturated *n*-butanol. One series of chromatograms was stained for phosphate with the reagent of Hanes & Isherwood (1949). Blue phosphate-containing spots appeared in the position of lecithin on the chromatograms of the samples incubated for 0 and 25 min and less intensively in the chromatogram of the 4 hr sample. This blue spot was absent in the chromatogram of the 14 hr

sample, and instead, in the chromatograms of the 4 and 14 hr samples, another blue spot appeared below the position of lecithin, as shown in Fig. 2 (left side). These lower spots corresponded to the position of lysolecithin.

A second series of chromatograms, which had been run in parallel, was exposed to osmium tetroxide vapour, whereby dark grey spots developed in the region of lecithin, which again showed the gradual disappearance of lecithin with increasing times of incubation. A second spot developed at the site of the solvent front where free fatty acids are to be expected (see right side of Fig. 2). This spot was absent in the chromatogram of the 0 min sample, but appeared on incubation and became more pronounced with longer times of incubation. These chromatograms thus showed that the egg lecithin, which contains one unsaturated and one saturated fatty acid, had been split by cobra venom into a free unsaturated fatty acid and into lysolecithin, the latter no longer demonstrable with osmium tetroxide.

Other chromatograms run parallel with the above were cut into five transverse slices, each corresponding (from above downwards) to the position of fatty acids (no. 5, Fig. 2), lecithin (no. 4), lysolecithin (no. 3) and then two apparently empty fractions (nos. 2 and 1). Eluates of these fractions were assayed on the isolated guinea-pig ileum in the following way. First, all eluates of the chromatogram from the 14 hr sample were tested, in order to locate the biologically active regions. As shown in Fig. 3 the fatty acid fraction (no. 5) gave the typical effect of SRS, that is, a slow contraction of the ileum, followed by slow relaxation and an increase in histamine-sensitivity. The lecithin fraction (no. 4) had no action on the guinea-pig gut. The lysolecithin fraction (no. 3) did not stimulate the ileum, but greatly reduced its sensitivity to histamine for several minutes. Fractions nos. 1 and 2 were inactive, or nearly so. The next procedure was to take corresponding eluates of the 0, 25 min, 4 and 14 hr samples, and to compare their activities on the ileum, in order to see whether the effects found in the active fractions of the 14 hr sample had resulted from incubation or had been present before. No effects were obtained with any fraction of the 0 min sample. On the other hand, the fatty acid fraction of all three incubated samples produced a slow contraction and increased the sensitivity of the ileum to histamine. The effect was strongest with the 14 hr sample and weakest with the 25 min sample (Fig. 4). Similarly, the lysolecithin fractions of all three incubated samples rendered the ileum less sensitive to histamine and again this effect was smallest with the 25 min sample and greatest with the 14 hr sample.

The eluates of the 14 hr chromatogram were tested for haemolytic activity. Two peaks of haemolytic activity were found, one corresponded to the lysolecithin fraction and one to the fatty acid fraction (Fig. 5). The haemolytic activity of the lysolecithin fraction, however, was much stronger than that of the fatty acid fraction. When both fractions were compared under equal

conditions, the fatty acid fraction produced 40% haemolysis in 10 min, whereas the lysolecithin fraction caused complete haemolysis in less than 1 min. A weak haemolytic effect was obtained with the fraction below lysolecithin apparently owing to some tailing of the substance in the chromatogram. This fraction elicited also a weak gut inhibitory effect (Fig. 3). The

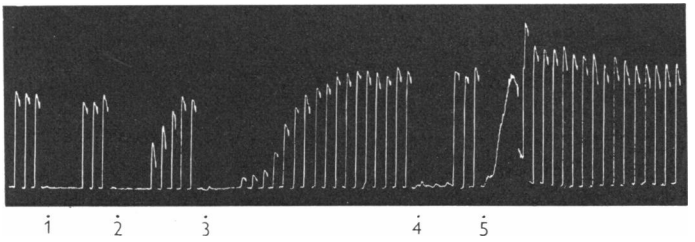

Fig. 3. Guinea-pig ileum, suspended in 15 ml. Tyrode solution. Assay of successive fractions of a chromatogram of lecithin run in parallel to those of Fig. 2 incubated with cobra venom for 14 hr. The numbers 1–5 refer to the different fractions obtained from slices numbered as in Fig. 2; each fraction was tested in a dose which corresponded to 1 mg of lecithin or to the equivalent amount of split products: other contractions are due to 0·05 μg histamine. Note the inhibitory effect in fraction 3 (lysolecithin) and the slow contraction with subsequent increase in histamine-sensitivity in fraction 5 (fatty acids).

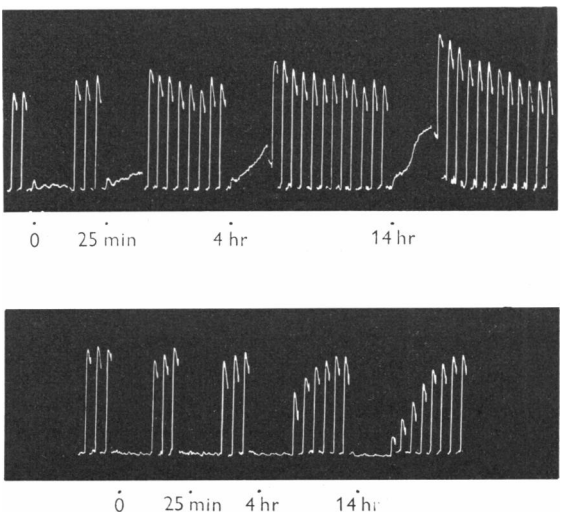

Fig. 4. Guinea-pig ileum, suspended in 15 ml. Tyrode solution. Upper tracing: assay of the fatty acid fractions (slice no. 5 in Fig. 2) from paper chromatograms of lecithin incubated with venom for varying times which are indicated below the tracing; the fractions were tested in amounts equivalent to 1 mg of lecithin. Lower tracing: assay of the corresponding lysolecithin fractions (slice no. 3 in Fig. 2), each fraction equivalent to 330 μg of lecithin. Other contractions in both tracings due to 0·05 μg histamine; both the slow contracting and the inhibitory effect are absent in the zero-time fractions and become stronger with increasing times of incubation.

haemolytic activity of the lysolecithin fraction, which was absent in the 0 min sample, increased with the time of incubation.

The haemolytic action of the fatty acid fraction accords with the known properties of unsaturated fatty acids. Some of them, as for instance *cis*11:12-octadecenoic acid (Laser, 1950) are highly haemolytic. The results obtained with pure lecithin thus prove that SRS is an unsaturated fatty acid liberated from lecithin and that the gut-inhibitory action is a property of the haemolytic lysolecithin.

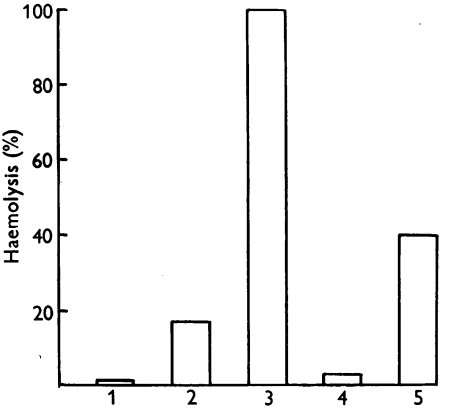

Fig. 5. Haemolytic activity of eluate of the same fractions as in Fig. 3 from the paper chromatogram of the 14 hr sample. Amounts of each fraction, corresponding to 500 μg of lecithin or equivalents of split products, were dissolved in 0·15 ml. of saline and added to 4 ml. of 5% red cell suspension. Haemolysis was measured photometrically after 10 min incubation at 22° C.

Formation of SRS by chemical hydrolysis of lecithin

Further evidence that SRS is an unsaturated fatty acid liberated from lecithin was obtained by hydrolysing lecithin chemically instead of enzymically.

A sample of 70 mg pure lecithin was saponified in N-NaOH at 37° C. No lysolecithin is produced by this procedure, because both fatty acids of lecithin are split off simultaneously. After complete hydrolysis the reaction mixture was acidified with HCl and the fatty acids were extracted with ether. The unsaturated fatty acids were isolated by the lead salt–ether method. For comparison another 70 mg from the same lecithin stock was incubated with cobra venom in ether solution and after 14 hr of incubation the liberated fatty acids were isolated. The effects of the chemically and enzymically prepared unsaturated fatty acids were tested on the guinea-pig ileum. Slow contractions were produced by both; using corresponding amounts the effects were qualitatively and quantitatively the same (Fig. 6). With repeated administration of the fatty acids of either source, the size of the contractions decreased and

their latencies increased. This can be seen in Fig. 6 which gives the times in seconds required for the development of the full contractions produced by repeated alternating doses of the two fatty acid samples.

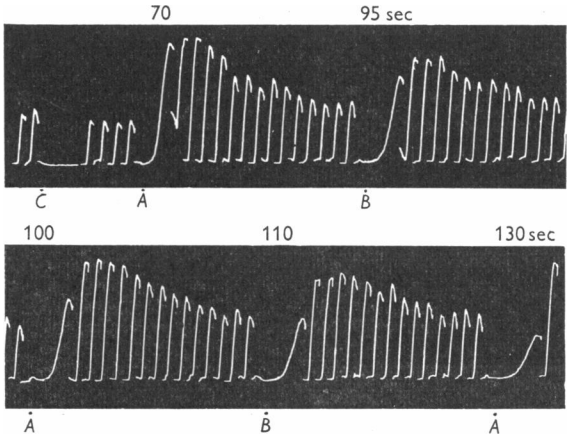

Fig. 6. Guinea-pig ileum, suspended in 15 ml. Tyrode solution. Comparison of effects of unsaturated fatty acids prepared from lecithin by chemical hydrolysis (*A*) and by enzymic hydrolysis with cobra venom (*B*); each dose equivalent to 3·5 mg of lecithin; *C*, 3·5 mg of unhydrolysed lecithin from the same stock; other contractions due to 0·05 μg histamine: the lower strip is a continuation of the upper. Figures given above the tracing indicate the times in seconds necessary for the development of the full contraction; note increase in latency and time required for full contraction with repeated application of SRS, owing to desensitization.

Effects of oleic, linoleic and stearic acids

In a few experiments an attempt was made to identify the unsaturated fatty acid derived from egg lecithin and responsible for the slow contraction and increased sensitivity to histamine. Neutralized oleic and linoleic acids both of which are unsaturated fatty acid constituents of egg lecithin, were tested on the guinea-pig ileum preparation, and so was the saturated stearic acid.

Stearic acid produced neither a slow contraction nor did it render the ileum preparation more sensitive to histamine (Fig. 7 *B*). Oleic acid was found by Feldberg *et al.* (1938) to be inactive on the guinea-pig ileum and was therefore no longer considered as the substance responsible for the slow contracting effect. In the present experiments oleic acid was also unable to imitate, or at least to imitate fully, the action of SRS. When 0·5–2 mg of oleic acid was added to the 15 ml. bath in which the ileum preparation was kept, it did not cause a contraction; occasionally it produced a slight elevation of the base line as illustrated in Fig. 7 *A*. Oleic acid, however, shared with SRS the ability to render the ileum more sensitive to histamine (Fig. 7).

Linoleic acid had usually no contracting effect on the guinea-pig ileum, sometimes it produced a very small gradual contraction and in one preparation

a contraction like that produced by SRS was obtained on the addition of 250 μg of linoleic acid to the bath. When given a second time the linoleic acid, however, was inactive. When the linoleic acid was washed out, the sensitivity of the ileum to histamine was found to be increased. However, the time course of sensitization was different from that seen after SRS. After SRS was washed out the increase was maximal during the first one or two minutes, and decreased with each subsequent histamine administration. After linoleic acid the increased sensitivity was not apparent, or only slight, during the first few minutes after it had been washed out, and only the third or fourth histamine contraction was increased.

Finally, mention may be made that, with repeated administration of large doses of either oleic or stearic acid, small, quick contractions were produced like those seen with SRS under similar conditions. They were probably due to precipitation of calcium, like those produced by SRS.

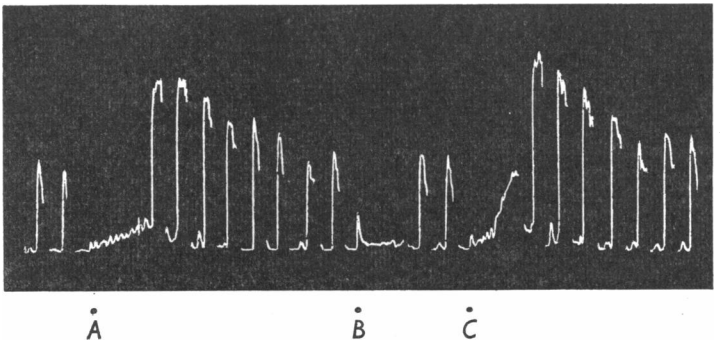

Fig. 7. Guinea-pig ileum (15 ml. bath): *A*, oleic acid; *B*, stearic acid; *C*, SRS; each dose = 0·5 mg free acid, neutralized with NaOH. Other contractions due to 0·05 μg histamine.

DISCUSSION

The results of the present experiments show that the pharmacological activities produced by incubating egg yolk with cobra venom are due to two different substances, which are both split-products of lecithin. The one is lysolecithin, the other SRS, which was identified as unsaturated fatty acid in nature. Lysolecithin is responsible not only for most of the haemolytic property of envenomed egg yolk, but also for the inhibitory effect on the guinea-pig ileum, which after lysolecithin becomes less sensitive to stimulating substances such as histamine. On the other hand, the slow contraction as well as the increased sensitivity to histamine are effects of the unsaturated fatty acids split off from lecithin.

Parallel development or disappearance of two different biological effects is usually regarded as an indication that both effects are due to the same substance. The simultaneous formation of lysolecithin and active unsaturated

fatty acids, however, provides an instance where such conclusions may be misleading.

The finding that lysolecithin is responsible for the inhibitory effect on the guinea-pig ileum confirms the conclusion of Feldberg *et al.* (1938), but is at variance with that of Boquet *et al.* (1950). It is not clear why these authors were unable to demonstrate this effect with lysolecithin, and, also, why they did not obtain an inhibitory effect when venom was incubated with plasma or lecithin, but only when it was incubated with egg yolk. The ineffectiveness of plasma as substrate for lysolecithin formation may be explained by its rather low lecithin content, as compared with egg yolk. Furthermore, plasma may have an inhibitory action on the splitting enzyme of cobra venom similar to that shown for the phospholipase A of bee venom (Neumann & Habermann, 1954). In the experiments of Boquet *et al.* in which lecithin was used as substrate, either the ionic milieu for the enzymic action of the venom may have been unsuitable, or their lecithin preparation may have contained $CdCl_2$, since $CdCl_2$ is used in the classical methods for purification of lecithin. It is known that $CdCl_2$ inhibits the effect of cobra venom (Habermann & Neumann, 1954). In the present investigations, the use of $CdCl_2$ was deliberately avoided by purifying the lecithin chromatographically (Hanahan *et al.* 1951).

The characteristic features of the contraction produced by SRS, that is to say the pronounced latency and the slowness of the contraction as well as of the relaxation after changing the bath fluid, were reproduced by the unsaturated fatty acid fraction of envenomed egg yolk or of lecithin. The unsaturated fatty acid fraction also caused a transient increase in histamine sensitivity of the guinea-pig ileum similar to that which follows the application of envenomed egg yolk once lysolecithin has been eliminated. Sensitization to muscle-stimulating substances may be a common property of lipid-soluble slow contracting acidic substances. For instance, Darmstoff, a phosphatidic acid, has this effect (Kuck & Vogt, 1950).

The SRS extracts used in the present experiments were a mixture of unsaturated fatty acids, and it is not certain whether the slow contraction produced by these extracts were the effect of one or several such acids. It is certain, however, that not all unsaturated fatty acids have this property; oleic acid, for instance, is ineffective. The exact chemical structure of the acid or acids which produce the slow contraction is as yet unknown. Linoleic acid according to Gabr (1956) produces a slow contraction on the guinea-pig ileum in doses of 20–50 μg in a 10 ml. bath. However, the present results suggest that linoleic acid at most contributes to a small extent only to the contraction produced by SRS, since it proved to be a much weaker smooth muscle-stimulating substance than was expected from the experiments of Gabr. When its activity was compared with that of SRS extracts, it was found that weight for weight linoleic acid had a much weaker effect than the SRS extract, although

the extracts contain, in addition, a proportion of inactive unsaturated fatty acids such as oleic acid and possibly other inactive components. Another possible candidate for SRS may be Δ-3:4-octadecenoic acid, which was found by Gabr to contract the guinea-pig ileum, but there is as yet no evidence of its presence in egg lecithin. Gabr suggested that this acid, or a similar unsaturated fatty acid, might be responsible for SRS effects.

From the present results it is not possible to conclude that the slow contraction and the sensitizing effect are produced by the same unsaturated fatty acids, and that SRS is thus also responsible for the sensitizing effect. For instance, oleic acid has this property and is certainly formed by the action of cobra venom on lecithin of egg yolk; therefore, part of the sensitizing effect produced by SRS-containing extracts may be due to this acid.

The sensitizing effect produced by unsaturated fatty acids has to be taken into account when perfusates of organs are assayed for their histamine content on the isolated guinea-pig ileum, as they may contain such acids. Fortunately, the sensitization persists for some time, so that it would be revealed by a subsequent histamine application. In fact, Feldberg & Kellaway (1937) considered the possibility that their histamine values of lung perfusates obtained after injection of cobra venom might have been too high, because of this sensitization.

The desensitization of the guinea-pig ileum to SRS after its repeated application differs from that observed with other substances such as bradykinin or 5-hydroxytryptamine. With these substances desensitization is relatively transient and occurs only with supramaximal doses or with smaller doses when given at short intervals, whereas with SRS desensitization occurs already with doses sufficient to give a small response and persists for many minutes. The following hypothesis would account for the desensitization to SRS. Unsaturated fatty acids are easily oxidized into hydroperoxides. It may be that the contraction produced by SRS is due not to the unsaturated fatty acid itself, but to its hydroperoxide, and that the oxidizing system in the intestinal wall becomes quickly exhausted after application of SRS. The residual effect that is sometimes seen after desensitization, might then be due to the presence of peroxides in the SRS sample itself, and in fact, estimation by the Wheeler (1932) method showed that the SRS extracts contained peroxides. It is interesting to note in this connexion that oleic acid, which does not produce the slow contraction, cannot be peroxidized by plant lipoxidases (Holman & Bergström, 1951).

The unsaturated fatty acids split off from lecithin by cobra venom were found not only to contract the guinea-pig ileum and render it more sensitive to histamine, but also to produce haemolysis. It is not known whether the acid or acids responsible for the haemolysis are identical with those responsible for the effects on the guinea-pig ileum. The haemolytic effect of the unsaturated

fatty acids is weak in comparison with that of lysolecithin. This was shown by comparing the haemolysis caused by equivalent amounts of lysolecithin and of the unsaturated fatty acid fraction derived from lecithin. The haemolytic effect of the unsaturated fatty acids, however, may explain findings such as that of Dunn (1934), that incubates of red cells, cephalinase and cephalin produced stronger haemolysis than was to be expected from the amount of lysocephalin that could have been formed.

SUMMARY

1. The pharmacological activities produced in egg yolk on incubation with cobra venom are shown by paper chromatography to be due to lysolecithin and to unknown unsaturated fatty acids.

2. Lysolecithin is shown to be responsible not only for the main haemolytic activity of envenomed egg yolk, but also for its ability to render the guinea-pig ileum less sensitive to histamine.

3. Envenomed egg yolk produces on the guinea-pig ileum a slow contraction and the substance responsible for it has been termed slow-reacting substance (SRS). It is shown that SRS is an unsaturated fatty acid, split off from lecithin: this unsaturated fatty acid, however, is not oleic acid.

4. The unsaturated fatty acid fraction of envenomed egg yolk produces on the isolated guinea-pig ileum not only the slow contraction but also an increased sensitivity to histamine. In addition, the unsaturated fatty acid fraction exerts some haemolytic activity. It is not known whether all three activities are due to the same or to different acids.

5. The slow contraction and the increased sensitivity to histamine can be produced on the guinea-pig ileum not only with the unsaturated fatty acid fraction obtained from envenomed egg yolk, but also with that obtained from enzymic or chemical hydrolysates of purified lecithin.

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REFERENCES

AMBACHE, N. (1956). Trigeminomimetic action of iris extracts in rabbits. *J. Physiol.* **132**, 49–50*P*.

BANDURSKI, R. S. & AXELROD, B. (1951). The chromatographic identification of some biologically important phosphate esters. *J. biol. Chem.* **193**, 405–410.

BERGSTRÖM, S. (1949). Prostaglandinets Kemi. *Nord. Med.* **42**, 1465.

BEVAN, T. H., GREGORY, G. I., MALKIN, T. & POOLE, A. G. (1951). Chromatographic separation of choline-containing phospholipids from phospholipid mixtures. *J. chem. Soc.* pp. 841–842.

BOQUET, P., DWORETZKY, M. & ESSEX, H. E. (1950). Physiologic responses of certain animals and isolated preparations to mixtures of snake venom and egg yolk. *Amer. J. Physiol.* **161**, 561–572.

DELEZENNE, C. & LEDEBT, S. (1912). Nouvelle contribution à l'étude des substances hémolytiques dérivées du sérum et du vitellus de l'œuf, soumis a l'action des venins. *C.R. Acad. Sci., Paris*, **155**, 1101–1103.

DUNN, E. E. (1934). The separation of the enzymes and toxic principles of the venom of Crotalus adamanteus. *J. Pharmacol.* **50**, 393–406.

FELDBERG, W. & KELLAWAY, C. H. (1937). Liberation of histamine from the perfused lung by snake venoms. *J. Physiol.* **90**, 257–279.

FELDBERG, W., HOLDEN, H. F. & KELLAWAY, C. H. (1938). The formation of lysocithin and of a smooth muscle-stimulating substance by snake venoms. *J. Physiol.* **94**, 232–248.

GABR, Y. (1956). Observations on a substance in human plasma which gives a slow contraction of guinea-pig gut *in vitro*. *Brit. J. Pharmacol.* **11**, 93–98.

HABERMANN, E. & NEUMANN, W. (1954). Die Hemmung der Hitzekoagulation von Eigelb durch Bienengift—ein Phospholipase-Effekt. *Hoppe-Seyl. Z.* **297**, 179–193.

HACK, M. H. (1953). Analysis of lipids by spot tests on filter paper disk chromatograms. *Biochem. J.* **54**, 602–605.

HANAHAN, D. J. (1952). The enzymic degradation of phosphatidyl choline in diethyl ether. *J. biol. Chem.* **195**, 199–206.

HANAHAN, D. J., RODBELL, M. & TURNER, L. D. (1954). Enzymatic formation of monopalmitoleyl- and monopalmitoyl-lecithin (lysolecithins). *J. biol. Chem.* **206**, 431–441.

HANAHAN, D. J., TURNER, M. B. & JAYKO, M. E. (1951). The isolation of egg phosphatidylcholine by an adsorption-column technique. *J. biol. Chem.* **192**, 623–628.

HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.*, **164**, 1107–1112.

HOLMAN, R. T. & BERGSTRÖM, S. (1951). In SUMNER, J. B. & MYRBÄCK, K. *The Enzymes*, vol. II, part I, p. 567. New York: Academic Press Inc.

HUENNEKENS, F. M., HANAHAN, D. J. & UZIEL, M. (1954). Paper chromatography of lecithins. *J. biol. Chem.* **206**, 443–447.

KUCK, H. & VOGT, W. (1950). Pharmakologische Wirkungen des Darmstoffs. *Arch. exp. Path. Pharmak.* **209**, 71–81.

LASER, H. (1950). The isolation of a haemolytic substance from animal tissues and its biological properties. *J. Physiol.* **110**, 338–355.

LÜDECKE, K. Dissertation Munich, 1905. Quoted from NEUMANN, W. & HABERMANN, E. (1954). *Hoppe-Seyl. Z.* **296**, 166–179.

NEUMANN, W. & HABERMANN, E. (1954). Über die Phospholipase A des Bienengiftes. *Hoppe-Seyl. Z.* **296**, 166–179.

PONDER, E. (1948). *Haemolysis and Related Phenomena*, p. 181. London: J. and A. Churchill Ltd.

ROCHA E SILVA, M. & BERALDO, W. T. (1948). Dynamics of recovery and measure of drug antagonism. Inhibition of smooth muscle by lysocithin and antihistaminics. *J. Pharmacol.* **93**, 457–469.

VOGT, W. (1955). Eigenschaften und Gewinnung von Darmstoffpräparaten. *Arch. exp. Path. Pharmak.* **227**, 224–233.

VON EULER, U. S. (1936). On the specific vasodilating and plain muscle stimulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin). *J. Physiol.* **88**, 213–234.

WHEELER, D. H. (1932). Peroxide formation as a measure of autoxidative deterioration. *Oil & Soap*, **9**, 89–97.