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THE CHEMICAL NATURE OF DARMSTOFF

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The term Darmstoff was given to a smooth-muscle-stimulating substance which was first observed with bath fluid of isolated intestine preparations (Vogt, 1949). The substance is easily soluble in many organic solvents and is acidic in nature. These properties distinguish Darmstoff from amines and polypeptides (Vogt, 1955).

The present experiments show that Darmstoff consists of acidic phospholipids, one of which is an acetalphosphatidic acid. The experiments were begun following a suggestion by Professor W. O. Kermack and Dr C. Long, Aberdeen, that Darmstoff might contain acetal groups because of the finding that Darmstoff was more resistant to alkali than to acid. This behaviour is peculiar to acetals.

METHODS

Preparation of Darmstoff. The sample of Darmstoff used in the present experiments was obtained from 10·5 kg of washed small intestine of the horse. The tissue was cut into pieces and boiled in 21 l. of 0·2 N-NaOH for 10 min. This treatment can be used because Darmstoff is stable in alkali, and it has the advantage over the method previously used (Vogt, 1955) in that it simplifies the extraction because the tissue is dissolved by the alkali. After cooling the solution it was made slightly acidic by adding HCl. A precipitate formed which, besides proteins, contained Darmstoff. The precipitate was collected by centrifugation and then extracted with 5 l. *n*-butanol. A clear butanol filtrate was obtained which was concentrated *in vacuo* after the addition of sufficient ammonia solution to prevent the filtrate from becoming acidic. The concentrate was purified by two counter current distributions in water–methanol–butanol–benzene and methylethylketone–water, as described earlier (Vogt, 1955). An amount of 418 mg dry weight of Darmstoff extract was obtained with an activity of 35,900 u./mg. The unit has been defined elsewhere (Vogt, 1953). The material was dissolved in butanol and stored for nearly a year before the present experiments were undertaken; some loss of activity may have occurred during this time. For use, suitable portions of this solution were taken and dried in a stream of N_2. All weights given refer to the dry material.

Preparation of Ba *salt of Darmstoff.* A sample of 18 mg of Darmstoff was dissolved in 5 ml. of a methanol–butanol mixture (1 + 1 vol.). On the addition of 0·3 ml. of a saturated $BaCl_2$ solution in methanol a small precipitate formed. It had no smooth-muscle-stimulating effect on the rabbit duodenum and was therefore discarded. When the filtrate was made alkaline by the addition of 0·1 ml. N-NaOH, a new precipitate formed, which was collected by centrifugation after

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standing at −7° C during the night. When dried the precipitate weighed 5·16 mg. The 5·16 mg of the precipitated Ba salt contained more than 75% of the original activity. The sample was thus about three times as active as the starting material. For the biological assay, the Ba was first eliminated by dissolving the Ba salt with Na_2SO_4.

Mild acidic hydrolysis of Darmstoff. A sample of 7·5 mg of Darmstoff was dissolved in 1 ml. of 50% aqueous acetic acid, and 0·1 ml. of 0·005 M-$HgCl_2$ was added to the solution which was then sealed in an ampoule filled with N_2 and kept for 48 hr at 37° C. The mercury was eliminated by H_2S and the solution dried in a desiccator *in vacuo* over NaOH. Portions of this hydrolysate were taken for estimation of biological activity and for paper chromatography. Occasionally, hydrolysis of Darmstoff was carried out in 90% acetic acid for 24 hr, but otherwise proceeding in the same manner.

Paper chromatography. Whatman paper No. 1 and No. 3 were used. The paper No. 1 was washed with 2 N-acetic acid, water and ammonia, and treated with H_2S. The paper No. 3 was kindly supplied by Dr T. S. Work after it had been washed according to the procedure of Connell, Dixon & Hanes (1955). The samples to be chromatographed were applied to the paper as single spots, usually in amounts corresponding to 350–700 μg of Darmstoff. The chromatograms were run in an ascending direction in a sealed tank in a N_2-atmosphere, and chromatography was stopped when the solvent front had reached a distance of 23–25 cm from the site of application of the spots. Usually two mixtures were used as solvents, the upper phase being either methylethylketone-diethylamine–water mixture (60 + 3 + 20 vol.) (Vogt, 1955) or *n*-propanol–NH_3–water prepared according to Hanes & Isherwood (1949). In addition, some chromatograms were developed with light petrol (50–60° C b.p.) or with *n*-butanol saturated with water.

Phosphate was detected on paper chromatograms by spraying with the molybdate reagent of Hanes & Isherwood (1949), heating the paper to about 80° C for 10 min and finally irradiating the paper with ultra-violet light for a further 10 min (Bandurski & Axelrod, 1951). With this procedure phosphate-containing compounds give blue spots.

Aldehydes were shown by the purple colour they give with Schiff reagent (fuchsin-sulphurous acid). The reagent was prepared by dissolving 1 g of parafuchsin in 700 ml. H_2O, adding 50 ml. of 2 N-HCl and 5 g of $NaHSO_3$ and adjusting to 1 l. with H_2O (Christl, 1953). The reagent was sprayed on to the paper chromatogram which was then placed either between two glass plates or in a sealed tank which contained some SO_2 solution and was filled with N_2. After the colour had developed, the paper was washed twice in SO_2 solution and finally in distilled water. Some of the chromatograms were treated with acetic acid to hydrolyse acetals before they were stained. For this purpose the chromatograms were either sprayed with 0·005 M-$HgCl_2$ in 90% acetic acid and incubated for 24 hr at 37° C, lying between two glass plates, or they were exposed for 24 hr to acetic acid vapour in a sealed tank.

Ninhydrin reaction. The chromatograms were sprayed with 0·1% ninhydrin in butanol and then left in the dark at room temperature until the violet colour had developed. These chromatograms could afterwards be used for the phosphate reaction.

Biological assay. Isolated strips of rabbit duodenum were used for assay in a 15 ml. bath of Tyrode solution at 37 °C, aerated with a mixture of 95% O_2 + 5% CO_2; 0·4 μg of atropine was added to the bath after each change. The fractions to be assayed were freed from solvents by drying at room temperature in a stream of N_2 and redissolving the residue in a suitable amount of saline or Tyrode solution. The position of smooth-muscle-stimulating compounds on single-dimension paper chromatograms was ascertained by cutting the paper into transverse sections and either immersing these directly in the organ bath, or eluting them with methanol containing some NH_3, drying the eluate as described, redissolving the residue in Tyrode solution, and adding equal portions to the bath.

RESULTS

Paper chromatography and colour reactions of Darmstoff

Phosphate. When the paper chromatograms of 500–1000 μg of Darmstoff on Whatman No. 3 paper developed in the methylethylketone-diethylamine mixture were treated with the molybdate reagent of Hanes & Isherwood (1949) and then irradiated with ultra-violet light, they showed two blue spots

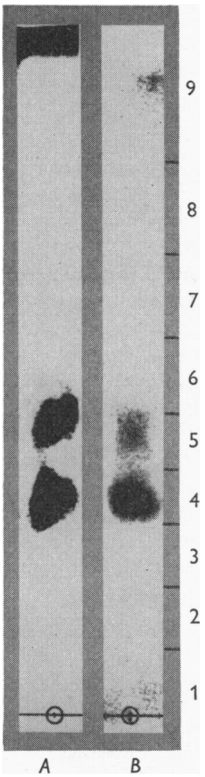


Fig. 1. Chromatograms of 1·5 mg of Darmstoff on Whatman No. 3 paper, developed with methylethylketone–diethylamine. At *A* the paper was stained with Schiff reagent, showing the position of aldehydes. At *B* the paper was stained with the molybdate reagent, showing phosphate spots. The numbers on the right margin indicate the sections of a parallel unstained chromatogram, which were eluted and tested for biological activity, as shown in Fig. 2.

corresponding to R_F/0·34 and 0·43 (Fig. 1*B*). The lower spot was stronger in colour and larger in size than the upper and could be seen even when as little as 75–150 μg of Darmstoff was used. With these small amounts of Darmstoff, the upper spot was not visible. When parallel chromatograms were tested for biological activity on the rabbit duodenum, only the eluate from the area

which corresponded to the lower of the two phosphate spots stimulated the intestine. This is shown in Fig. 2.

In chromatograms run on Whatman No. 1 paper instead of Whatman No. 3, the active phosphate spot was formed in a slightly higher position (R_F 0·4). The same R_F value had previously been found for Darmstoff on Schleicher and Schüll paper No. 2043*b* (Vogt, 1955).

In chromatograms of Darmstoff developed with propanol–ammonia mixture two phosphate spots appeared near the solvent front (R_F 0·75–0·8 and 0·85–0·9) (see Fig. 4*A*). Sometimes the separation of the two spots was incomplete. In those chromatograms in which the two spots had clearly separated, both yielded smooth-muscle-stimulating activity, but the activity obtained from the two areas showed the following differences. When the paper section corresponding to the lower spot was immersed in the organ bath there was a short initial relaxation of the intestinal preparation followed by contraction.

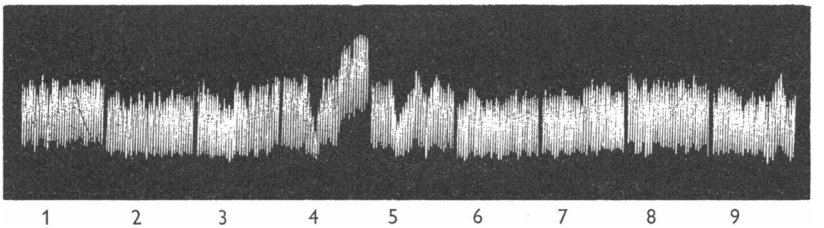


Fig. 2. Rabbit duodenum in 15 ml. Tyrode solution. Assay of eluate from the nine sections of the paper chromatogram of Darmstoff shown in Fig. 1. Each time 2% of the eluate was administered to the bath.

The section corresponding to the upper phosphate spot did not produce the initial inhibition but it caused only contraction, and this contraction proceeded more slowly than that obtained from the lower spot. These differences indicate that the substances present in the two spots have different pharmacological properties, although the differences in the time courses of the contractions may have been due to differences in the speed of extraction of the substances from the paper by the bath fluid.

In chromatograms developed with water-saturated *n*-butanol, the phosphate-containing compounds formed a long streak which occupied about one-third of the whole chromatogram, reaching from R_F 0·3 to 0·7. Again, this phosphate-containing area, when eluted and tested, exerted the smooth-muscle-stimulating activity.

In one experiment the Ba salt of Darmstoff was used; 200 μg were run in a chromatogram with *n*-butanol and a phosphate spot of 5 cm length was obtained with its centre at R_F 0·26. Apparently the movement of the Ba salt was retarded as compared with that of free Darmstoff. In another experiment

free Darmstoff was run with *n*-butanol on a paper which had first been sprayed with 1% $BaCl_2$ solution at the site of application of the sample. Again the phosphate spot as well as the site of biological activity was found in a lower position (R_F 0·08) than in the chromatograms obtained with free Darmstoff.

Since the biological activity coincided in all experiments with areas where the positive phosphate reactions were obtained, it is concluded that the active principle, Darmstoff, is a phospholipid itself.

Aldehyde. (Schiff reaction.) A sample of 3·7 mg of Darmstoff was dissolved in 0·5 ml. of 90% acetic acid containing 0·5 m-mole $HgCl_2$ and was incubated at 37° C for 24 hr in a sealed ampoule filled with N_2. When 0·5 ml. of fuchsin-sulphurous acid was then added, a deep violet colour developed after about half a minute, indicating the presence of aldehydes.

In paper chromatograms the aldehyde reaction with fuchsin-sulphurous acid was given in the same regions which reacted with the phosphate reagent (Fig. 1 *A*). The violet colour developed in about a minute when the chromatogram had been exposed for 24 hr to acetic acid before staining. When this was omitted, a positive reaction was seen only after the chromatogram had been exposed to the fuchsin-sulphurous acid solution for 6–10 hr. This different time course in the development of the colour suggests that the aldehyde group was originally bound as acetal, and had to be liberated by hydrolysis. In the experiments in which the chromatograms had been exposed to acetic acid this hydrolysis had occurred before the addition of the Schiff reagent. In the experiments without previous treatment with acetic acid the hydrolysis occurred after the addition of the Schiff reagent, owing to its acidic reaction, and this hydrolysis then proceeded gradually.

A chromatogram of the Ba salt of Darmstoff developed with *n*-butanol also gave a positive Schiff reaction, which coincided with the phosphate spot obtained.

Amino groups. (Ninhydrin reaction.) Paper chromatograms of Darmstoff developed with the methylethylketone mixture were unsuitable for ninhydrin-reaction because of interference with traces of the solvent mixture which remained in the paper after drying. Chromatograms were therefore developed with propanol-NH_3. They showed ninhydrin-reacting spots in the areas which contained the biologically active phosphate compounds. The colour was always weak and sometimes hardly visible, although Darmstoff was applied to the paper in amounts sufficient to give a distinct phosphate reaction.

A chromatogram of 200 μg of the Ba salt of Darmstoff did not show any ninhydrin spot. The ninhydrin-reacting material remained in the supernatant from which the Ba salt had been precipitated. In the supernatant it was found combined with unprecipitated phospholipids.

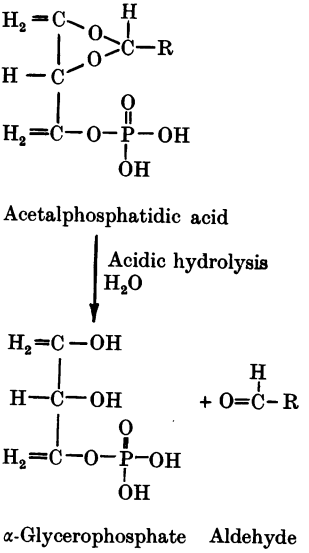
' These results suggest that some base-containing compounds are present in

the acidic phospholipids of Darmstoff extract, but that they form only a small part of the whole phospholipids. The bulk of smooth-muscle-stimulating compounds in the extract is probably free of amino groups.

Mild acidic hydrolysis of Darmstoff

The following experiments show: (1) that mild acidic hydrolysis of Darmstoff leads to the appearance of a free lipid-soluble aldehyde and of α-glycerophosphate, suggesting that the parent compound of these split products is acetalphosphatidic acid; and (2) that Darmstoff owes its biological activity partly to the acetalphosphatidic acid and partly to other acidic phospholipids.

Separation of an aldehyde from the phosphate part. The presence of phosphate in the lipid-soluble and acidic Darmstoff suggested phosphatidic acids as a possible structure, and since acetal groups were also found, it seemed likely that the sample contained acetalphosphatidic acid. According to Feulgen & Bersin (1939) acetalphosphatidic acids are cyclic acetals formed by a fatty aldehyde with the two hydroxyl groups of glycerophosphate. If such compounds were present in the Darmstoff sample, then selective scission of the acetal linkage should give rise to the formation of free fatty aldehyde and glycerophosphate according to the equation



The separation of an aldehyde from the phosphate of Darmstoff after acetal cleavage was demonstrated in the following manner. A sample of Darmstoff was hydrolysed in acetic acid + $HgCl_2$ at 37° C. After elimination of the Hg by H_2S, portions of the hydrolysate corresponding to 750 μg of Darmstoff were applied in single spots to Whatman No. 1 paper. As a control the same

amount of unhydrolysed Darmstoff was applied to the same paper. The chromatograms were run in light petrol (50–60 °C b.p.). Darmstoff does not move on paper in this solvent. Thus in the chromatograms of unhydrolysed Darmstoff both phosphate and aldehyde reaction were obtained at the origin, where the spots had been applied (Fig. 3*A*). However, a weak narrow band giving a positive Schiff reaction was also present at the solvent front indicating some free aldehyde; probably some destruction of the Darmstoff had occurred. In the chromatograms of the hydrolysate, the phosphate was again found at the origin, but now the aldehyde reaction was given exclusively at the solvent front, forming a bright violet band in the front line (Fig. 3*B*). This finding

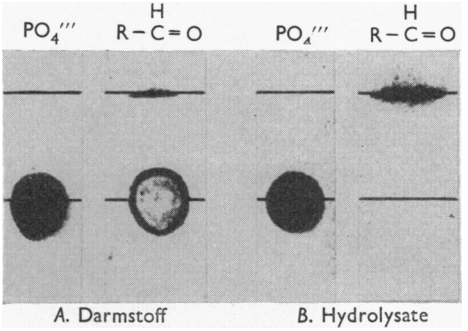


Fig. 3. Paper chromatograms of Darmstoff (*A*), and of acidic hydrolysate of Darmstoff (*B*), with light petrol and stained for either phosphate or for aldehyde as indicated above the chromatograms. (For details see text.)

shows that the aldehyde in Darmstoff is bound as acetal to a phosphate compound from which it is liberated by mild acidic hydrolysis. Further, since the aldehyde moved on paper with the solvent front, when developed with light petrol, it is a lipid-soluble aldehyde, as would be expected from an acetal-phospholipid.

Non-lipid-soluble phosphate fraction of Darmstoff hydrolysate. To characterize the phosphate-containing split products the material obtained by hydrolysis of Darmstoff in 50% acetic acid was dried at room temperature and separated into a lipid-soluble and a non-lipid-soluble fraction by distribution between water and butanol. Equal portions of both fractions, equivalent to 1·5 mg of Darmstoff, were applied to Whatman No. 1 paper and chromatograms were run in propanol–NH_3. For comparison, unhydrolysed Darmstoff, α- and β-glycerophosphate, and inorganic phosphate, were run on the same paper sheets.

Chromatograms of the non-lipid-soluble fraction, i.e. of the material contained in the water phase after distribution of the hydrolysate between water and butanol, showed only one phosphate spot (Fig. 4*B*). This spot was in the position of α-glycerophosphate (R_F 0·25) (Fig. 4*C*), whereas β-glycerophosphate

had a higher (0·31) (Fig. 4*D*) and inorganic phosphate a lower (0·08) R_F value. In chromatograms obtained from a mixture of hydrolysate and α-glycerophosphate again only one phosphate spot appeared, showing that the non-lipid-soluble phosphate compound is α-glycerophosphate itself. No other non-lipid-soluble phosphate compounds were found.

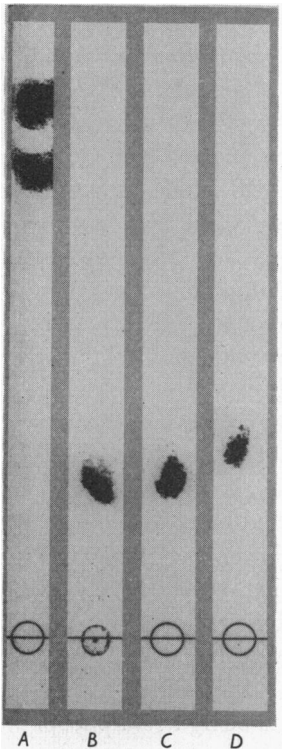


Fig. 4. Chromatograms of fractions of Darmstoff hydrolysate and of α- and β-glycerophosphate, run with propanol-NH_3 and stained for phosphate. *A*, lipid-soluble; *B*, non-lipid-soluble fractions of hydrolysate; *C*, α-glycerophosphate; *D*, β-glycerophosphate. (For details see text.)

Since no glycerophosphate was observed in chromatograms of intact Darmstoff it must have been formed as the result of the mild acidic hydrolysis. The following experiment proves that it is a split product derived from the active principle of the Darmstoff extract. A chromatogram of intact Darmstoff was run with methylethylketone–diethylamine. When the biologically active region of this chromatogram, i.e. the lower of the two phosphate-containing zones, was eluted, α-glycerophosphate was obtained on hydrolysis of this eluate.

Lipid-soluble phosphate fraction of Darmstoff hydrolysate. Paper chromatograms of the lipid-soluble fraction of hydrolysed Darmstoff, i.e. of the material

contained in the butanol phase after distribution of the hydrolysate between butanol and water, showed two phosphate spots in the same positions as unhydrolysed Darmstoff, whether propanol–NH_3 or methylethylketone–diethylamine was used as solvent (Fig. 4*A*). Apparently these phosphate compounds of the Darmstoff extract were resistant to the mild hydrolysis in acetic acid at 37° C. The two spots also contained the ninhydrin-reacting bases.

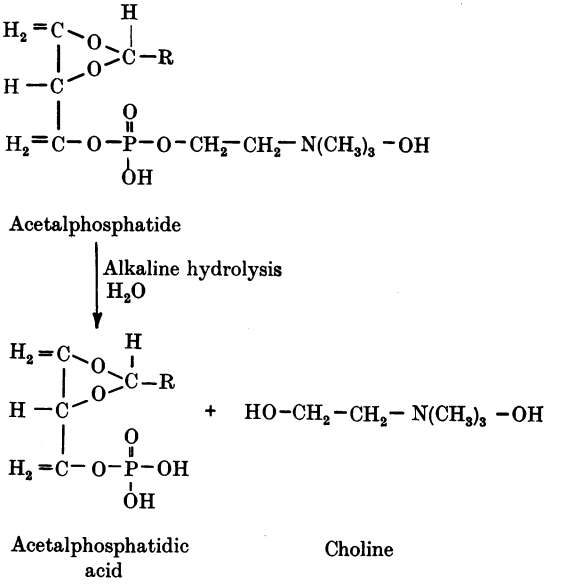
Biological activity of Darmstoff after mild acidic hydrolysis. The treatment with acetic acid led to a 50% loss of the smooth-muscle-stimulating activity. Since the compound destroyed in the treatment was shown to be acetalphosphatidic acid, this compound is a biologically active part of the Darmstoff extract. On the other hand, the whole biological activity of Darmstoff cannot be attributed to acetalphosphatidic acid, since the remaining activity of the hydrolysate was not due to the split products formed, i.e. aldehyde and α-glycerophosphate. Both compounds were found to be inactive on the rabbit duodenum, at least in doses comparable to active doses of Darmstoff.

When the hydrolysate was distributed between butanol and water, the residual activity was found in the butanol layer, i.e. in the lipid-soluble fraction. Evaluation of the chromatograms of this fraction on the rabbit duodenum showed that the biological activity coincided with the position of the two phosphate spots obtained in the chromatograms developed with propanol–NH_3. When the chromatograms had been developed with the methylethylketone–diethylamine mixture, the lower of the two phosphate spots corresponded to the zone of biological activity. Thus, the phospholipids of Darmstoff extract, which were not split by mild acidic hydrolysis and moved on paper like unhydrolysed Darmstoff, were responsible for the residual effects after mild acidic hydrolysis, indicating that Darmstoff is a mixture of acidic phospholipids, one active component being acetalphosphatidic acid.

Chemical preparation of acetalphosphatidic acid

The conclusion that part of the Darmstoff activity is due to acetalphosphatidic acid is substantiated by the finding that chemically prepared acetalphosphatidic acid acts like Darmstoff on the rabbit duodenum. A sample of acetalphosphatide was hydrolysed with alkali under conditions which, according to Feulgen & Bersin (1939), lead to formation of the corresponding acetalphosphatidic acid. The sample, prepared from beef heart, was kindly supplied by Professor Klenk. It contained choline as base and was contaminated with lecithin which was removed in the following way: 300 mg of the acetalphosphatide was emulsified in 3 ml. of N/3-NaOH in an ampoule, which was filled with N_2 and sealed. The emulsion was kept at 37° C for 2 hr. This treatment destroys the lecithin by cleavage of its fatty acids. After cooling, the hydrolysate was adjusted to pH 6 with HCl and extracted with 3 ml. of

butanol. The butanol extract was washed with 2 ml. of water and evaporated to dryness at room temperature in a stream of N_2. The residue was redissolved in 0·5 ml. of chloroform and 4 ml. of acetone were added. A precipitate of acetalphosphatide formed, which when dried weighed 45 mg. It had no effect on the rabbit duodenum when tested in a dose of 10 μg. This precipitate was used as starting material for the preparation of the acetalphosphatidic acid. For this purpose 13 mg was emulsified in 1 ml. of 3·3% KOH, sealed in an ampoule filled with N_2, and kept in a boiling water-bath for 4 hr to cleave the base from the phosphatide (Feulgen & Bersin, 1939). This results in the formation of acetalphosphatidic acid, according to the following reaction:



The hydrolysate was then acidified to pH 5 with HCl and the phosphatidic acid extracted with butanol, to free it from K and choline. The butanol extract contained 8·97 mg of dry material. This material was tested on the rabbit duodenum; it caused contraction when added in a dose of 2 μg to the 15 ml. bath. Another preparation obtained by hydrolysis under similar conditions contracted the gut even in a dose of 0·45 μg. This effect is illustrated in Fig. 5.

The acetalphosphatidic acid obtained in this way was not in a pure state; other split products might have been present in the hydrolysate. However, the following two findings show that the smooth-muscle-stimulating effect must have been due to the formed acetalphosphatidic acid. (1) The action was absent in the starting material, i.e. it was not due to accidental impurities or to the acetalphosphatide itself. (2) The active principle behaved as a lipid-soluble acid. When the hydrolysate was distributed between water and

methylethylketone at acidic pH, the smooth-muscle-stimulating activity was found in the organic layer, whereas after distribution at alkaline pH it was found in the aqueous phase. This excludes all split products of acetalphosphatide other than acetalphosphatidic acid. Only fatty acids derived from the destroyed lecithin would show the same behaviour in distribution. The possibility that they were responsible for the biological activity is further excluded by the following findings. The activity was destroyed after standing for 48 hr in 50% acetic acid at room temperature, i.e. after mild acidic hydrolysis. In paper chromatograms of the alkaline hydrolysate developed with methylethylketone–diethylamine the biological activity was found in an area which gave a positive phosphate and Schiff reaction. Thus the only compound in the alkaline hydrolysate which has all the properties of the active principle is acetalphosphatidic acid. Finally, the conclusion that part of the Darmstoff activity is due to acetalphosphatidic acid is supported by the fact that the position of the biologically active phosphate- and aldehyde-containing zone of the alkaline hydrolysate of acetalphosphatide, in paper chromatograms developed with methylethylketone–diethylamine, was the same as that of natural Darmstoff.

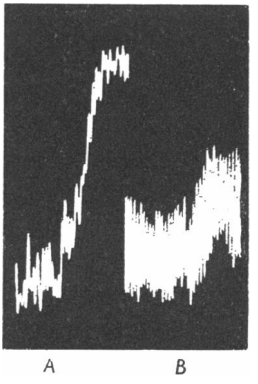


Fig. 5. Contractions of the rabbit duodenum, suspended in 15 ml. of Tyrode solution to 1·35μg (at *A*) and to 0·45μg (at *B*) of chemically prepared acetalphosphatidic acid.

DISCUSSION

The experiments described show that the smooth-muscle-stimulating activity of Darmstoff is not due to a single substance but to a mixture of acidic phospholipids. Two different active fractions were obtained in chromatograms developed with propanol–NH_3 but these two fractions certainly contain more than two smooth-muscle-contracting phospholipids. This is evident from the fact that after acidic hydrolysis of Darmstoff at least one compound was destroyed, leaving two other active compounds unaltered, as shown in the chromatograms of the acidic hydrolysate developed with propanol–NH_3. On the other hand, not all phospholipids present in the extract appear to be biologically active. In paper chromatograms developed with methylethylketone one inactive phospholipid compound was found. This compound had either no smooth-muscle-contracting effect or the amount available for testing was too small to give an effect.

Evidence that part of the Darmstoff extract consists of acetalphosphatidic acid was derived from the demonstration of its split products, namely a lipid-soluble aldehyde and α-glycerophosphate. These split products were obtained

by very mild acidic hydrolysis; this treatment only cleaves the acetal linkage in phospholipids. Since no other non-lipid-soluble phosphate compound but α-glycerophosphate was found in the acidic hydrolysate, acetalphosphatidic acid is the only acetal-containing phospholipid in Darmstoff extract.

The finding that chemically prepared acetalphosphatidic acid stimulates smooth muscle further supports the conclusion that part of the Darmstoff activity is due to this compound. This does not necessarily mean that both the chemically prepared and the natural acid are identical. They may contain different fatty aldehydes and probably both of the acetalphosphatide preparations contain compounds with various aldehydes. It can, therefore, not be expected that the two preparations have quantitatively the same potency. Nevertheless, natural Darmstoff and chemically prepared acetalphosphatidic acid were active on the rabbit duodenum in amounts of the same order.

Recently, Klenk & Debuch (1954), as well as Debuch (1956), have shown that genuine acetalphosphatides do not have the cyclic acetal structure as suggested by Feulgen & Bersin (1939), but that they are semiacetals in which only one hydroxyl-group of the glycerol is combined with a fatty aldehyde, the other being combined with a fatty acid. The cyclic acetal structure of Feulgen results from treatment with alkali which splits off the fatty acid. This treatment is the procedure commonly used for purification of acetalphosphatides. Since in the present experiments treatment with alkali was the first step in preparing the Darmstoff extract, its acetalphosphatidic acid may also have originally been a semi-acetal containing one fatty acid.

The following tentative conclusions can be drawn about the polar and fatty portions of the phospholipids of Darmstoff which remained unaltered by mild acidic hydrolysis. Regarding the polar part one may assume that it consists not only of phosphatidic acids, but also of inositol- or serine-containing phospholipids. Both inositol-phosphatides and serine-phosphatides are acidic in nature, having one net negative charge, whereas phosphatidic acids have two free negative charges. No evidence for or against the presence of inositol-phosphatides in Darmstoff extract is as yet available. On the other hand, the finding of a positive, though rather weak, ninhydrin reaction with phospholipids of Darmstoff may indicate the presence of serine-containing phospholipids. Concerning the fatty part, it is possible that the yet unidentified phospholipids are of the sphingomyelin type, because they are not only stable to mild acid but also to alkali treatment. This stability is peculiar to sphingomyelin. Ester-phosphatides which also would resist the mild acidic hydrolysis used in the present experiments cannot be present in the Darmstoff extract since they would have been destroyed by the boiling in 0·2 N-NaOH which was the first step in preparing the sample. On the other hand, the absence of esterphosphatides in the Darmstoff extract does not exclude their presence in the tissue, and they may be pharmacologically active. They would be detected,

however, only under more suitable conditions of extraction, avoiding treatment with alkali.

Further work on the nature and occurrence of smooth-muscle-stimulating acidic phospholipids will have to deal with the difficulty, known in phospholipid research, of separating single compounds without unintentionally selecting, losing or altering some of them by the mode of preparation and purification.

The acidic phospholipids form a new class of pharmacologically active of substances natural occurrence. The fact that several of them have a similar effect suggests that their activity is mainly dependent on properties which are common to all of them: acidic nature and lipid-solubility. The special nature of the fatty portion, which is the cause of the lipid-solubility and the nature of the acidic groups, may be of minor importance. This suggestion is supported by the fact that other lipid-soluble acids, namely certain unsaturated fatty acids, also have smooth-muscle stimulating properties (Gabr, 1956; Vogt, 1956).

The finding that acidic phospholipids have muscle-stimulating properties calls attention to their possible role in cell excitability in general. Excitability and excitation are associated with cation movements across the cell membranes; these movements seem to be dependent on negatively charged membrane constituents. Since phosphatides occur in membranes, the phosphatidic acids, which can be regarded as split products of phosphatides, may play a role as carriers of such negative charges. This problem has been discussed elsewhere (Vogt, 1957).

SUMMARY

1. The chemical nature of Darmstoff was investigated using an extract prepared from horse small intestine.

2. With paper chromatography and mild acidic hydrolysis it could be shown that Darmstoff consists of several acidic phospholipids, one of which is an acetalphosphatidic acid. The other acidic phospholipids have not been identified.

3. Chemically prepared acetalphosphatidic acid was shown to have smooth-muscle-stimulating activity similar to that of natural Darmstoff.

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