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ON THE PREPARATION OF SECRETIN AND PANCREOZYMIN

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The existence in the mucosa of the small intestine of a material, distinct from secretin, which influences the external secretion of the pancreas, was first demonstrated by Harper & Raper (1943). They showed that secretin stimulated the production of water and bicarbonate only, and that the other material, which they named pancreozymin, increased the enzyme content of the pancreatic juice.

In their method of separating secretin and pancreozymin Harper & Raper followed closely the procedure adopted by Mellanby (1932) for the preparation of secretin, with the exception that they added a definite amount of bile salt to ensure complete precipitation of the secretin. The mucous membrane from the first metre of the small intestine of freshly killed pigs was extracted with alcohol. Calcium chloride was added to the extract, which was then filtered and concentrated to a quarter of its original volume. At this stage, to each 500 c.c. concentrate was added 1 g. bile salt (commercial tauroglycocholate) dissolved in 10 c.c. water followed by 15 c.c. 1% acetic acid. The precipitate obtained, containing the secretin, was separated in a centrifuge and purified by extraction with alcohol and re-precipitation with acetone.

The pancreozymin was not adsorbed on the bile acid precipitate but remained in the supernatant liquid. This was saturated with sodium chloride and allowed to stand in the dark at room temperature for 2–3 days. The sticky precipitate which separated contained the pancreozymin. It was dissolved in a small quantity of water and allowed to dry in a vacuum desiccator. This 'NaCl precipitate' was extracted with absolute alcohol and the material thus obtained was called the 'alcohol-soluble' preparation.

The bile salt used in these experiments came from a bottle of commercial tauroglycocholate in the laboratory and during the 3 years that this supply

lasted no difficulty was experienced in separating secretin and pancreozymin. Later, when new commercial samples of bile salts were used, low and variable yields of secretin were obtained. Appreciable amounts of secretin remained in solution and were precipitated with the pancreozymin when sodium chloride was added.

The unreliability of the method and the lack of uniformity in these results was attributed to variation in the composition of commercial samples of bile salts. In his earlier method of secretin preparation Mellanby (1928) added bile salt at one stage. He was aware of the variability of bile salt preparations but claimed that his method was adequate for 'all commercial preparations of bile, which, when dissolved in water, became opalescent on adding acetic acid to the extent of 0·1 %'. He later (1932) modified his method, and merely acidified the concentrated intestinal extract in order to obtain a precipitate, thus avoiding the addition of bile salts. The success of this method, however, seems to depend on the very variable amount of bile present in the intestine. If the intestinal mucosa is washed before extracting with alcohol the amount of precipitate at the stage of acidification with acetic acid is greatly reduced and the yield of secretin correspondingly low. It was for this reason that Harper & Raper added a definite amount of bile salt before acidification of the extract.

Difficulties with commercial bile salt preparations have also been encountered by the American workers Mortimer & Ivy (1929). They followed Mellanby's (1928) description, with the exception that they added 'Merck's technical sodium glycocholate'. They found that the method gave inconsistent results. Still (1931), who also attempted unsuccessfully to repeat Mellanby's experiments, suggested that the poor yields by this method were due to the use of alcohol as the agent for extraction of the mucosa.

It was obvious that a modification of Harper & Raper's original procedure was desirable. An investigation of the conditions for the adsorption of secretin by bile acids and a revised method for the preparation of secretin and pancreozymin were required. It was to these ends that the following experiments were directed.

METHODS

All the preparations were tested on cats which had been fed a few hours previously. The animals were anaesthetized with chloralose (0·075 g./kg. body weight intravenously). The splanchnic nerves were cut extraperitoneally and the pylorus occluded. The pancreatic duct was cannulated, the ligature passing round the bile and pancreatic ducts so that no further bile could enter the duodenum.

A continuous flow of pancreatic juice was maintained by injections of secretin intravenously at intervals of 12 min. in amounts sufficient to give a flow of 1·0–1·2 c.c./12 min. At intervals doses of the pancreozymin to be tested were given, together with the 'background' secretin. An injection of pancreozymin was always followed by two separate control doses of secretin alone before another injection of pancreozymin was given. The injections of secretin were given over a period of 10–20 sec. and the pancreozymin injections in 2 min. The amylase content of successive samples of pancreatic juice was measured by Wohlgemuth's method and the minute output of amylase, $D(J/T)$, calculated. The methods used were described by Harper & Vass (1941).

Bile salts were prepared from ox and pig bile by a slight modification of the method described by Cole (1933). 100 c.c. bile and 25 g. 'norite' charcoal were mixed and evaporated to dryness on a water-bath. The dry residue was extracted with 'methylated spirit' (95% ethyl alcohol, 4% methyl alcohol) in the proportion of 175 c.c. alcohol to 100 c.c. bile, on a boiling water-bath for 20 min. After cooling, the suspension was filtered through paper pulp and two volumes of ether added to the filtrate. The curdy white precipitate was allowed to settle overnight at 0° C. The supernatant liquid was then siphoned off and the residue separated by centrifuging. The precipitate was washed several times with ether and dried in a desiccator. From 100 c.c. ox bile the average yield of bile salts by this method was 2·14 g. A heavier average yield, 3·6 g., was given by the same amount of pig bile.

RESULTS

Secretin and pancreozymin were prepared by various modifications of Harper & Raper's orginal method. As a basis for comparison of the different secretin preparations 1 unit of secretin was defined as the activity present in 0·1 mg. of an arbitrarily chosen standard preparation. In a number of experiments the average amount of pancreatic juice secreted in the 12 min. period following intravenous injection of 0·1 mg. of this preparation was 1·2 c.c. In assaying samples of secretin, the preparations being tested were injected in doses which stimulated a flow of juice similar in volume to those produced in that particular experiment by injections of the standard preparation. By this method, the potency of any secretin fraction could be measured in units per mg. By combining this measurement of potency with the weight of the preparation per 100 g. of wet mucosa scraped from the intestine, the yield of secretin could be calculated as units per 100 g. of mucosa: e.g. in a fraction with a potency of 7·8 units/mg. and in which 57 mg. had been obtained per 100 g. mucosa, the total yield of secretin was $7{\cdot}8 \times 57 = 445$ units/100 g. mucosa. As the activity of secretin preparations gradually diminishes even when they are kept in the form of a dry powder, freshly made preparations were from time to time adopted as the standard. At each change over to a new standard the activities of the new and old standards were carefully compared and a conversion factor worked out so that the activity of all the preparations tested throughout the investigation could be expressed in terms of the original standard.

At the same time an attempt was made to gauge roughly the amount of any residual 'secretin activity' present in samples of pancreozymin, as indicated by the slight increase in volume of juice secreted in response to a combined secretin and pancreozymin injection, compared with the response to the same amount of secretin alone. The amounts of juice produced by the pancreozymin were very much less than those produced by injections of the standard secretin, but it was assumed that the amounts of juice would be proportional to the amounts of 'secretin activity'. Combining such measurements with the weight of pancreozymin preparations per 100 g. of intestinal mucosa an estimate could be made of the total amount of 'secretin activity' in any pancreozymin extract.

In comparing the pancreozymin activity of different preparations 1 unit of pancreozymin was defined as the activity of 1 mg. of an arbitrarily chosen

standard preparation. Five mg. of the standard was the yield from 3·8 g. of intestinal mucosa (wet weight). The preparations to be tested were injected in doses equivalent to 3·8 g. of intestinal mucosa, and the increase in enzyme output in the following 12 min. compared with the increases produced by injections of the standard preparation at the beginning and at the end of the experiment. As pancreozymin preparations, like secretin, show a gradual loss of activity even in the dry state, freshly made preparations were from time to time adopted as the standard and their activity related to that of the original standard.

When either pig or ox bile salts were used alone for preparing secretin and pancreozymin by Harper & Raper's (1943) method, an unsatisfactory separation of the two materials was obtained. With pig bile salts a thick curdy precipitate appeared when the secretin-pancreozymin concentrate was brought to pH 4·2–4·3 with acetic acid. On the other hand, with ox bile salts, the slight precipitate which appeared during concentration disappeared on the addition of the bile salt and the solution became clearer. Commercial samples of bile salts gave variable results with low yields of secretin, much being left behind with the pancreozymin.

Observations were made on the solubility at different pH's of mixtures of ox and pig bile salts in the ratio of 600 mg. ox bile salt to 200 mg. pig bile salt per 500 c.c. of solution (the proportion finally used to precipitate secretin). In these experiments all the various samples of ox and pig bile salts used in preparing secretin and pancreozymin were tested. It was found that the solubility varied with the composition of the buffer solutions used. Citrate and phenylacetate ions seemed to increase the solubility of cholic and other related acids, so that no precipitation of bile acids occurred in buffer solutions containing these ions even when the pH was as low as 3·4. On the other hand, when different pig and ox bile salts combined in the same proportions were added to a series of acetic acid/acetate buffer solutions, differing by 0·05 of a pH unit (checked by the glass electrode) it was found that all were precipitated to the same extent at a pH of 3·85, although they remained in solution at pH 3·95. It was also found that when peptone was added to the bile salt preparations, precipitation occurred in all cases at pH 4·05, both with acetic acid/acetate buffer and with acetic acid alone. This suggests that protein derivatives in the solution obtained by the extraction of the mucosa may also facilitate precipitation of the bile acids.

It seemed clear that to ensure a satisfactory separation of secretin and pancreozymin a very fine bile acid precipitate with a large adsorptive surface was required. By trying various mixtures of pig and ox bile salts a method was finally evolved whereby about 95% of the total secretin could be precipitated on adding a mixture of pig and ox bile salts. Any residual secretin was removed by the addition of more bile salts. The total amount added was 1 g./500 c.c.

concentrate, i.e. the amount originally prescribed by Harper & Raper. When larger amounts, up to 1·5 g./500 c.c., were used, no better separation of the two materials was obtained. The pancreozymin remained in the supernatant liquid and was precipitated when the solution was saturated with sodium chloride.

The following is a detailed description of the method. The mucous membrane from the first metre of the small intestines of freshly killed pigs is scraped off, ground with sand and extracted for 30 min. with four times its weight, expressed in c.c., of 'methylated spirit' (95% ethyl alcohol, 4% methyl alcohol). At this and all subsequent stages the material is protected from the light. The extract is filtered and stored in the ice chest until it is to be concentrated. Before concentration an equal volume of 0·1 M-$CaCl_2$ solution is added and the mixture allowed to stand for 15 min. It is filtered under slight suction through paper pulp and the filtrate is concentrated in 500 c.c. portions to a quarter of its original volume. The concentration is carried out by distillation under reduced pressure in the shortest possible time, i.e. $1\frac{1}{2}$–$2\frac{1}{2}$ hr. The temperature of the water-bath is controlled at 40° C.

A mixture of 600 mg. ox and 200 mg. pig bile salts, dissolved in 10 c.c. water, is added to each 500 c.c. concentrate followed by sufficient 2% acetic acid (usually about 20 c.c.) to bring the pH to 4·0–4·2, measured by the glass electrode. The mixture is allowed to stand for 10–15 min. and precipitate I, containing most of the secretin, separated in a centrifuge. At this stage some secretin remains in solution. A 10% solution of a mixture of bile salts is added in the proportion of 67 mg. ox and 133 mg. pig bile salt per 500 c.c. concentrate. (Precipitate II thus obtained may be treated as precipitate I if it is desired to assay its secretin content, or, since its potency is low, it may in routine preparations be discarded.)

Precipitate I is extracted with absolute alcohol. The alcoholic solution is spun off and the active secretin precipitated by the addition of four volumes of acetone. The precipitate is separated in a centrifuge, washed with acetone and dried in a desiccator over sulphuric acid.

The supernatant liquid from precipitate II, now almost free from secretin, is poured into a conical flask and saturated with NaCl (30 g./100 c.c.). It is allowed to stand 2–3 days at room temperature in the dark while a sticky precipitate settles out. From this the 'NaCl precipitate' is prepared by dissolving the precipitate in the minimum amount of water and evaporating to dryness in a vacuum desiccator over H_2SO_4. When this is dry it is extracted by shaking for 30 min. at room temperature with absolute alcohol (50 c.c./l.g.). The residue is separated in a centrifuge and the alcoholic solution evaporated to dryness in a vacuum desiccator. This is the 'alcohol-soluble' preparation which contains the pancreozymin. It is almost free from secretin.

The potency and yield of secretin from ten preparations made by this method were estimated, and the results are set out in Table 1. Weights of secretin and

pancreozymin per 100 g. wet mucosa varied considerably; the variation in the ten secretin preparations (precipitate I) was from 34 to 109 mg. (average 62 mg.), and in twelve pancreozymin preparations from 112 to 236 mg. (average 179 mg.). This variation may be due to differences in the composition of the bile salts used in the preparations, and in the amount of bile salt initially present in the mucosa before extraction.

TABLE 1. The potency, yield and percentage distribution of secretin in ten preparations

	Potency (units/mg.)	Yield (units/100 g. mucosa)		Percentage distribution
Secretin, precipitate I	6·8 (4·1–10·0)	380 (289–450)	402 (294–476)	94·5 (88·5–98·5)
Secretin, precipitate II	0·8 (0–2·3)	18 (0–50)		4·5 (0–11)
Pancreozymin	—	4 (0–8·5)		1·0 (0–2)

The figures in brackets indicate the range of results.

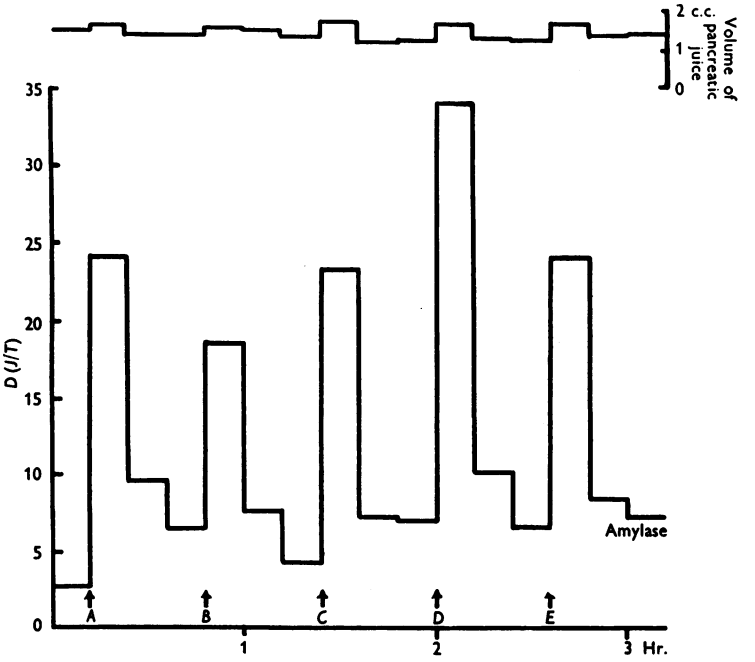

Fig. 1. Pancreatic secretion was maintained by injections of secretin at 12 min. intervals. Injections of the standard pancreozymin in amounts equivalent to 3·8 g. mucosa were given at *A* and *E*. Injections of the test pancreozymin were given at *B*, *C* and *D*. At *B* 3·8 mg. (=1·9 g. mucosa) were injected; at *C*, 7·6 mg. (=3·8 g. mucosa); and at *D*, 15·2 mg. (=7·6 g. mucosa). The increase in enzyme output following the injection at *C* is equal to that produced by the injections of the standard pancreozymin.

In testing the pancreozymin preparations it was found that there was a reasonably constant yield of pancreozymin when samples were injected equivalent

in terms of g. of wet mucosa to the doses of the standard preparation, i.e. 3·8 g. mucosa (Fig. 1). The weights of the different pancreozymin preparations of course varied considerably. In twenty-nine such tests on twelve pancreozymin preparations the average minute output of amylase in the 12 min. period after injection was 14·2 compared with an average minute output in the preceding 12 min. control period of 4·3. Such results were obtained only if the amounts of residual 'secretin activity' present in the pancreozymin was small. If the separation of the secretin and pancreozymin had been inadequate there was an apparently greater activity in the pancreozymin preparations, as the increase in volume of juice produced by the secretin increased the calculated minute output of amylase, e.g. in twelve tests on three earlier preparations which contained appreciable amounts of secretin there was an average post-pancreozymin amylase output of 32·0, compared with an average output of 5·0 in the preceding 12 min. period. The yield of pancreozymin per 100 g. of mucosa was about 130 units.

To compare the potency and yield of secretin by our modified method with that obtained by a method which did not involve the addition of bile salts, one batch of secretin was prepared by the S1 method of Greengard & Ivy (1938). In order to make the results comparable with our method the first metre of ten intestines was used for the experiment instead of the first 6 ft. described by Greengard & Ivy. In five tests the potency of this S1 secretin was found to be 2 units per mg. The total amount of S1 obtained from the ten lengths of intestine was 292 mg. The mucosa is not scraped from the intestine in Greengard & Ivy's method, but assuming the average amount of mucosa per intestine (40 g.) this would give a yield of secretin by the S1 method of 146 units per 100 g. mucosa.

DISCUSSION

Now that a satisfactory procedure has been worked out for the separation of secretin and pancreozymin it is of interest to note the differences in behaviour between various bile-salt preparations. It seems from our experiments that it is important that the bile acids from both pig and ox bile should be present in the mixture used for precipitation. Ox bile contains principally cholic acid conjugated with glycine and taurine, while the chief bile acid in pig bile is hyodesoxycholic acid conjugated with glycine. There is little or no taurine in pig bile. The presence of the sulphonic acid group of taurine in ox bile salts probably explains their greater solubility in acid solutions. It has been stated by Hammarsten (1895) that taurocholates will keep glycocholates in solution at low pH. This property may be an attribute of the taurine-conjugated acid and may be its only function in this precipitation, where it prevents the pig bile acid from forming an immediate coarse precipitate with poor adsorptive properties.

In their original experiments Harper & Raper were fortunate in having a commercial sample of bile salt available which apparently contained the

right proportions of ox and pig bile acids. Later, during the war, British commercial preparations of bile may have been manufactured almost exclusively from ox bile, since our pig population was so greatly reduced. It is possible that Still (1931) and Mortimer & Ivy (1929) had similarly prepared bile salts. Failure to get good yields of secretin in all these cases may have been due to lack of the pig bile acid. Mellanby's criterion for the effectiveness of bile salt in his (1928) method of preparation is obviously valueless. We have found that several commercial preparations which on this standard should have been effective in precipitating secretin gave poor yields and left much of the secretin in solution with the pancreozymin. Our own experiments have shown that the solubility of bile salts at various pH's is of little value at present in determining their effectiveness in the precipitation of secretin.

The modified method of preparation described above has been used with different batches of ox and pig bile salts prepared by ourselves. In all cases good separation has been obtained. Secretin and pancreozymin prepared by this method have been found to be sufficiently potent for use in human subjects.

The standardization of secretin and pancreozymin has recently been discussed by Burn & Holton (1948). The methods suggested by Ivy and his colleagues for standardizing secretin (Ivy, Kloster, Drewyer & Leuth, 1930), and pancreozymin (Greengard & Ivy, 1945) are straightforward measurements of the volume and enzyme content respectively of pancreatic juice, without reference to the activity of a standard preparation of secretin or pancreozymin. Still (1931) and Wilander & Ågren (1932), on the other hand, express the activity of their secretin preparations in terms of the activity of a standard preparation.

The main difficulty in measuring the activity of secretin and pancreozymin by their effects on the output of juice or of enzymes is the great variability in the response of the pancreas in different animals to these materials. In acute experiments this variability may be due to the effects of the anaesthetic, to the amount of operative trauma in preparing the animal, to variations in the previous diet of the animal or to other unrecognized factors. To eliminate this source of error preparations would have to be tested on a number of animals and an average response obtained; a procedure which would be wasteful and impracticable when large numbers of preparations have to be compared. For this reason, in the present investigation, we adopted the measurement of secretin and pancreozymin activity in terms of arbitrarily chosen standards.

Another difficulty in measuring the activity of secretin and pancreozymin is that the responsiveness of the pancreas may vary in a single experiment in the course of a few hours, the variation usually being a gradual decrease in responsiveness. To minimize this source of error we have compared test preparations of secretin with injections of standard secretin given 12 min. before or 12 min. after the test preparation, and injections of standard pancreozymin have been given at the beginning and end of the experiment so that any

decrease in responsiveness might be apparent. The gradual diminution in responsiveness of the pancreas may be due to the preparation of the animal in these experiments. The animals were routinely fed before the experiment, and the splanchnic nerves were cut extraperitoneally before the abdomen was opened. This ensured in most experiments that the pancreas was very responsive to both secretin and pancreozymin, but the responsiveness did tend to decrease in many experiments during the period of injection. In other experiments in the laboratory on fasting animals in which the splanchnic nerves have not been cut, the pancreas has been found to be less responsive to secretin but the responsiveness has varied little over 4 or 5 hr. For the routine assaying of secretin and pancreozymin it might be advisable to use the less sensitive but also less variable fasting animal with the splanchnic nerves intact.

Measuring secretin and pancreozymin activity in terms of a standard preparation is made more difficult by the gradual loss of potency of dry preparations of secretin and pancreozymin, which becomes obvious after a year or two. This difficulty, which has been experienced also by Lagerlöf (1942) with secretin, was avoided by changing to freshly made standards every few months. It would be very useful to have standard preparations of secretin and pancreozymin which maintained their potency indefinitely. Burn & Holton suggest that their acetone-dried powders may fulfil this requirement.

It was suggested by Still (1931) that the yield of secretin was small if alcohol was used for the extraction of the intestinal mucosa. In our hands an extraction with N/10 HCl (S1 preparation of Greengard & Ivy) gave a less potent secretin preparation and a smaller total yield of secretin than extraction with alcohol and adsorption of secretin on a bile acid precipitate. Our unfamiliarity with the S1 method of preparation probably explains the poor yield obtained, but the result suggests that alcohol is at least as effective as N/10 HCl in extracting secretin.

SUMMARY

1. Inconsistent results which have been encountered in the original method of Harper & Raper for the separation of pancreozymin and secretin have been investigated, and found to be due to the nature of the bile salts originally used in the process.

2. A modified method of preparing secretin and pancreozymin, sufficiently potent for use in man, is described. Secretin and pancreozymin are extracted from the intestinal mucosa by alcohol. The secretin is separated from the pancreozymin by adsorption on a precipitate of mixed pig and ox bile acids, and the pancreozymin precipitated from the supernatant fluid by saturation with NaCl.

3. The conditions governing the adsorption of secretin on bile acid precipitates have been investigated.

4. 'Units' of secretin and pancreozymin are defined, and the assaying of secretin and pancreozymin is discussed.

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