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Preparation and assay of urogastrone, an inhibitor of gastric acid secretion

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Summary

1. Urogastrone, an acid gastric secretion inhibitor extracted from human urine, was assayed in the anaesthetized rat by circulating through its stomach a succinic-propionic buffer and recording electrometrically the secretion of acid in response to carbachol, and inhibition of carbachol secretion by urogastrone.

2. Quantitative assay procedures are described requiring the administration of only one or two doses of urogastrone to each rat.

3. An extraction procedure for urogastrone is described based on the use of zeocarb, a cationic polystyrene resin.

4. The most purified samples contained 40–80 times the amount/milligram which was just detectable by the assay method.

Introduction

Urogastrone is an active principle extracted from human urine which inhibits gastric acid secretion. Methods for the extraction and assay of urogastrone have previously been described by workers from this laboratory (Mongar & Rosenoer, 1962 ; Rosenoer & Schild, 1962), in which the extraction procedure was based on the absorption of urine on a naturally occurring zeolite, cabunite, followed by desorption. When attempts were made more recently to use cabunite from the same commercial source for the extraction of urogastrone, unexpectedly low yields were obtained. We therefore investigated a variety of extraction procedures for urogastrone and developed a procedure based on absorption and desorption from a polystyrene resin, zeocarb, which gave high and reproducible yields of urogastrone. This method of obtaining urogastrone from urine and purifying it will now be described in detail.

We have modified the bioassay of urogastrone: we continued to use the preparation of the perfused rat stomach in which acid secretion is recorded electrometrically (Ghosh & Schild, 1958), but instead of using the earlier 'differential' procedure in which the rate of secretion is measured, an alternative was adopted which provided an 'integrated' record of total acid secreted during a test period. The method, which is based on reperfusion of the rat's stomach, has already been described (Smith, Lawrence, Colin-Jones & Schild, 1970). The assay design has been changed and two designs for comparative assays are described in this paper which do not necessitate injecting four doses of urogastrone in a single animal. In addition, a

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rapid assay is described, based on an 'animal unit', in which the activity of urogastrone is expressed in terms of the log dose-response curve of carbachol in that animal.

This work has been communicated to the British Pharmacological Society (Lawrence, Schild & Smith, 1970).

Methods

A preparation of perfused rat stomach was used which has previously been described in detail (Smith *et al.*, 1970). Briefly, a measured quantity of succinic-propionic buffer solution was continuously circulated through the stomach of an anaesthetized rat. Secretagogue drugs were injected intravenously and the amount of acid secreted above the basal concentration was recorded electrometrically. In this way, a cumulative pH change in the linear range of the buffer was produced after each dose of stimulant and recorded on a multichannel recorder. The acid output from four rats was usually recorded concurrently. After a period of 45–60 min following injection of stimulant drug, the buffered recirculating solution was replaced and the preparation was then ready for a further dose of stimulant. Inhibitory (urogastrone) solutions were injected 5 min before stimulant carbachol doses. All carbachol injections were carried out at constant speed by automatic burette.

The inhibitory effect of urogastrone was measured in terms of a logarithmic 'carbachol index' (C.I.) defined as follows: $C.I.=0{\cdot}3\ \frac{C_1-C_u}{C_1-C_2}$ where C_1=response to standard dose of carbachol, C_2= response to half the standard dose of carbachol, and C_u=response to standard dose of carbachol preceded by a dose of urogastrone.

Expressing inhibition in terms of the dose-response curve of carbachol gave more consistent results than expressing it simply in terms of pH changes.

Materials

Ion-exchange resin. Sulphonated polystyrene resins from the permutit range zeocarb 225 were used in the sodium form. These resins are subdivided according to particle size and cross-linkage and classified by the numbering system SRC 1–SRC 22. We used 1% cross-linked (SRC 2, 3 and 4) and 2% cross-linked (SRC 6 and 7) resins. These had a similar capacity for absorbing the activity. The resins of smaller particle size (SRC 3, 4 and 7) were used in the later stages of the preparation. The resins will be referred to in the text by the SRC classification alone.

In all of the absorption and desorption procedures of urogastrone in which the zeocarb 225 resins were used the pH of solutions was raised by addition of N NaOH and lowered by addition of N HCl. Activity was absorbed by the resin at pH 6 and eluted from it at pH 9·5. During absorption or desorption the resin was maintained in suspension in the solution for 15 min by mechanical stirring. The resin was removed from the liquid by vacuum assisted filtration using Whatmans No. 90 filter sheets on a Buchner funnel. Resin was washed on the filter with distilled water and the washings added to the filtrate.

Source of materials. Zeocarb (Zeo-karb) was obtained from the Permutit Co. Ltd. Cabunite was obtained from Ghent & Co., Copenhagen.

Results

Bioassay of urogastrone

Urogastrone was assayed by its effect in reducing carbachol induced gastric acid secretion. The activity of urogastrone was expressed in terms of the log dose-response curve of carbachol by the carbachol index as previously defined. Part of an assay is shown in Fig. 1. The first two intravenous doses of 0·25 and 0·5 μg carbachol served to establish a dose-response curve for the carbachol index. The next dose of 0·5 μg carbachol, preceded by an intravenous injection of urogastrone administered 5 min earlier, produced reduced carbachol stimulation. The next dose of 0·5 μg carbachol produced full recovery.

When the effects of carbachol were expressed by the carbachol index, results in different animals were fairly consistent. Figure 2 shows an experiment in which increasing doses of a crude urogastrone preparation were administered to five rats, each of which received the same dose twice in succession. The relationship between log dose and response was approximately linear.

Whenever possible we used a comparative 2+2 assay method in which four randomized doses of standard and test urogastrone were administered in one rat. For a complete assay block it was necessary to give two or three preliminary doses

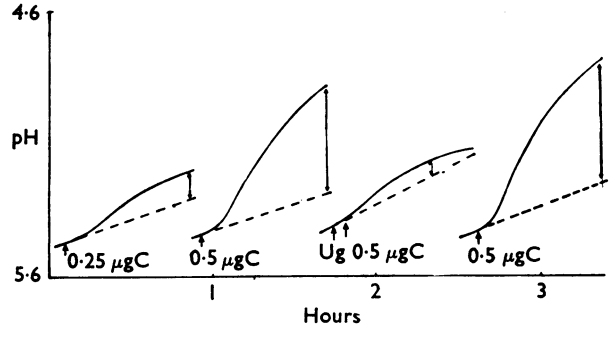

FIG. 1. Inhibition of carbachol induced gastric acid secretion. See text. Preparation of recirculated rat stomach in this and subsequent Figs. (1–4). Responses (pH change) measured 45 min after injection of carbachol (C), except third response which was measured at maximum.

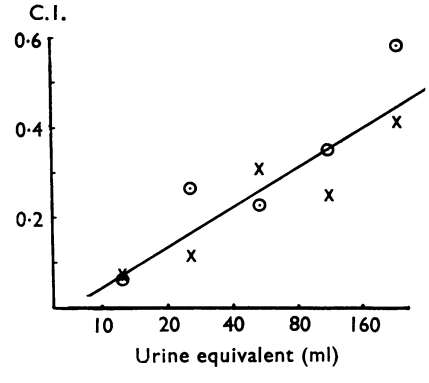

FIG. 2. Responses in terms of carbachol index (C.I.) in five rats given increasing doses of a crude sample of urogastrone ; two successive identical doses (×, ⊙) given to each rat. Dose expressed in terms of equivalent urine volume.

of carbachol in order to establish a regression, followed by at least two further doses of carbachol in conjunction with each urogastrone injection to provide control and inhibited responses. With each carbachol effect lasting at least 45 min, it was frequently impossible to achieve a complete assay block in one rat. Several alternative shorter assay procedures were therefore used.

Single dose assay. A single dose of urogastrone was given to each rat and its effect expressed in terms of the carbachol index. This procedure, which lacks the element of comparison with a standard and is therefore theoretically deficient, was nevertheless extensively used because of its convenience. It was particularly useful during column fractionation procedures, each result showing activity usually being checked on several animals.

The remaining assay procedures involved comparison with a standard.

A 1+1 'staircase' assay. In this design varying combinations of one dose of standard and one dose of test were given to different rats. The activity ratio of standard and unknown was calculated graphically as shown in Fig. 3, where the ordinate represents the log ratio of responses and the abscissa the log ratio of doses of standard and test; the intercept of the regression line with the abscissa represents the equivalence point. This assay method has certain practical advantages since every experiment in which at least one response to standard and one to test has been recorded contributes to the final result. But the efficiency of the design seemed low. Thus in the series shown in Fig. 3, a significant slope ($P<0{\cdot}01$) was obtained after thirteen pairs of injections, but when the results were analysed earlier, after only seven injection pairs, the slope was not significant.

A 2+2 assay in the form of a twin cross-over design of incomplete blocks of two. An assay of this kind (actually a uniformity trial using two samples of the same preparation) is shown in Fig. 4. In this design each rat received a combination of two doses, consisting either of a large dose of standard and small dose of test or *vice versa.* By reversing the order of administration and repeating each combination twice, the assay was carried out with eight rats. The analysis of variance of the assay of Fig. 4 is presented in Table 1. It shows a highly significant ($P<0{\cdot}01$) dose regression and also a significant ($P<0{\cdot}02$) difference between responses to the first and second dose of urogastrone (the first being more effective). Differences between rats were significant ($P<0{\cdot}05$) but there was no significant difference between

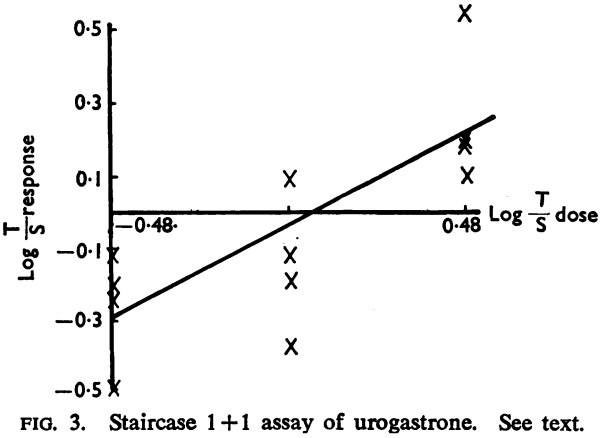

FIG. 3. Staircase 1+1 assay of urogastrone. See text.

preparations (as expected). The 5% fiducial limits were 0·84 and 1·32, which is a reasonably narrow range. This design was the most satisfactory for measuring urogastrone activity quantitatively.

Urogastrone antagonized the stimulant effects of carbachol, histamine, gastrin and pentagastrin on rat gastric acid secretion. Histamine was not used as stimulant in the present experiments owing to its variable activity. Carbachol in doses of 0·25–1·0 μg produced consistent stimulation; it was used as the standard stimulant in spite of causing occasionally disturbing circulatory side-effects. In a few of the later experiments pentagastrin was used as stimulant. It produced consistent secretory stimulation provided that submaximal doses were given. Pentagastrin is likely to prove a useful stimulant if used in conjunction with a sensitive secretion assay such as the conductivity assay described by Lawrence & Smith (1969).

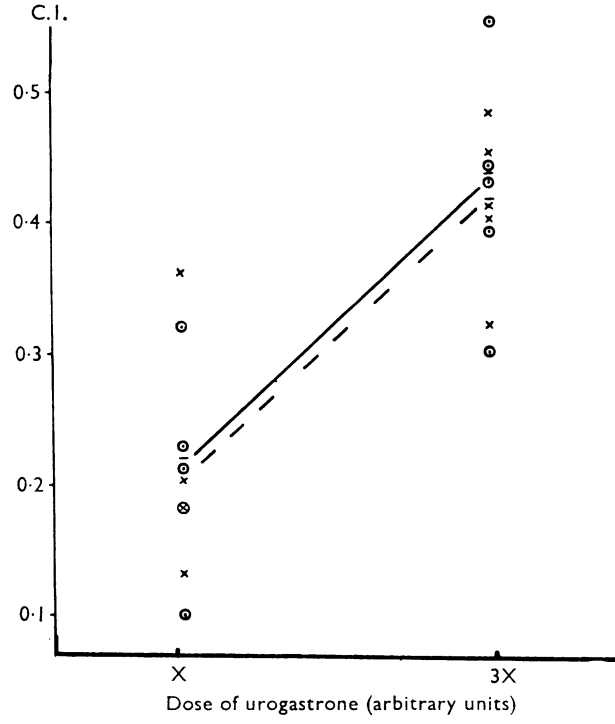

FIG. 4. Twin cross-over 2+2 assay of urogastrone. Incomplete blocks of two . Ratio large/small dose=3. Eight rats. See text.

TABLE 1. *Analysis of variance of twin cross-over assay of urogastrone by incomplete blocks of two*

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*
Rats	687	7	98	5·3*
Preparations	4	1	4	
Regression	1,849	1	1,849	100†
1st & 2nd injection	272	1	272	15‡
Error within rats	92	5	18·4	
Total	2,904			

* $P<0·05$; †$P<0·01$; ‡$P<0·02$.

Extraction of urogastrone

In earlier work in this laboratory urogastrone was extracted from human urine by absorbing it onto cabunite, a naturally occurring zeolite. In the present series of experiments, using the same batch of cabunite as earlier workers here, satisfactory yields were obtained, but when a new batch of cabunite was purchased from the makers the yield decreased sharply. After several trials a new extraction procedure, using a sulphonated polystyrene resin, zeocarb, was worked out. The procedure which was finally evolved seemed fully reliable and gave consistent yields.

Details of zeocarb extraction

The starting material was urine collected from male students. Receptacles for about 20 l. of urine containing a few millilitres of 0·1% chlorhexidine were distributed and replaced twice daily.

Stage 1 : Extraction from urine. The urine was stored in a stainless steel vat (120 l.) in a cold room. When the vat was full (1–2 days) it was acidified with 10 N H_2SO_4 to pH 3·0 ; a precipitate formed, which settled rapidly, one hour after acidification. The top 110 l. of clear liquid was pumped through a column (30·5 × 10·2 cm) of 1% cross-linked zeocarb resin SRC 2 (equilibrated at pH 3) at a rate of 5–10 l./hour. The column was finally allowed to run dry, and the resin removed from the column for elution of the activity.

Stage 2 : Elution of the activity from the resin. The resin was suspended in 2 l. of water and the pH raised to 5·5 by addition of normal NaOH. This removed impurities but left the activity on the resin. Activity was recovered from the resin by desorption at pH 9·5 in 2 l. of water. The active supernatant was neutralized to pH 6 with N HCl and stored in the cold. Five extracts (from 550 l. of urine or one week's supply) were pooled at this stage and the subsequent account refers to this large batch. A precipitate formed in the solution during storage which was removed by decantation and low speed centrifugation. At pH 6 the precipitate contained little activity, but at lower pH it could contain a substantial fraction of the activity.

Stage 3 : Batch extraction with zeocarb. The activity was re-extracted from the solution by absorption onto 200 g of the resin SRC 3 or SRC 4. It was important to wash the resin carefully before the desorption step. The activity was recovered by the standard desorption process at pH 9·5 with the resin suspended in 200 ml of water. The resin was recovered by filtration and added back to the mother liquor for further absorption of activity. This process was repeated three times to give three active supernatant solutions which were pooled, refiltered and neutralized to pH 7.

Stage 4 : Acetone precipitation. The activity was precipitated by the addition of five volumes of acetone and the solution was stored in the cold overnight. The precipitate was collected by decantation and filtration through glass fibre filters (Whatman GF/A). An unusual feature of this precipitation was a requirement for chloride to enable the precipitate to coagulate and adhere to the glass ; in the absence of chloride it stayed as an intractable suspension. This step was included as a convenient method of concentration. (There was some evidence that it removed toxic components.)

Stage 5 : Solvent extraction. The precipitate was dissolved in 200 ml of water and cooled to 0° C. Activity was extracted by shaking the solution with 500 ml of

a cold 1% solution of trichloracetic acid (TCA) in N-butanol for 5–10 min. The activity entered the upper (predominantly butanol) phase. The phases were separated by low speed centrifugation and the clear, dark upper phase was removed. The lower phase and a layer of sludge, which floated on it, were re-extracted with 500 ml of TCA-butanol solution. The upper phase was removed and pooled with the first upper phase. Activity was extracted into aqueous solution by addition of 100 ml of ice-cold water and titration of the suspension to pH 7·0 with N NaOH. The solution was cooled in an ice-bath during titration. The dark aqueous solution was recovered by low speed centrifugation and washed twice by shaking with ether.

Stage 6: Removal of TCA. Most of the original TCA was present as its sodium salt in this solution. TCA inhibited gastric secretion when the solution was assayed for urogastrone at 100–200 ml of original urine. The sodium trichloracetate and other electrolytes were removed by a further batch extraction onto SRC 3 or SRC 4 as in stage 3. Anionic species were not absorbed on the resin and were removed as sodium salts. The solution was extracted three times with 100 g of SRC 3 as described in stage 3 above. The resin was carefully washed with distilled water on the filter before elution of the activity.

Stage 7: Rotary evaporation. Urogastrone in neutral solution was stable to boiling for at least 10 min and could be concentrated by rotary evaporation under a wide range of conditions without loss of activity. Samples were concentrated by evaporation in a Buchi Rotavapor which had a 50° C water bath. In this apparatus the solution temperature stayed close to freezing point until most of the water had evaporated. The active solutions were concentrated to the smallest possible volume commensurate with low handling losses and stored cold or frozen.

The sequence of steps described above gives a good purification with little loss of total activity and can be carried out routinely with no need for biological assay. The remaining purification by gel-filtration required close monitoring by biological assay.

Preliminary experiments with Sephadex gels showed that the activity was almost completely excluded by G 25, but much of the pigment was irreversibly absorbed on the gel. The activity tended to trail and yields were less than 100%. In contrast, with G 50 gels there was no indication of trailing, the gel absorbed very little pigment and the yields were quantitative. The active material eluted roughly half way between the first (macromolecular) peak and the electrolyte peak. It was considered that this was the optimum gel size for molecular sieve fractionation.

Stage 8: Gel-filtration with Sephadex G 50. The gel was packed in Pharmacia columns and washed with distilled water. Columns were run in the cold with flow rates of the order of 1 ml/min and the ultraviolet absorption of the effluent at 2537 Å was continuously monitored. Fractions were collected and the electrical conductance of the fractions was measured.

A K 50/100 column was generally used for the first gel filtration step and one of the K 25 range of columns thereafter. The samples were put on the column in the smallest possible volume and eluted with distilled water. For the first column the sample volume was in the order of 20–30 ml. During elution the material separated into two pigmented zones. The optical density curve showed two peaks and the activity was found on the ascending shoulder of the second peak. All the activity was eluted before the conductivity began to rise to the major peak. Figure 5 shows a typical result at this stage.

Yield of activity

In the absence of a standard preparation of urogastrone, activity was expressed in terms of an arbitrary 'unit', defined as the specific inhibitory activity which gave rise to a carbachol index of 0·15 in the rat assay method. This corresponds roughly to the threshold urogastrone activity which could be detected reliably.

Examples of two separate extractions and the relevant activity expressed in terms of the above unit are given in Table 2. Experiment 1 refers to a dried urine sample which was obtained through the kindness of Messrs. Leo Ltd. (Copenhagen), who prepared for us a sample of urine according to our instructions to stage 2. The subsequent extraction stages were carried out in this laboratory. Experiment 2 represents an extraction procedure carried out entirely in this laboratory on normal male urine. In experiment 1 a salt-free but otherwise crude dried sample of 150 g, extracted from 6000 l. urine, was tested. It contained activity corresponding to approximately 20 U/l. of original urine. During purification two-thirds of total activity was lost but at the same time specific activity increased 50-fold. Activity of the final sample in terms of weight was of 40 U/mg. In experiment 2 activity was

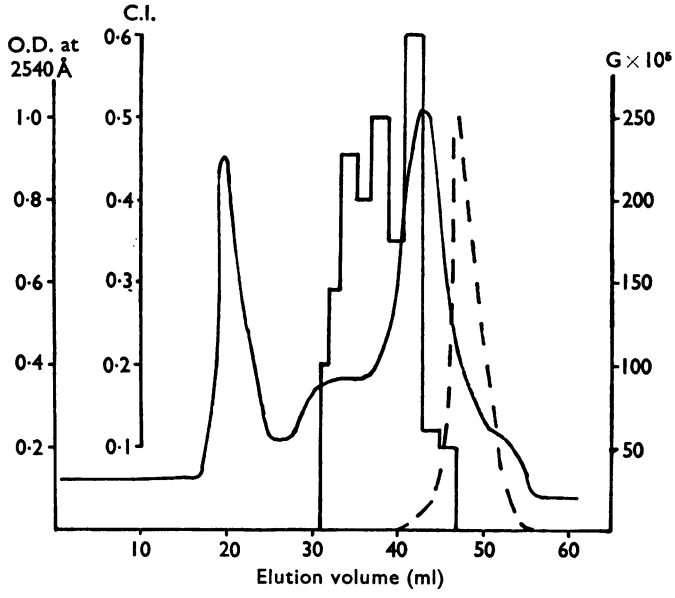

FIG. 5. Elution of urogastrone from Sephadex G50. (——), Optical density curve at 2,540 Å ; (– – –), area of high electrolyte concentration (conductivity G in Ω^{-1}). Blocked area indicates urogastrone activity in terms of carbachol index (C.I.).

TABLE 2. *Activity of extracts of human urine in the course of purification*

Experiment 1				
Stage	Vol. of original urine (l.)	Weight of Sample (g)	U/mg	U/l.
2	6,000	150	0·8	20
8		1	40	6·6

Experiment 2				
Stage	Vol. of original urine (l.)	Weight of sample (g)	U/mg	U/l.
4	1,200	2	12	20
8		0·3	80	20

Activity is expressed in terms of units as defined in text (U).

extracted from 1200 l. male urine. The first assay was performed at stage 4 (i.e. at a later purification stage than in experiment 1). Activity corresponded to 20 U/l. of original urine. The sample was further purified to 80 U/mg, specific activity increasing about 6-fold between steps 4 and 8. After gel filtration the preparations seemed notably less toxic than earlier and had lost most of their pigment. Electrophoretically they were not homogeneous.

Optimum pore size for zeocarb absorption

A series of experiments was performed to investigate which of the available forms of zeocarb would be most effective. The extracts were tested after the initial extraction process. Using resins cross-linked between 1 and 10% the amount of urogastrone activity extracted from urine was related to the degree of cross-linkage as shown in Fig. 6 where degree of cross-linkage is plotted against units of activity per litre of urine. The order of effectiveness was SRC 2>6>10>14>22.

Discussion

The term urogastrone is ill-defined and is likely to remain so until urogastrone becomes chemically identified. From the start of investigations of a gastric inhibitory principle derived from urine some workers have considered urogastrone to be an antiulcer factor (Sandweiss, Saltzstein & Farbmann, 1939) but others have considered it to be an antisecretory factor (Gray, Wieczorowski & Ivy, 1939 ; Necheles, Hanke & Fantl, 1939). Sandweiss & Friedman (1942) concluded that the two activities were separate and named the antiulcer factor anthelone and the antisecretory factor urogastrone, but Lugaro, Lupi & Corbellini (1965) who studied the chemical and biological properties of a commercial preparation called Urogastrone considered that the two activities were probably due to the same substance. Even the definition of an antisecretory factor is uncertain, since some workers have assessed it by measuring inhibition of spontaneous secretion (e.g. in the Shay rat, Lugaro *et al*, 1965) while others have measured inhibition of secretion caused by histamine (Howat & Schofield, 1954) or other stimulants. For the purpose of the present work urogastrone can be defined as a factor extracted from human urine which inhibits carbachol induced secretion in the anaesthetized rat. The same material also in-

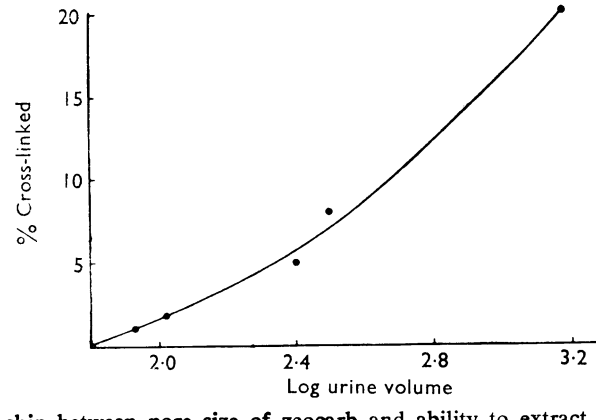

FIG. 6. Relationship between pore size of zeocarb and ability to extract urogastrone. Pore size plotted as degree of cross-linkage of resin. Abscissa, log of urine volume from which activity corresponding to C.I.=0·3 was extracted.

hibited secretion induced by histamine and pentagastrin, although there is no proof that a single substance was responsible for these various effects.

One of the most pressing problems is to establish a viable quantitative bioassay for urogastrone. In the present work two types of assay have been investigated. One type involved comparison with a standard, a procedure which is theoretically desirable since comparative assays are known to be less subject to animal variation than assays based on 'animal units'. Comparative assays tend to be time-consuming and laborious, but we have shown that it is possible to carry out a satisfactory comparative assay, with narrow fiducial limits, by the use of an incomplete block design involving only eight rats. A limitation of comparative assays at this stage is that no international standard of urogastrone exists. A stable standard, even though impure, would be of considerable value to workers in this field.

Comparative assays are not always convenient, especially when many samples have to be tested in the course of purification procedures. We have found the use of an animal unit to be of practical convenience and have used it extensively in this work. Urogastrone activity was measured by reference to the log dose-response curve of carbachol, by means of the 'carbachol index', and a unit of activity derived from this index has been adopted. The unit is defined as the amount of urogastrone which, when injected intravenously into an anaesthetized rat, produced a carbachol index of 0·15. This degree of activity corresponds roughly to the threshold quantity of urogastrone which can be reliably detected by the present method.

Before adopting the method of extraction of urine with zeocarb, we carried out investigations with two other extraction procedures, the cabunite method of Mongar *et al.* (1962) and the charcoal method of Gregory (1955). Both procedures were eventually abandoned for similar reasons, namely that their yields seemed to depend on the particular batch of absorbent material used. With cabunite variability was extreme, some samples giving an excellent yield and others none. The charcoal samples at our disposal all gave lower yields than the best cabunite or the standard zeocarb samples and with some samples of charcoal recovery was very low.

Extraction by weakly cross-linked zeocarb polystyrene resins gave reproducible yields when applied to successive samples of urine. This method could be used with large or small quantities of male urine. It would be of interest to apply it to other types of urine, for example to test the urogastrone content of pregnancy urine or of urine from patients with abnormalities of gastric secretion. Some of the details of our extraction procedure are inessential and could probably be modified. For example the initial column process (stage 1) could be replaced by a simpler batch absorption process which is likely to be as effective. The operant pH values could also be varied provided that the critical region of absorption and desorption of urogastrone from the zeocarb resin of pH 6–pH 8·5 is maintained. It is useful, however, to acidify crude urine initially to pH below 4 since it then forms an inactive precipitate which can be decanted.

Attempts at further purification of urogastrone by Sephadex gel and other chromatographic procedures produced only relatively small increases in specific activity although the samples became less pigmented and apparently less toxic. When the most highly purified samples were tested by thin layer chromatography they were found still to contain at least ten separate components.

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