Some Experiments on Intrinsic Factor

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Latner, Merrills & Raine (1956) reported that an apparently homogeneous preparation of intrinsic factor from dried pig-stomach powder could be resolved into an active and an inactive fraction by chromatography on the cationic-resin Amberlite IRC-50. The inactive ('peak 1') material, moreover, inhibited absorption of cyanocobalamin in the normal rat.

Pyloric secretion, from a blind pyloric pouch in the pig, freed from muco-substance by filtration had been shown (Heatley *et al.* 1954) to possess intrinsic factor activity in the patient with pernicious anaemia in remission, and since a supply of this material in the fresh state was available it was decided to see whether a concentrate from it could be fractionated by the chromatographic process of Latner *et al.* (1956); it was hoped that a purer final product might be obtained from the secretion than from the mucosal starting materials generally used. It was also hoped to examine the part played by the inactive 'peak 1' of pig and human material in the lack of response when crude preparations from these sources are tested in gastrectomized rats. Results of some interest along these and other lines have been obtained; though incomplete they are presented now since dispersal of the authors precludes further work at the present time.

MATERIALS AND METHODS

Measurement of intrinsic-factor activity. The earlier tests were done on patients with pernicious anaemia in remission, by using radioactively labelled vitamin B₁₂, and collecting faeces in cardboard cartons. The radioactivity was measured by placing the unopened cartons in turn within a ring of Geiger counters and making suitable geometrical corrections (Bradley, 1957); the unpleasant homogenizing and sampling of the faeces was thus avoided. Towards the end of the work tests were made as well on gastrectomized rats by the method of Taylor, Mallett, Witts & Taylor (1958), activity in 8-day collections of faeces being measured in a scintillation counter.

For the human experiments [⁶⁰Co]vitamin B₁₂ of specific activity about 0.3 µCi/µg. was used, and, for the rat tests,

vitamin labelled with ⁵⁸Co having a specific activity of 1.0–3.2 µCi/µg. Dilutions of these materials were stored at 4° and fresh batches of the ⁵⁸Co labelled material were obtained from The Radiochemical Centre, Amersham, Bucks., each month to reduce the risk of deterioration.

Human gastric juice. This was obtained partly from the authors and partly from patients undergoing investigation of gastric function. All subjects were fasting; juice from some was collected after stimulation by a maximal dose of histamine (Kay, 1953). Only juice containing free hydrochloric acid was used. The different products were not prepared separately.

Pig-mucosal-powder concentrate. This material had been kindly supplied by Dr L. Ellenbogen of Lederle Laboratories and was a concentrate (45774–133) prepared by ammonium sulphate precipitation of an aqueous extract from desiccated and defatted hog pylorus.

Pig pyloric secretion. The pig was one of a number prepared with gastric or duodenal fistulae by Professor Florey and Dr M. A. Jennings. It had a blind pyloric pouch, opening to the surface, which had been formed when it was a few weeks old (Jennings, Florey, Stebbins & French, 1960). Even shortly before the pig's death, 7 years and 2 months later, it was producing juice of the usual appearance and consistency of pyloric-fistula juice, and as the pouch had grown with the pig the rate of secretion was over 250 ml. in 12 hr. Pouches of this kind rarely remain usable, for one reason or another, for more than 6 months. Pyloric secretion was collected in a special Perspex container (devised by Mr J. Kent) which was applied in a broad bandage while the pig was feeding and was left in position for several hours. To reduce bacterial action, 20 mg. each of penicillin and streptomycin sulphate were put in the container before it was fixed to the pig. The collecting pot was not kept on continuously, and since the pig was so large (its weight some months before death was estimated at 200 kg.) the pot could be put in position only when the pig would co-operate, and only a part of the total juice secreted was actually collected and used.

Treatment of pyloric secretion. The secretion, faintly alkaline and of the consistency of egg-white, was filtered under suction through a 5-on-3 fritted glass filter (Heatley, 1959), or latterly through an Oxoid membrane laid on such a filter. This had the advantage that, with the help of a strip of cellophane to lift its edge, the membrane could be stripped off and replaced by a fresh membrane, thus avoiding both the need to clean the funnel and the loss of the 5–6 ml. of filtrate left in its pores.

When it had been established that intrinsic-factor activity was adsorbed at pH 4.9 on to Amberlite resin XE-64, whereas pepsinogen passed straight through, the

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routine treatment of the crude secretion was modified as follows: the secretion was brought to about pH 5 by whisking in 1.1 ml. of *N*-acetic acid/100 ml. before placing on the filter. Attached to the stem of the latter, as shown in Fig. 1, was a small column of resin XE-64 previously equilibrated with the 'buffer A' of Latner *et al.* (1956) and rinsed just before use with buffer A from which gas had been removed. The polythene tube allowed the air pressure over the column to equalize with that in the outer flask when this was evacuated or filled with air at the completion of filtration. The rate of flow through the columns varied, but if it was less than that of filtration (as happened with most columns initially) the level of fluid in the stem of the funnel would rise and flow rate through the column would be correspondingly increased. When filtration was complete, the small column was detached, well washed with buffer A, then the bound intrinsic factor was eluted by percolation with 0.5 *M*-phosphate buffer, pH 6.8. Elution was considered to be complete when the pH of the eluent had risen above 6.0.

At this or other stages of purification the active material was concentrated by fully saturating with ammonium

sulphate, spinning down the precipitate, usually in a Spinco model L centrifuge, taking it up in the minimum amount of water or 0.01 *N*-HCl and dialysing exhaustively, first in 0.01 *N*-HCl and then in water. The granular grey precipitate remaining was removed by centrifuging and the supernatant pooled with other batches and submitted to more detailed fractionation on the same resin.

Amberlite resin. Amberlite resin XE-64, a cationic-exchange resin chemically identical with Amberlite IRC-50. Particles passing a 90-mesh sieve were freed from fines and then prepared as described by Latner *et al.* (1956).

Conduct of chromatographic separation. The concentrate, usually well dialysed, was brought to the ionic composition of Latner's buffer A by adding the calculated amounts of sodium phosphate and sodium hydroxide; it was then passed through the column of resin (packed in the usual way) before continuing percolation of the latter with buffer A. The effluent was collected on a fraction cutter and the volume and adsorption at 275 m μ of each fraction or of several, pooled, fractions were measured. Buffer A was replaced by other buffers and further fractions were collected. The whole operation was carried out in a cold room at about 4°.

Electrophoresis. The micro-method of Kohn (1958), with strips of Oxoid membrane filter, was used with dilute nigrosine as stain. The apparatus was copied from a minor modification of Dr R. Consden's apparatus (personal communication). All runs were made at about 4°.

RESULTS

All the tests on pernicious-anaemia patients have been summarized in Table 1 and are individually referred to in the text by the numbers in column 1. Since tests were done singly and on a series of different patients (who could in any case be given no more than six doses of the [⁶⁰Co]vitamin B₁₂) only a qualitative interpretation of results could be made.

Nearly all the work was done on pig pyloric secretion; that a filtrate of it was still very active was shown by a preliminary experiment (1, Table 1). Fractions of the filtrate precipitated by 25–56 and by 56–100% saturation with ammonium sulphate were also active (2, 3, Table 1). It was noticed that saturation of the acidified secretion with ammonium sulphate always resulted in a strong odour of caprylic acid.

Precipitation with cetylpyridinium chloride. Small increments of aqueous 1% cetylpyridinium chloride were added to an exhaustively dialysed ammonium sulphate precipitate of filtrate from pig pyloric secretion (Scott, 1955; Blumberg & Ogston, 1958). The precipitate and supernatant were separately freed from excess of reagent by shaking with chloroform, a process which, applied more vigorously for 30 min. to another sample, did not destroy all activity (4, Table 1). The supernatant remained active (5, Table 1). The precipitate was inactive (6, Table 1) but was so scanty that purification would have been negligible.

Fig. 1. Arrangement for filtration of pig pyloric secretion and for trapping intrinsic-factor activity in Amberlite resin XE-64. For details see text.

Fractionation of pig pyloric secretion on Amberlite resin XE-64. An exhaustively dialysed ammonium sulphate precipitate of filtrate from pig pyloric secretion was passed through a column of resin XE-64 exactly as described by Latner *et al.* (1956). The absorption at 275 m μ of the successive eluate fractions closely resembled that obtained by these authors with a preparation from pig-stomach powder. 'Peak 2' and 'peak 1' were found to have pronounced and slight activity respectively (8, 7, Table 1), though the latter was tested at nearly double the equivalent concentration of the former. Similar 'peak-2' material prepared in the same way on other occasions, after ammonium sulphate precipitation and exhaustive dialysis, was found to

give marked activity in the amounts shown in test nos. 11, 12, 14, 17 and 21 of Table 1).

Adsorption of intrinsic-factor activity from crude secretion filtrate. When resin XE-64 had been shown to adsorb activity from concentrates, a test was made to see whether it could do so also from the crude filtrate of pig pyloric secretion. About 90 ml. of filtrate was adjusted to about pH 4.9 with acetic acid before passing it through a small column of the resin. After brief washing of the column with buffer A, the eluate with buffer B was tested and found to be highly active (9, Table 1). After this, most material was collected in resin at the time of filtration (see Materials and Methods section), the secretion being acidified with 1.1 ml. of *N*-acetic

Table 1. *Tests of intrinsic-factor activity in pernicious-anaemia patients in remission*

For details of test see text.

Test no.	Patient	Material tested	Wt. tested (mg.)	Equivalent volume of original secretion (ml.)	Percentage of vitamin B ₁₂ (dose 0.5 μ g.) excreted	
					Given alone	Given with test preparation
1	I.G.	Filtrate from pig pyloric secretion dialysed against 0.01 <i>N</i> -HCl and water	—	110	86.7	25.6
2	F.T.	Filtrate from pig pyloric secretion precipitated by 25–56% saturation with ammonium sulphate	11.6	100	85.2	38.8
3	W.J.	As 2, but precipitated by 56–100% saturation	41	250	77.2	22.8
4	L.W.	Redissolved precipitate from ammonium sulphate shaken 30 min. with chloroform	27	100	81.8	52.1
5	L.W.	Well-dialysed concentrate not precipitated by cetylpyridinium chloride	—	100	81.8	66.6
6	L.W.	Precipitate from 5	—	100	81.8	91.6
7	W.R.	'Peak 2' from resin XE-64, from concentrate of pig pyloric secretion	—	109	94.6	49.7
8	W.R.	'Peak 1' from resin XE-64, from concentrate of pig pyloric secretion	—	209	94.6	64.8
9	W.R.	Eluate from resin XE-64, through which crude filtrate from secretion had been passed	—	90	94.6	40.0
10	W.R.	Eluate from charged column with buffer A containing 2 <i>M</i> -NaCl	—	405	94.6	82.1
11	W.R.	'Peak 2' fraction from pig pyloric secretion	—	100	94.6	41.3
12	E.A.	'Peak 2' fraction from pig pyloric secretion	0.49	100	79.1	20.0
13	E.A.	'Peak 2' fraction from human gastric juice	—	100	79.1	70.9
14	E.A.	'Peak 2' fraction from pig pyloric secretion	0.31	20	79.1	50.1
15	W.O'C.	'Peak 2' fraction from human gastric juice II	—	100	79	47.0
16	W.O'C.	Material not precipitated by Rivanol	0.22	52.5	79	70.9
17	F.W.	'Peak 2' fraction from pig pyloric secretion	0.75	30	72.1	39.0
18	F.W.	Material precipitated by Rivanol	1.2	52.5	72.1	29.8
19	H.D.	Concentrate from pig pylorus (Lederle 45774–133)	50	—	95.6	52.9
20	H.D.	'Peak-2' fraction from pig pyloric secretion, heated for 5 sec.	0.6	60	95.6	78.7
21	W.W.	'Peak-2' fraction from pig pyloric secretion, unheated	0.6	60	89.7	50.7

acid/100 ml., whisked in before filtration. The pepsinogen occurring in the filtrate passed through the resin.

Properties of 'peak-2' material

Several batches of partially purified pyloric secretion were pooled and passed once more through resin XE-64, the 'peak-2' fractions being

reprecipitated with ammonium sulphate, dialysed and finally freeze-dried and stored at 4° in the dark in an atmosphere of nitrogen. For each 100 ml. of original secretion the final product weighed 1.05 mg. This is the 'peak-2' concentrate referred to in this paper.

When dried on a watch-glass a solution of it left a shiny brittle transparent almost colourless glaze,

Table 2. *Activity of intrinsic-factor preparations in totally gastrectomized rats*

Figures in parentheses, when given, are control values (without intrinsic factor) for the rats concerned. The mean excretion in 43 gastrectomized rats without intrinsic factor was $95.3 \pm 8\%$. Volumes (marked *) in the third column, when given, represent volumes of original secretion equivalent to the material given. 'Peak 2' is pig pyloric secretion 'peak 2'.

Test no.	Preparation	Volume (ml.) for Test 1 or Weight (μ g.) for Tests 2-7	Percentage of vitamin B ₁₂ excreted
1	'Peak 2'		
	Precipitated by 20% (v/v) of ethanol	1*	81.7 (104.4); 83.3
	Precipitated by 20-30% (v/v) of ethanol	1*	88.4 (95.0); 85.2 (96.0); 80.7 (80.5)
	Precipitated by 20-30% (v/v) of ethanol	2*	80.1; 80.0 (93.5)
	Not precipitated by 30% (v/v) of ethanol	1*	82.3 (96.2); 90.3
2	'Peak 2'	28	64.1 (89.3); 74.5 (80.5); 60.6 (100.0)
	'Peak 2'	1	79.3 (101.1); 77.4 (98.0)
3	'Peak 2' another preparation	10.5	81.8 (93.5); 65.0 (95.0); 75.2 (104.4); 79.3 (96.0); 89.2 (80.5)
	Precipitate of 'peak 2' after heating for 5 sec. in a boiling-water bath	Portion	103.7; 100.8; 94.5; 94.7 (89.3); 96.1
	Supernatant of 'peak 2' after heating for 5 sec. in a boiling-water bath	Portion	103.7; 100.8; 94.5; 94.7 (89.3); 96.1
4	Fractions of Fig. 2		
	Fraction I	20	87.2; 99.9 (80.5)
	Fraction II	20	101.6 (97.0); 94.0
	Fraction III	20	89.0 (95.0); 90.1 (96.2)
	Fraction IV	20	91.4 (101.1); 100.5 (104.4)
	Fraction V	20	83.9; 82.6 (96.0)
	Fraction VI	2	81.1; 96.0 (98.0)
		10	69.0 (92.6)
		20	76.1; 98.0; 79.1 (97.8)
		38	83.6
	Fraction VII	20	87.4; 88.5
	Pooled fractions I-VII	20	79.0; 89.5 (97.0)
5	Concentrate from pig pylorus (Lederle)	500	95.3; 92.1 (104.4); 96.6 (101.1); 93.2; 92.4; 100.2
		250	90.3 (92.6); 98.7 (98.9); 104.4 (99.4)
6	Fractions from 5 (see Table 4)		
	110-115	0.61	99.1 (96.2); 105.1 (97.0)
		0.91	102.5 (97.8); 97.8
	116-134	0.35	67.5 (95.0); 72.7 (101.0); 70.0 (100.0)
	135-144	1.10	93.3 (80.5); 103.0 (96.0)
		1.65	92.0 (89.0); 96.0 (104.2)
	145-149	1.52	106.0 (98.0); 104.5
		2.28	95.0; 93.0 (89.3)
7	'Peak 2' from normal human gastric juice	0.58	87.0 (102.2); 84.4 (85.0); 87.7 (98.0); 83.0 (101.1)
		1.15	77.5 (80.5); 96.2 (99.5); 93.3 (89.3); 99.7 (100.0)
		2.3	89.0 (80.5); 101.2 (99.5); 102.0 (89.3); 100.2 (100.0)
		11.3	79.1 (100.0); 76.3 (96.2); 72.8 (93.3); 80.1; 79.8 (86.5); 78.8 (80.0); 78.0 (80.5); 78.2
		56.5	100.0; 98.0; 100.0; 101.7; 81.8

which was not markedly hygroscopic and dissolved readily in water. The solution, which was neither mucoid nor very viscous, gave no precipitate with 'dialysed iron' or with a final concentration of 10% (w/v) of trichloroacetic acid or 0.6% of perchloric acid. When mixed with cyanocobalamin, no precipitate was formed. A heavy, greyish, waxy precipitate formed with 1% phosphotungstic acid, and an abundant, yellow precipitate with a small volume of 25% (w/v) phosphomolybdic acid in 6.5N-hydrochloric acid. With calcium pterate it gave an amorphous yellow precipitate at neutral pH. A spot of solution dried on Oxoid membrane gave a strong periodic acid-Schiff reaction. Membrane electrophoresis (see below) suggested that the material was far from homogeneous.

Heat coagulability of 'peak-2' concentrate. A neutral, 1% solution of the concentrate sealed in thin-walled glass capillaries was heated in a water bath at various temperatures and for varying lengths of time. Immersion in a boiling-water bath for 5 sec. precipitated about 30% of the material; when this was centrifuged and the supernatant again heated no further precipitate formed, however long the heating. The 5 sec. immersion in boiling water appeared to destroy most (20, 21, Table 1) or all (3, Table 2) of the activity.

Ethanol fractionation of 'peak-2' concentrate. Fractionation of a 1% solution with ethanol at -10° to -2° showed that 27% of the dry weight was precipitated by a final concentration of 20% (v/v) of ethanol, and a further 32.5% on increasing the ethanol concentration to 30% (v/v). These two fractions and the 40.5% not precipitated were tested in gastrectomized rats (1, Table 2). All three were slightly active and ethanol fractionation at this stage did not appear to be a fruitful purification step.

Fractionation with Rivanol. Steinbuch & Quentin (1959) stated that Rivanol (2:5-diamino-7-ethoxy-acridine) preferentially precipitates some proteins under carefully controlled pH conditions. When a 1% solution of the 'peak-2' concentrate mentioned above was mixed with 0.2% of Rivanol adjusted to

pH 8.0 only a faint turbidity appeared. Cautious addition of alkali caused a marked turbidity at pH 8.4 and a definite precipitate at about pH 8.8. Further alkali caused more precipitate, but there was the risk that Rivanol itself, or rather the free base, might be precipitated as well, as the pH was raised. Since the pH was so critical and at the same time difficult to control in the small volume of unbuffered solution used (0.4 ml.) this method would not be easy to reproduce. Two successive precipitates (together 60–70% of the total) and a non-precipitated portion (30–40% of the total) which were obtained in one fractionation were freed from Rivanol by dialysis and tested. The combined precipitates appeared strongly active, but the non-precipitated material was not (18, 16, Table 1).

In a second Rivanol fractionation about 90% of the starting material was precipitated and was not tested.

Attempts to resolve 'peak 2'. Slight as is the difference between the buffers A and B of Latner *et al.* (1956), it seems quite clear that the first does not, and the second does, easily, elute intrinsic-factor activity from a column of Amberlite XE-64 resin. Their pH is almost identical (A, 4.92; B, 4.96; see below), but that a high ionic concentration itself is not responsible for elution is shown by the fact that buffer A containing 2M-sodium chloride does not elute activity (10, Table 1); however, this may well be because the sodium chloride lowers the pH of buffer A considerably. At the pH level used the resin is only slightly ionized.

The first empirical attempt to resolve 'peak 2' (from pig secretion) was by gradient elution from buffer A to buffer B. The peak was spread, but no significant resolution of material absorbing at $275\text{ m}\mu$ was obtained, and no tests for intrinsic-factor activity were made. Later, when a provisional rat assay became available, an attempt was made with buffers intermediate between A and B, as well as A and B, with the results shown in Fig. 2. The composition and pH, measured according to British Standards Specification 1647 (1950), of buffers carefully made up with boiled water is given in Table 3. It will be noted that buffer A2 is

Table 3. Composition and pH of buffers used for elution

Buffer A2 is buffer B diluted 1 in 5. Note marked effect of dilution on pH. The phosphate was in the form of sodium dihydrogen phosphate dihydrate, recrystallized twice.

Buffer	Molarity of phosphate	Vol. of N-NaOH added/l. (ml.)	pH
A of Latner, Merrills & Raine (1956)	0.2	3.0	4.92
A1	0.2	10.0	5.32
A2	0.2	14.0	5.50
B of Latner <i>et al.</i> (1956)	1.0	70.0	4.96
B diluted 1 in 2	—	—	Approx. 5.26
B diluted 1 in 10	—	—	Approx. 5.64

buffer B diluted 1 in 5, and that the pH of buffer B differs considerably from the 5.2 reported by Latner *et al.* (1956). This discrepancy, and the marked effect of dilution (Table 3), is perhaps less surprising in view of the fact that the solutions concerned are almost outside the buffering range.

Fractions I-VII (Fig. 2) were separately precipitated by full saturation with ammonium sulphate and were exhaustively dialysed. Their total dry weight might be expected to be roughly the same as if buffer B had been used for elution instead of the sequence A1, A2 and B. The total weight, 27.34 mg., i.e. 2.24 mg./100 ml. of original secretion, is only a little higher than usually obtained for 'peak 2', as eluted by buffer B. However, only fraction VI has unequivocal activity in gastrectomized rats (4, Table 2), and if all the activity were to reside in this fraction the dry weight would be equivalent to only 0.18 mg./100 ml. of original secretion. Unfortunately it has not been possible to make more tests on this material.

A similar fractionation with a crude mucosal extract is described in the next section.

Behaviour towards resin chromatography of human gastric juice. When dialysed human gastric juice was passed through a column of resin by the procedure of Latner *et al.* (1956) without prior concentration, the 'peak 2' finally obtained was active both in a pernicious-anaemia patient (15, Table 1) and in gastrectomized rats (7, Table 2), though only, in the rats, at certain dose levels. The dry weight of this preparation was not known, but that of a similar one was 1.85 mg./100 ml. of original juice.

An earlier resin fractionation of an ammonium sulphate precipitate of human gastric juice had

given an inactive 'peak 2' (13, Table 1), perhaps because the strongly acid juice had not been neutralized before precipitation.

Behaviour towards resin chromatography of crude concentrate from pig pylorus. The crude concentrate from pig pylorus (Lederle) was shaken with 0.02 M-phosphate buffer, pH 6.8, for 2-3 hr., a few drops of 0.2 N-NaOH being added from time to time to maintain the pH. The faintly turbid solution was centrifuged, dialysed, centrifuged again and the supernatant made up to the ionic composition of buffer A and passed through the resin in the usual way. The column was then eluted with buffers A, A1, A2 and B in turn, and the absorption, at 275 m μ , of the fractions was measured. The rate of flow was roughly constant, but the fractions were collected at different time intervals and therefore

Table 4. Condensed summary of fractionation of 1.4 g. of pig pylorus concentrate (Lederle) on Amberlite resin XE-64

For composition of buffers, see Table 3. Rate of flow was roughly constant but fractions were collected at different time intervals and therefore varied in volume. For assays, see 6, Table 2.

Fractions	Buffer	Volume (ml.)	Dry wt. (mg.)	Intrinsic-factor activity in gastrectomized rats
1-14	A	1182	—	Not tested
15-61	A1	186	2.4	Not tested
62-90	A1	461	41.6	Not tested
91-109	A1	352	11.6	Not tested
110-115	A1	74	1.69	Inactive
116-134	A2	58	0.97	Active
135-144	A2	110	3.07	Inactive
145-149	B	88	4.2	Inactive

Fig. 2. Absorption at 275 m μ (1 cm. path) of successive eluates from a column of Amberlite resin XE-64 loaded with a concentrate derived from 1220 ml. of pig pyloric secretion. For composition of buffers A, A1, A2 and B see Table 3. Eluates were pooled in fractions I-VII; the dry weights were those of the dialysed ammonium sulphate precipitates.

varied considerably in volume. Table 4, giving the results in condensed form, and 6, Table 2, shows that only the first part of the A2 eluate was active in gastrectomized rats. The dry weight of this fraction was 0.97 mg., and as it is derived from about 1.4 g. of the crude Lederle powder, the purification would be nearly 1500-fold, if all the activity had collected in this one fraction. Further, it would appear that had only buffers A and B been used as recommended by Latner *et al.* (1956), the dry weight of the active 'peak 2' would have been nearly 70 times as great.

The dose of the active fraction given to the gastrectomized rats was 0.35 μ g., which would have been equivalent to 0.5 mg. of the original powder. Yet the crude powder had been shown to be inactive in gastrectomized rats at this dose and at half this dose (5, Table 2). Some inhibitory factor or factors have possibly been removed, and this has been confirmed by M. A. Sheikh & K. B. Taylor (unpublished work).

Membrane electrophoresis. Various fractions were tested in 0.1M-veronal-acetate buffer (Oxoid), pH 8.6, and/or in 0.1M-phosphate buffer, pH 6.8 or 7.0. Half-neutralized 0.1M-acetic acid was also used in a few cases.

On electrophoresis a sample of 'peak 1' gave several distinct nigrosine-staining spots, suggesting that the electrophoresis technique was adequate. However, 'peak 2' preparations, apart from a spot moving towards the cathode (at pH 6.8 or 8.6), gave an indistinct blur spreading in both directions from the point of application. The cathodal spot is not associated with intrinsic-factor activity since, of fractions I-VII of Fig. 2, only the inactive fraction VII contained this component. The membrane-electrophoretic pattern of VI (which was active) was indistinguishable from those of I-V (which were inactive), and the technique has so far been disappointing. The cathodal spot might, however, serve as a marker during elution from Amberlite XE-64 if its appearance immediately after the active fraction can be confirmed under other conditions. It is fully realized that intrinsic factor itself may not stain with nigrosine.

DISCUSSION

The fact that the three different starting materials (pig pyloric secretion, a concentrate from pig pylorus and normal human gastric juice) behave similarly on chromatography on Amberlite resin XE-64, suggests that this purification procedure, described by Latner *et al.* (1956), is a reliable and reproducible one. By choosing buffers graded more finely than their buffers A and B a product many times more active than their 'peak 2' can be obtained. Thus if the recovery in the A-A1-A2-B

procedure were to be the same as in the simple A-B system, a 12-fold purification would have been obtained with the pyloric secretion, and one of 70-fold with the mucosal concentrate. It is possible that choice of slightly different buffers for elution might give still further purification, and washing of the column with buffer A containing 2M-NaCl (which does not remove intrinsic factor) might also be worth testing. A technical point needing investigation is whether intrinsic-factor activity would eventually be eluted by buffer A1 if a sufficient volume were used, for this could lead to poor recovery. In the two runs reported here buffer A1 has not removed detectable intrinsic factor, nor has much tailing occurred in the elution with A2, another possible cause of low recovery. Whether the slight activity observed in the single test of 'peak 1' (8, Table 1) is genuine has not been investigated.

Measurement of the ultraviolet absorption, which is a useful guide to the course of elution when buffers A and B only are used, is of little or no value in the more refined elution process described here, and a blind application of the process would seem to be the only course available at present, with the possible exception that a nigrosine-staining spot, which moves fast towards the cathode on membrane electrophoresis, has so far emerged from the Amberlite column just after the active material and may have limited value as a marker. In this connexion Jacob, Williams, Howe & Glass (1959), purifying pig intrinsic factor by the totally different procedures of high-speed centrifuging and zone electrophoresis, suggest (with marked reservation) that intrinsic factor may be associated with a cathodal component. It would be interesting to see whether chromatography of their product on Amberlite XE-64 would separate intrinsic factor from their cathodal component. It would appear that their purest product is of much the same activity as the 'peak 2' concentrate obtained here by the procedure of Latner *et al.* (1956). Fraction VI of Fig. 2 and 116-134 of Table 4 should be several times more active, though the opportunity has not occurred of testing these directly on a pernicious-anaemia patient.

Pyloric secretion would not appear to be a more favourable starting point for purification of intrinsic factor than a mucosal extract, which is, of course, very much easier to obtain.

Membrane electrophoresis still indicates that the purest products are grossly impure, yet the 'peak-2' concentrate is active at 0.3 mg. in pernicious-anaemia patients (14, Table 1), and the purer products should be several times as active.

A difficulty which has always beset work on intrinsic factor is the, at best, semi-quantitative nature of the assays. Should the use of gastrecto-

mized rats for assay be possible, the easy replication of tests would go some way to meet this difficulty. However, the hope of an accurate assay will be somewhat dashed by (a) the presence in partly purified intrinsic-factor preparations of unknown amounts of inhibitor or inhibitors, (b) the marked dependence, in some cases, of activity on the size of the dose (e.g. 7, Table 2), (c) the possibility of quantitative differences in response of a test animal to intrinsic factor (and inhibitors) from a different animal species and (d) the possibility of a test animal becoming refractory. These points are discussed more fully by M. A. Sheikh & K. B. Taylor (unpublished work).

SUMMARY

1. Preliminary attempts to purify and examine the properties of intrinsic factor are reported.

2. Fractionation by chromatography on an exchange resin, as described by Latner, Merrills & Raine (1956) for a concentrate from pig gastric mucosa, gives closely similar results with pig pyloric secretion and human gastric juice, and appears to be a reliable and reproducible method.

3. The active fraction can be obtained several times purer by modifications of the exchange-resin process. There are reasons for thinking that the final product might be active in pernicious-anaemia patients in a dose of about 30 μ g.

4. Membrane electrophoresis suggests that the purest product is still far from homogeneous.

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Synthesis of the α - and γ -Isomers of Glutamylcystinylvaline

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It has been shown (Arnstein & Morris, 1959, 1960b) that L-cystinyl-L-valine is available for penicillin biosynthesis in washed mycelium of *Penicillium chrysogenum* by a pathway other than that involving breakdown to free amino acids. Following this observation, the mycelium of *P. chrysogenum* was examined for the presence of cystinylvaline or of similar peptides containing both cystine and valine; at an early stage in this work there was some evidence of the presence of glutamylcystinylvaline in mycelial extracts. In order to assist in the identification of this peptide the α - and γ -isomers of glutamylcystinylvaline were synthesized.

The main problem in the synthesis of glutamyl peptides is that of producing, unequivocally, either peptides in which the α -carbonyl group is a member of the peptide chain, i.e. with part structure (A), or those in which the γ -carbonyl group participates in peptide-bond formation, as shown in part structure (B) below. Amiard, Heymès & Velluz (1956) have reported the synthesis of γ -glutamylcystinylglycine (glutathione), in which the desired γ -linkage of glutamic acid to the rest of the molecule was