dinin, and their isomers, constituted 80–90 % of the total carotenoids in the organisms which contained these pigments.

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Intrinsic Factor: Active and Inhibitory Components from the Mitochondria of Human Gastric Mucosal Cells

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Cell particles, separated by high-speed ultracentrifuging from preparations of human and rat gastric mucosa, have been shown to contain as much as half of the intrinsic-factor activity of the whole preparation (Taylor, 1955). The activity of the human particles may be removed by disruption with water, in which the active material subsequently dissolves, but in the rat the activity remains bound to the disrupted particles (Taylor, Mallett, Witts & Taylor, 1958*b*). The activity from the human particles may be further concentrated into a 'substance E' (O'Brien, Taylor, Turnbull & Witts, 1955), which on paper electrophoresis, electrophoresis in the Tiselius apparatus and Svedberg ultracentrifuging cannot be resolved into more than one component. Substance E promotes the absorption of ^{60}Co -labelled vitamin B_{12} in patients with pernicious anaemia in doses of about 10 mg., but this represents a low order of activity and it has been provisionally suggested that substance E may be a precursor of intrinsic factor (O'Brien *et al.* 1955). Further investigations into

the activity of substance E and related studies are now reported.

METHODS

Substance E was prepared as described by O'Brien *et al.* (1955) from human gastric mucosa.

Paper electrophoresis was carried out at room temperature for 16 hr. in a horizontal bath with a current of 1 mA/4 cm. width of paper strip and diethylbarbituric acid-sodium diethylbarbiturate buffer (pH 8.6, 1.0.06). The paper strips were stained with 1% bromophenol blue in ethanol saturated with mercuric chloride.

Ultracentrifugal studies were carried out in the Oxford Svedberg ultracentrifuge under the guidance of Professor A. G. Ogston, F.R.S.

Intrinsic-factor activity was assayed in patients with pernicious anaemia in remission by giving 0.5 μg . of ^{60}Co -labelled vitamin B_{12} in aqueous solution by mouth, alone and then with preparations of intrinsic factor, and comparing the amounts of radioactivity appearing in the stools. Measurements of activity were made in a ring of Geiger counters, similar to that described by Bradley (1957). In order to compare quantitatively results obtained with different fractions and patients, the increase of absorption

of radioactive vitamin B₁₂ promoted by any fraction, over that occurring with a control dose of vitamin B₁₂ alone, is expressed as a percentage of the oral dose of the vitamin (0.5 µg.)/mg. of the fraction under test. Not more than five assays were performed in any one patient. Two control assays with radioactive vitamin B₁₂ alone were sometimes done in order to ensure that the patient was both a reliable collector of faeces and not subject to spontaneous fluctuations in absorption of vitamin B₁₂. In the inhibitor experiments, duplicate estimations of the activity of hog intrinsic factor were made for the same reason.

Nitrogen was estimated by a micro-Kjeldahl technique and absorption spectra in a Unicam SP. 500 spectrophotometer.

To establish another criterion of homogeneity, attempts were made to make antibodies to substance E. This material was made up with complete Freund adjuvant and injected by multiple intramuscular and subcutaneous puncture into healthy rabbits of mixed strain (Taylor & Morton, 1958). Antibodies to substance E were detected in the sera by simple tube precipitation tests and by an agar-gel-diffusion technique (Ouchterlony, 1948). The capacity of the antisera to inhibit intrinsic-factor activity was measured by mixing an amount of substance E known to be active by the faecal excretion test with antiserum, keeping it overnight and then administering supernatant and precipitate separately, with the usual 0.5 µg. test dose of labelled vitamin B₁₂, to a patient with pernicious anaemia, one test following the other.

Blood-group-substance activity was measured by adding serial dilutions of substance E to 1:3 dilutions of anti-A and anti-B grouping sera, leaving the mixtures at room temperature for 30 min., then adding a 5% suspension of A₁ and B cells and recording agglutination. Purified blood-group substances, kindly provided by Dr W. T. J. Morgan of the Lister Institute, were used for comparison.

RESULTS

Physical and chemical properties of substance E.

The nitrogen content of seven batches of substance E, prepared during 1955–1958, varied from 11.7 to 12.4%, mean 12.0%, with one exception, the nitrogen content of which was only 9.4%. Qualitative tests were also carried out on five batches of substance E. The biuret reaction, Millon's test for tyrosine and Molisch's test for carbohydrate were positive in all. Tests for organic sulphur and phosphate were negative. It is therefore concluded that substance E is a glycoprotein containing tyrosine but not sulphur-containing amino-acids.

All four batches tested at pH 6–7 showed an identical absorption spectrum, with a slight maximum at 405 mµ, a prolonged minimum rising to a sharp maximum at 277 mµ, a minimum at 251 mµ and increasing absorption to 220 mµ, the limit of the spectrophotometer. The ratio of extinction at 277 mµ to that at 405 mµ is 7.97. At pH 2.4 the maxima shift slightly from 405 to 398 mµ and from 277 to 275 mµ. These observa-

tions also suggest that substance E is a protein containing phenylalanine or tyrosine or both.

All batches on electrophoresis at pH 8.6 in barbiturate buffer showed a single protein-staining band migrating to the anode. At pH 3–5, however, in sodium acetate–hydrochloric acid buffer, two bands were shown. Both moved very slowly to the anode at the latter pH but to the cathode at the former pH (Fig. 1).

During fractionation with ammonium sulphate a pH of 5 is attained and two electrophoretic bands (A and B) then appear and persist, even though the electrophoresis be carried out at pH 8.6. The impression is that dissociation of substance E into two components takes place below pH 5. The faster-moving component A can be recognized

Fig. 1. Paper electrophoresis of substance E and of fractions prepared from it. (a) Substance E at pH 8.6 in barbiturate buffer. Only a single band is seen, which has moved to the anode. (b) Substance E at pH 5.2 in 0.2M sodium acetate–0.1M hydrochloric acid buffer. Two bands moving to the anode are now seen. (c) 61–69% Ammonium sulphate fraction of substance E at pH 8.6, showing one band, component B. (d) 41–50% Ammonium sulphate fraction of substance E at pH 8.6, showing one band, component A. The paper strips shown in (c) and (d) were obtained simultaneously. In all strips the anode is on the right side of the paper.

readily by the fact that upon staining with bromophenol blue it has a more yellow tint than the greener component B. Component B migrates slightly faster than the original substance E but stains with the same dark colour.

In the Svedberg ultracentrifuge at pH 6-7, substance E sedimented as a single substance, the schlieren pattern of the boundary appearing symmetrical, but dispersed (Fig. 2*a*). At pH 4.55, the boundary pattern was no longer symmetrical so that at least two components were present, (Fig. 2*b*). Again the impression is one of dissociation below pH 5. After fractionation of batch 5 with ammonium sulphate, the 41-50% and 61-69% fractions, containing only the electrophoretic components A and B respectively (Fig. 1), sedimented as single substances at pH 4.55 (Fig. 2*c, d*).

Biological activity of substance E and fractions from it. Fig. 3 relates the dose of substance E, from all seven batches, to its ability to promote the absorption of radioactive vitamin B₁₂. The determinations were carried out upon nine different

patients. The correlation coefficient is +0.204 and is not statistically significant. The dotted line is that representing an increased absorption of 1% of the radioactive vitamin for each milligram of substance E and it is seen therefore that the activity of the batches varied, the increase in absorption of vitamin B₁₂/mg. of substance E ranging from 0.7 to 3.2%. The three continuous lines join determinations made from the same batches of substance E. They indicate that for any given batch of substance E a direct dose-response relationship exists, so that the lack of correlation noted above arises from the varying activity of the different batches.

Five different batches of substance E were fractionally precipitated with ammonium sulphate at pH 4-5 in an attempt to separate the two components observed at this pH on paper electrophoresis and thus produce a more active material. The precipitates were redissolved in water, dialysed free of sulphate ion, and tested for activity. The results (Table 1) do not show precise uniformity either in the fractions with activity or in the degree of activity exhibited, but in general activity occurs in two zones. Below 50% saturation, the results on batches 1, 3, 4 and 5, taken together, would suggest that the most active material is likely to be precipitated between 33 and 40% saturation. In the second zone the results are more variable, the active agent sometimes being precipitated by 65% saturation and sometimes not. In all subjects the fractionation procedures increased the activity of substance E, by amounts varying from 1½ to 10 times.

The relationship of intrinsic-factor activity to the electrophoretic components is unclear, the more

Fig. 2. Ultracentrifugal analysis of substance E and of fractions prepared from it. (a) Substance E at pH 7.18 in 0.067 M-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer. Left-hand photograph at 43 min.; right-hand photograph at 83 min. Speed, 918 rev./sec. A symmetrical schlieren pattern is seen in both. (b) Substance E at pH 4.55 in 0.2 M-sodium acetate-0.1 M-hydrochloric acid. Both photographs were taken at the same times as in (a), but at 83 min. the pattern is no longer symmetrical, indicating the presence of more than one sedimenting component. Speed, 950 rev./sec. (c) 41-50% Ammonium sulphate fraction of substance E at pH 4.55. Only a single component is present after 95 min. Speed, 902 rev./sec. Photograph taken at 65 min. (d) 61-69% Ammonium sulphate fraction of substance E at pH 4.55. Only a single component is present after 84 min. Speed, 931 rev./sec. Photograph taken at 54 min.

Fig. 3. Relationship between the amount of substance E fed to patients with pernicious anaemia in remission and the resulting increased absorption of radioactive vitamin B₁₂. The continuous lines join observations made with the same batch of substance E. The broken line is that for an increased absorption of 1% dose of vitamin B₁₂/mg. The three values for 18.8 mg. of substance E were carried out with the same batch on three different patients.

Table 1. *Intrinsic-factor activity and paper-electrophoretic analysis of fractions of substance E obtained after precipitation by ammonium sulphate*

Batch of substance E	Saturation with ammonium sulphate	Intrinsic-factor activity	Increased absorption of radioactive B ₁₂ (% of dose/mg.)	Electrophoretic bands (A, B), pH 8.6
1	Before	Active	3.2	—
	0-33 %	Inactive	—	A
	35-50 %	Active	13.0	A and B
	50-67 %	Inactive	—	B
	Supernatant	Inactive	—	No bands
2	Before	Active	1.3	—
	0-33 %	Inactive	—	—
	33-50 %	Inactive	—	B
	50-65 %	Inactive	—	B
	Supernatant	Active	13.2	No bands
3	Before	Active	0.7	—
	0-33 %	Inactive	—	B
	33-40 %	Active	—	A and B
	40-50 %	Inactive	—	B
	50-65 %	Active	0.9	B
	Supernatant	Active	2.8	No bands
4	Before	Active	0.6	—
	0-67 %	Active	1.9	A
	Supernatant	Inactive	—	—
	35-50 %*	Active	2.5	A
	50-67 %*	Inactive	—	—
5	Before	Active	2.1	—
	0-40 %	Active	2.2	A
	41-50 %	Active	1.1	A
	50-57 %	Active	3.1	A and B
	57-61 %	Active	2.3	A and B
	61-69 %	Inactive	—	B
	69-75 %	Inactive	—	B
	Supernatant	Inactive	—	—

* 0-67 % fraction, refractionated.

so as the active material in the active supernatants of batches 2 and 3 repeatedly failed to stain at all with bromophenol blue on paper. As a general rule it seems that fractions containing component A alone, or with B, were active, those containing component B alone were not. Of the two exceptions, the inactive 0-33 % fraction of batch 1, containing component A, was available in only small quantities, and the amount fed, 2 mg., may have been insufficient to give a significant increase in B₁₂ absorption; the weak activity of the 50-65 % fraction of batch 3, containing component B, may have been due to contamination with the non-staining active agent of the supernatant of this batch.

Inhibition of absorption of vitamin B₁₂ by substance E. One explanation of the variable activity of substance E and of its fractions might be that it contains a factor inhibiting the absorption of vitamin B₁₂. The least active preparation of intrinsic factor (batch 4) was therefore investigated. Inhibition was measured by determining the ability of the substance under test to reduce the vitamin B₁₂ absorption produced by feeding a given

amount of a known active preparation of hog or human intrinsic factor. A definite inhibitory effect was observed with this batch of substance E (Table 2) and led to a more exhaustive investigation of batch 5. The 61-69 % fraction from ammonium sulphate precipitation (containing electrophoretic component B) inhibited the intrinsic-factor action of both the hog material and of the 41-50 % ammonium sulphate fraction derived from the same batch (containing electrophoretic component A). The 69-75 % fraction, containing component B only, also inhibited hog intrinsic factor. Intrinsic factor is thus associated in gastric mucosal cell particles with a material which will inhibit its activity when both are fed orally.

Antibody to substance E. Antisera prepared to substance E yielded a single, weak ring of precipitation by the agar-diffusion technique, whereas preparations of pig intrinsic factor tested at that time yielded more than one ring (Taylor & Morton, 1958). Both anti-hog and anti-human-intrinsic-factor rabbit sera inhibited substance E activity and anti-human-intrinsic-factor serum precipitated with both substance E and hog intrinsic factor.

Table 2. *Inhibitory action of substance E and of a fraction derived from it, upon the intrinsic-factor activity of a hog gastric concentrate*Vitamin B₁₂ represents 0.5 µg. of radioactive vitamin B₁₂; I.F., intrinsic factor.

Substances fed	Absorption of radioactive vitamin B ₁₂ (%)	Inhibition of B ₁₂ absorption (% of B ₁₂ /mg.)
Vitamin B ₁₂	2.7, 6.6	—
Vitamin B ₁₂ + hog I.F. (25 mg.)	61.4	—
Vitamin B ₁₂ + hog I.F. (25 mg.) + 32 mg. of substance E (batch 4)	36.9	0.8
Vitamin B ₁₂	22.2	—
Vitamin B ₁₂ + hog I.F. (25 mg.)	83.8, 77.6	—
Vitamin B ₁₂ + hog I.F. (25 mg.) + 14 mg. of 61–69% fraction (batch 5)	65.2	1.1
Vitamin B ₁₂	13.7	—
Vitamin B ₁₂ + 24.5 mg. of 41–50% fraction	40	—
Vitamin B ₁₂ + 41–50% fraction (as above) + 14 mg. of 61–69% fraction	19.6	1.5
Vitamin B ₁₂	19.8	—
Vitamin B ₁₂ + hog I.F. (25 mg.)	43.5, 45.4	—
Vitamin B ₁₂ + hog I.F. (25 mg.) + 5 mg. of 69–75% fraction (batch 5)	32.8	1.1

Blood-group activity of substance E. It is known that human gastric juice may contain A, B, or H blood-group substances according to the group of the individual. A sample of substance E possessed slight A-substance activity corresponding to about 0.2% by wt.

Intrinsic-factor activity of other ultracentrifugal fractions. Microsomal preparations from gastric mucosal extracts were prepared on three occasions and each preparation was free from intrinsic-factor activity. The supernatant fluids from these preparations, after further ultracentrifuging to ensure that they were completely free from particles, did, however, possess intrinsic-factor activity. Approximately half of the intrinsic-factor activity of the mucous membrane enters the supernatant, and half remains attached to the mitochondria. Further investigation of the supernatant fluid has shown that after concentration by dialysis against gum arabic, the active material is completely precipitated by 100% saturation with ammonium sulphate, but is not precipitated by 50 or 75% saturation.

DISCUSSION

The results show that intrinsic-factor activity may be extracted from human gastric mucosa in two forms, one attached to mitochondria and the other dissolved in the particle-free supernatant obtained after repeated ultracentrifuging. In a previous communication (O'Brien *et al.* 1955) the question was discussed as to whether intrinsic factor was an integral part of mitochondria or merely adsorbed fortuitously on the mitochondrial surface, and evidence was presented favouring the

former view. This evidence has since been reinforced by the discovery that, in the rat, intrinsic-factor activity is so intimately connected with mitochondrial structure that it is not released when the particles are disrupted with water and is therefore likely to be a true mitochondrial component. The general behaviour of substance E during its preparation from human gastric mucosa, and the reproducibility of the procedures involved, also support this view.

The intrinsic-factor activity of the particle-free supernatant has not been investigated further. It may possibly be derived from the mitochondrial factor, either by disintegration during preparation, or as a cytoplasmic storage form. Its existence, together with that of possibly two active mitochondrial components, indicates that none of the fractions prepared in the present experiments represent the pure substance. Although notable advances have been made by several groups (Latner, Merrills & Raine, 1956; Williams & Ellenbogen, 1958; Heatley, Sheikh & Taylor, 1960; Holdsworth, 1960) the preparation of pure intrinsic factor, whether from hog or human, seems far off, for whatever method of separation of gastric constituents is used—salt precipitation as above, electrophoretic separation (Latner & Ungley, 1953; Glass, Stephanson, Rich & Laughton, 1957; Jacob, Williams, Howe & Glass, 1959) or column ion-exchange chromatography (Johnson, 1959)—intrinsic-factor activity often appears in more than one physically different fraction and even the most active material is impure (Jacob *et al.* 1959; Heatley *et al.* 1960). Since the main methods of assaying intrinsic-factor activity measure the

whole process of vitamin B₁₂ absorption, the possibility should not be overlooked that two or more factors from the stomach influence the mechanism (as suggested by Gräsbeck, 1960) and that preparations may contain one or other or both of these but that feeding either gives rise to apparent 'intrinsic-factor' activity.

The present results show though that intrinsic factor can be prepared from human gastric mitochondria in a form (substance E) that has a reasonably constant composition, is always active and that behaves on electrophoresis and ultracentrifuging as a single molecular aggregate. Moreover when injected into rabbits it stimulates the production of only one serum antibody. This molecular aggregate splits into two components at a pH of 5 or less and fractions obtained by precipitation with ammonium sulphate contain one or other or both of these. One of the components tends to be associated with intrinsic-factor activity and the other with inhibition of this activity. Both behave as homogeneous aggregates on ultracentrifuging and electrophoresis. Minor variations in the precipitability of these components probably arises from differences in initial concentration and from the fact that as ammonium sulphate is added the pH falls, and splitting into two components occurs.

One of the many puzzling features about intrinsic factor is that preparations of it from human gastric juice are relatively inactive, when compared with material from hog gastric mucosa (Glass, Boyd, Stephanson & Jones, 1955; Latner *et al.* 1956; Glass *et al.* 1957; Williams & Ellenbogen, 1958; Holdsworth, 1960) or from hog pyloric juice (Heatley *et al.* 1960). This is one of the reasons why few investigators work with human material. Human gastric mucosa has in our hands similarly yielded material with relatively low activity, but a possible explanation is suggested by the observation that intrinsic factor is intimately associated within the cells with a substance inhibiting its action. Certainly, as in batches 1 and 2, a material with activity approaching that of the best hog preparations may be obtained from human material, but its preparation is, as Table 1 shows, quite unpredictable.

The presence, in hog-intrinsic-factor concentrates, of a vitamin-B₁₂-binding material which inhibits intrinsic-factor activity has been suggested in order to explain the inhibition of vitamin B₁₂ absorption which may occur, in both normal subjects and those with pernicious anaemia, and in normal and gastrectomized rats, when high test doses of intrinsic factor are used (Glass *et al.* 1955; Taylor, Mallett & Spray, 1958*a*; Taylor *et al.* 1958*b*). An alternative explanation of these observations has been proposed (Taylor *et al.* 1958*a, b*; Herbert, 1959), which does not involve a

hypothetical inhibitor. It supposes that when high doses of intrinsic factor are fed, the excess of it which fails to combine with vitamin B₁₂ attaches to receptor substances on the intestinal mucosal surface and thus blocks the attachment of the vitamin B₁₂-intrinsic-factor complex and the absorption of vitamin B₁₂. This latter hypothesis is the more plausible in that it explains why inhibition should occur only with high intakes of intrinsic factor, for a specific inhibiting substance should inhibit at low dosage too. It has, however, been pointed out that the two hypotheses are not mutually exclusive (Taylor *et al.* 1958*a, b*). The present work indicates that so far as human gastric mucosal extracts are concerned, an inhibitor of vitamin B₁₂ absorption is present, but this does not necessarily mean that a similar inhibitor occurs in the hog, nor that the inhibitor is secreted into human gastric juice. Nor does it invalidate the receptor-blocking hypothesis.

Because of the relatively gentle methods by which cell particles, substance E and its two components have been obtained, it is felt that the present observations have physiological significance. They show that intrinsic-factor activity, or some part of it, originates in the mitochondria of the gastric mucosal cells (from patients with peptic ulcer) in association with an inhibitor. After removal of the inhibitor, activity of the remaining material is enhanced when tested in patients with pernicious anaemia. The intrinsic-factor-inhibitor complex is itself mildly active, either of its own accord or because dissociation of the inhibitor occurs in the gut of such patients. *In vitro*, a small shift of pH from 7 to about 5 will induce this dissociation. If these results can be applied to normal subjects they would suggest that, when mitochondrial intrinsic factor is secreted, the inhibitor is either left behind in the mucosal cells, for it is not yet known to occur in gastric juice, or is dissociated from the active factor by hydrogen ions in the stomach. In this last event there would be a certain similarity with the secretion of pepsinogen and its conversion at low pH into pepsin and pepsin inhibitor, but the resemblance should not be overstressed for intrinsic-factor inhibitor is a protein, whereas pepsin inhibitor is a peptide, and the mechanism by which intrinsic-factor inhibitor acts is not yet known.

SUMMARY

1. Intrinsic-factor activity is present in both the mitochondria and the particle-free supernatant of human gastric mucosal extracts after repeated differential ultracentrifuging.

2. A single molecular aggregate can be prepared from the mitochondria, which dissociates below pH 5 into two components. One component

possesses intrinsic-factor activity, the other inhibits this activity and that of hog intrinsic factor.

3. It is concluded that a part of the intrinsic-factor activity of gastric mucosal cells is located on the mitochondria in association with a substance which inhibits its action.

4. The existence of this inhibitor may account for the relative inactivity of intrinsic-factor preparations from human material as compared with those from hog pyloric mucosa.

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Amino Acid-Activating Enzymes in Rat-Liver Mitochondria

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Studies on the incorporation of labelled amino acids into mitochondria both in intact animals (McLean, Cohn, Brandt & Simpson, 1958) and in cell-free systems (McLean *et al.* 1958; Reis, Coote & Work, 1959; Greengard, 1959; Greengard & Campbell, 1959; Korner, 1959; Fraser & Gutfreund, 1958; Mager, 1960) provide evidence for a role of these subcellular particles in protein synthesis. It was of interest to determine whether amino acid activation in mitochondria occurs by a mechanism similar to that which has been shown to exist in other parts of the cell (Hoagland, Keller & Zamecnik, 1956) and in many micro-organisms (De Moss & Novelli, 1956). In the series of experiments reported here, rat-liver preparations were used.

If the amino acid-activating enzymes play a role in protein synthesis in mitochondria, they must be associated with the mitochondria themselves because, although the addition of the soluble frac-

tion of the cell enhances amino acid incorporation (McLean *et al.* 1958), this effect is almost fully retained after heat treatment and can be accounted for, at least in part, by the amino acid content of this fraction (Reis *et al.* 1959; J. Lucas & M. V. Simpson, unpublished work). The methods used in testing for the activating enzymes included amino acid-dependent hydroxamic acid formation and amino acid-dependent pyrophosphate-adenosine triphosphate exchange (Hoagland *et al.* 1956). The results obtained with an amino acid mixture suggest the presence of activating enzymes, in confirmation of the results of Reis *et al.* (1959) on rat-liver mitochondria and of Davis & Novelli (1958) on pea-seedling mitochondria. Further work with individual amino acids revealed the presence of an activation mechanism for tryptophan and leucine. However, enzyme activities for most of the other amino acids could not be demonstrated, and the question of the general mode of amino acid activation in mitochondria is left unsettled.

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