The stability of the radiotriolein bond in intestinal secretions

A. G. COX AND Z. HINCHLIFFE

From the University Department of Surgery, Royal Infirmary, Sheffield

SYNOPSIS The radiotriolein test has been reported to be unreliable in the diagnosis of steatorrhoea. A possible explanation is provided by these studies in which labelled triolein was incubated in human gastric juice, duodenal juice, and faeces; the bond was not stable on duodenal juice.

Radio-iodinated triolein (RIT) has been widely used in the investigation of intestinal malabsorption. The method is essentially a tracer test in which a known dose of RIT is given by mouth and the percentage of the administered isotope appearing in the plasma or excreted in the faeces is measured. This provides an assessment of fat digestion and absorption by the alimentary tract.

The reliability of the method was not seriously questioned until Pimparkar, Tulskey, Kalser, and Bockus (1960) reported a large series of cases tested by concurrent faecal chemical fat and faecal RIT measurements. Taking a daily faecal chemical fat excretion of over 7.0 g. as the criterion for steatorrhoea, they found that the RIT test failed to diagnose the condition in 25% of cases. In a personal series of 33 patients found to have steatorrhoea by the faecal chemical fat excretion, the RIT test failed in 50% (Cox, 1961). A possible explanation of these findings is that the radio-iodine label is unstable in the alimentary tract. Since the metabolic pathway of iodine differs from that of fat, instability of the RIT bond would clearly vitiate the test.

Experimental testing of the RIT bond in intestinal secretions has been reported by Beres, Wenger, and Kirsner (1957) and by Balint, Pendower, and Ramsey (1960). The former group incubated RIT with gastric juice, pancreatic juice, and bile for one hour, and, by measuring the activity in the aqueous fraction after extraction with petroleum ether, they concluded that the bond was stable over the period of the experiment. Using a similar method, Balint and his colleagues incubated RIT in a wider range of media. Instability was not detected during four hours' incubation in gastric or duodenal secretions, but a small amount of free radio-iodine was liberated in faeces over a two-day period of incubation.

The use of an anion exchange resin to determine the percentage of free I^{131} in radio-iodinated lipids (Cox and Hinchliffe, 1961) offers a simple means of repeated testing of the stability of the bond, permits the behaviour of the bond to be followed over a long period of time, and allows a comparison of the characteristics of different batches of RIT. This paper reports the results of such a study.

METHOD

The experiments were conducted in the following way. The batch of RIT under test was emulsified with Tween 80 and water, and incubated with controls and intestinal secretions. Water at 20°C. and 37°C. served as controls. The intestinal secretions studied were gastric juice, duodenal juice, and faeces, each incubated at 37°C. Samples were taken from the incubation mixtures at intervals of 0, four, eight, 24, 48, 72, and 96 hours and tested for the amount of free I^{131} present. A rise in the percentage of free I^{131} was taken to indicate instability of the bond under the conditions of the test.

MATERIALS

RADIOTRIOLEIN Radioiodine (I^{131}) in two different chemical forms was used to label triolein.

Triolein labelled with I^{131} as elementary iodine The fat in ethereal solution was mixed with elementary I^{131} in ether. After allowing 48 hours for iodination, any remaining free I^{131} was removed by washing three times with a solution of sodium thiosulphate and potassium iodide. This was followed by three washes with distilled water. The ether was evaporated on a water-bath at 50°C.

Triolein labelled with I^{131} as iodine monochloride Iodine monochloride ($I^{131}Cl$) is generally accepted as the more suitable label. Four batches of triolein, differing according to labelling procedure or source, were selected for test. Brief details of these are given in the following paragraphs. In the description of the results they will be referred to by the letters given here.

A RIT was purchased from the Radiochemical Centre at Amersham. Full details of the labelling procedure are not available; the Hormel Foundation supplies triolein to Amersham.

B Triolein from the Aldrich Chemical Company was labelled by the method of Lubran and Pearson (1958).

C Synthetic triolein from Unilever Ltd. was labelled in the following way. Elementary iodine, liberated from an aqueous solution of NaI^{131} and carrier KI by oxidation with potassium iodate and sulphuric acid, was extracted into carbon tetrachloride and dried on a column of anhydrous sodium sulphate. A solution of chlorine in carbon tetrachloride, dried in the same way, was added dropwise to the I^{131} solution until it turned brown, the colour of iodine monochloride in carbon tetrachloride (Sidgwick, 1950). This iodinating solution was added to triolein dissolved in carbon tetrachloride. After the mixture had been allowed to react in the dark at 20°C . for three hours, any remaining free I^{131} was extracted into a weak solution of sodium thiosulphate and converted to I^{131}Cl in carbon tetrachloride as before, thus making it available for further iodination. After standing overnight, the solution was washed with sodium thiosulphate and potassium iodide solution, followed by water. The carbon tetrachloride was evaporated on a water-bath at 50°C . This two-stage iodination procedure was used in order to increase the percentage tag (Dossett, 1960).

D Triolein from the Aldrich Chemical Company was labelled in the same way as C.

INCUBATION MEDIA Four were used.

1 *Water at 20°C . and 37°C .* These served as controls and to assess the stability of the bond when RIT is stored as an emulsion for the purpose of dosing patients.

2 *Human gastric juice at 37°C .* Specimens were obtained during maximal histamine stimulation from duodenal ulcer patients undergoing routine pre-operative augmented histamine tests (Kay, 1953).

3 *Human duodenal juice at 37°C .* Samples were obtained by duodenal aspiration during secretin and pancreozymin stimulation. A pooled specimen, containing bile and pancreatic juice, was used. No attempt was made to exclude contamination with gastric secretions, since the intention was to mimic as closely as possible the secretions with which ingested RIT comes into contact. Most specimens were obtained from duodenal ulcer patients and their secretions will be referred to as 'hypersecretor' juice. One specimen was from a patient with a Polya partial gastrectomy, who failed to secrete acid on maximal histamine stimulation; his secretions will be referred to as 'achlorhydric' juice.

Duodenal juice was used both untreated and after boiling for 30 minutes; these will be referred to as 'fresh' and 'boiled' juice respectively. When not required for immediate use, the duodenal juice was stored at -40°C . and thawed out slowly at 2°C . immediately before the start of an experiment.

4 *Homogenized human faeces at 37°C .* Specimens were obtained immediately after defaecation from a healthy subject.

RESULTS

STABILITY OF THE BOND IN WATER The I^{131}Cl labelled triolein remained stable in water at 20°C . and 37°C . throughout 96 hours (Table I). By contrast the simple I^{131} label was not completely stable.

STABILITY OF THE BOND IN GASTRIC JUICE Two batches of I^{131}Cl labelled triolein were tested and each showed stability in gastric juice at 37°C . over the 96-hour period of study (Table II).

STABILITY OF THE BOND IN DUODENAL JUICE Since the first few experiments revealed appreciable instability of the bond in duodenal juice, this

TABLE I
STABILITY OF THE RADIOTRIOLEIN BOND IN WATER

<i>At 20°C.</i>		<i>Free I¹³¹ as Percentage of Total I¹³¹</i>							<i>No. of Specimens</i>
		<i>Hours of Incubation</i>							
		<i>0</i>	<i>4</i>	<i>8</i>	<i>24</i>	<i>48</i>	<i>72</i>	<i>96</i>	
I¹³¹ Cl label	A	0.5	1	1	0	8.5	5	2.5	2
	B	2	0	0	9	0	2	5	1
	C	0.7	0.3	2.3	3.1	4.0	0.3	1	3
	D	1	0	2	4	5	10	6	1
	Mean	1	0.3	1.3	4.0	4.4	4.4	3.8	7
Simple I¹³¹ label		5	8	3	6.5	9.5	13.5	15.5	2
<i>At 37°C.</i>									
I¹³¹ Cl label	A	7	0	0	0	8	0	2	2
	B	0	0	0	8	0	4	2	1
	C	2	0.3	2	0	2	2.3	2	3
	D	5	0	0	0	0	5	4	1
	Mean	2.9	0.1	0.9	2	1.9	3.3	3	7
Simple I¹³¹ label		2	4.5	1	9	13	8	12	2

TABLE II

STABILITY OF RADIOTRIOLEIN BOND IN
HUMAN GASTRIC JUICE

At 37°C.		Free I ¹³¹ as Percentage of Total I ¹³¹						
		Hours of Incubation						
		0	4	8	24	48	72	96
I ¹³¹ Cl label	A	0	0	0	0	4	0	0
	B	0	1	1	3	0	7	0

medium was tested more extensively. The results of the tests with RIT in fresh and boiled hypersecretor duodenal juice are given in Table III, and Fig. 1 illustrates the rise in the percentage of free I¹³¹ of each batch in the fresh juice. The findings may be summarized as follows:—

The instability of the I¹³¹ Cl bond first becomes apparent on 24 hours' incubation in fresh, hypersecretor juice and increases thereafter.

The I¹³¹ Cl bond is stable in boiled, hypersecretor juice.

All batches of I¹³¹ Cl labelled triolein behave in the same way in duodenal juice.

FIG. 1. Diagram to show the rise in the percentage of free I¹³¹ in seven batches of radio-iodinated triolein incubated at 37°C. with fresh, hypersecretor duodenal juice over a period of 96 hours.

TABLE III

STABILITY OF RADIOTRIOLEIN BOND IN DUODENAL SECRETIONS

At 37°C.		Free I ¹³¹ as Percentage of Total I ¹³¹							No. of Specimens
		Hours of Incubation							
		0	4	8	24	48	72	96	
Fresh hypersecretor juice									
I ¹³¹ Cl label	A	7	4	7	18	30	36	47	2
	B	3	4	5	15	30	36	41	1
	C	2	4	5	12	21	28	33	2
	D	1	3	9	15	22	29	29	1
	Mean	3.2	3.8	6.5	15	25.8	32.7	35	6
Simple I ¹³¹ label		1	30	46	69	78	80	82	1
Boiled hypersecretor juice									
I ¹³¹ Cl label	A	3.5	0	0	1.5	1	3.5	5	2
	B	4	2	7	6	4	2	3	2
	C	2.5	4	2.5	2.5	4.5	4	9	2
	D	2	5	0	4	7	6	5	1
	Mean	3	2.8	2.4	3.5	4.1	3.9	5.5	7
Simple I ¹³¹ label		5	2	4.5	4.5	24	29	36	2

The simple I¹³¹ bond shows marked instability after four hours' incubation with fresh, hypersecretor juice.

The simple I¹³¹ bond is unstable after 48 hours' incubation with boiled hypersecretor juice.

One batch of each of the I¹³¹ Cl and the simple I¹³¹ labelled triolein was tested in the achlorhydric duodenal juice. Both bonds were more stable in the fresh achlorhydric juice than in the fresh hypersecretor juice. Each bond behaved in the same way in boiled achlorhydric juice as in boiled hypersecretor juice; the I¹³¹ Cl bond remained stable over 96 hours of incubation, whereas

instability of the simple I¹³¹ bond appeared after 48 hours' incubation (Fig. 2).

STABILITY OF THE BOND IN FAECES The I¹³¹ Cl labelled triolein showed a minor degree of in-

TABLE IV

STABILITY OF RADIOTRIOLEIN BOND IN HUMAN FAECES
At 37°C.

		Free I ¹³¹ as Percentage of Total I ¹³¹						
		Hours of Incubation						
		0	4	8	24	48	72	96
I ¹³¹ Cl label	A	3	5	2	5	6	7	8
	B	0	7	1	3	10	12	9

FIG. 2. The stability of radio-iodinated triolein in hypersecretor duodenal juice (X) and achlorhydric duodenal juice (Y). The unbroken lines represent experiments with samples of the fresh juice and the broken lines experiments with the boiled juice.

stability after 96 hours' incubation in faeces at 37°C. (Table IV).

DISCUSSION

The conventional fat balance is generally held to be a difficult and tedious investigation. The introduction of the RIT test raised hopes that a simpler and more reliable means of diagnosing steatorrhoea had been found, and early reports appeared to justify this optimism. More recently, however, evidence has accumulated to show that the method is less sensitive than the fat balance in the diagnosis of steatorrhoea. Several groups of workers have found that the RIT test fails to diagnose steatorrhoea in a substantial number of cases in which the condition has been confirmed by concurrent faecal chemical fat determinations (Pimparkar *et al.*, 1960; Vaughan Jones, 1960; Cox, 1961). While technical errors, such as incomplete stool collection, may account for the failure of RIT in some cases, it is unlikely that these provide the complete explanation.

The RIT test relies for its validity on at least two fundamental assumptions which require verification before the method can be accepted. First, ingested RIT can at best be expected to follow the fate of dietary fat in the alimentary tract, and it is therefore fundamental to the RIT test that faecal chemical fat should be of predominantly dietary origin. However, Cooke, Thomas, Mangall, and Cross (1953) have reported patients with steatorrhoea in whom the faecal chemical fat excretion was appreciably higher than the dietary fat intake. In these cases, the lipid content of the stool may originate in part from the bile (McClure, Huntsinger,

and Fernald, 1934), synthesis of fats by the cells of the intestinal mucosa (Clark and Hübscher, 1960), active fat secretion by the bowel mucosa (Lewis and Partin, 1954), or by bacterial synthesis (Sammons, Vaughan, and Frazer, 1956). In patients with a normal faecal RIT excretion, a high faecal chemical fat excretion might be explained if the faecal lipids were of endogenous origin and not derived from unabsorbed dietary fat. The second fundamental assumption is that the radio-iodine label remains firmly attached to triolein in the alimentary tract; appreciable instability of the bond would annul any value that RIT might have as a diagnostic or research tool. The studies reported in this paper are concerned with this facet of the problem and have been designed to extend the observations of previous workers who found that the bond was stable over a relatively short period of time in the intestinal secretions they used (Beres *et al.*, 1957; Balint *et al.*, 1960).

Triolein labelled with radio-iodine in the simple elementary form has been used recently in clinical studies by Siurala, Lamberg, Turula, and Härkönen (1959). Our experiments with this material showed that 46% of the radio-iodine is split off after four hours' contact with fresh duodenal juice and that prolonged incubation resulted in the liberation of increasing amounts of the radio-isotope. Boiling the duodenal juice greatly diminished its bond-splitting property. In the water controls, only a minor degree of instability of the bond was detected. These findings support the view of Veall and Vetter (1958) that the simple radio-iodine label is unstable in the alimentary tract and therefore unsuitable for clinical use.

Triolein labelled with I^{131} as iodine monochloride is the more widely used material and has therefore been studied more extensively in these experiments, in order to compare the characteristics of different batches of RIT and to determine its stability in a wider range of intestinal secretions.

Thirty-six experiments were carried out using different incubation media and different batches of RIT. Each batch behaved similarly in a given set of conditions. This may be taken as evidence that the stability of the I^{131} Cl bond is not materially affected by varying the method of the labelling procedure and that each type of triolein used for labelling had similar properties.

Incubation for 96 hours showed that the I^{131} Cl label is stable in water at 20°C. and 37°C. This confirms the findings of Cheers, McCraw, Tyor, and Ruffin (1958), and demonstrates that RIT for clinical use may be stored as an emulsion. In gastric juice free I^{131} was not released from RIT, indicating that the bond is stable in the stomach. In faeces, however, a small rise in the percentage of free I^{131} was detected over the four-day incubation period. This is in keeping with the finding of Balint *et al.* (1960), but the degree of instability is so small that it seems unlikely to invalidate the clinical use of RIT.

The short period of incubation in duodenal juice used by other workers did not reveal any instability of the I^{131} Cl bond. In the experiments reported here it was found that extending the period of contact with duodenal juice caused an appreciable rise in the percentage of free I^{131} . In fresh duodenal juice, 15% of the I^{131} Cl label was split off after 24 hours' incubation and 35% at the end of 96 hours. The same juice, when boiled, had no effect on the bond, suggesting that the bond-splitting property of duodenal juice rests in its enzyme content.

The relevance of these findings to the use of RIT in the investigation of malabsorption is difficult to assess. The view may be held that the clinical use of the material is not jeopardized, since ingested RIT does not stay in the small intestine for the period of 24 hours required to demonstrate instability of the bond *in vitro*. However, these experiments suggest that the enzymes of duodenal juice are responsible for the breakdown of the bond, and Lagerlöf (1942) has shown that the lipolytic activity of duodenal juice falls by as much as 70% when kept at 37°C. for a period of 30 minutes. Whether this decline in activity occurs as rapidly in the alimentary tract is uncertain. If the enzyme activity of duodenal juice is maintained throughout the small intestine, it is possible that its bond-splitting potency is greater in the bowel than in the

test-tube. If this is so, the instability of the bond may reduce the reliability of the RIT test in the assessment of malabsorption.

If the breakdown of the radio-iodine linkage accounts for the failure of RIT to diagnose steatorrhoea in some cases, its success in others remains to be explained. In this connexion, the finding that duodenal juice from an achlorhydric, post-gastrectomy patient had markedly reduced bond-splitting properties was of considerable interest. It may be that biliary and pancreatic secretions from different subjects differ in their power to split the bond and that failure to diagnose steatorrhoea occurs where bond-splitting potency is high. On the evidence available, it would be speculative to carry the argument further than the statement that the behaviour of RIT in the alimentary tract may vary from patient to patient. These results *in vitro* can best be interpreted in the light of studies *in vivo* which are now in progress.

SUMMARY

The purpose of the study was to investigate the stability of the radio-iodinated triolein bond in intestinal secretions.

Labelled triolein was incubated in gastric juice, duodenal juice, and faeces. At intervals over a period of 96 hours, samples were taken from the incubation mixtures and the percentage of liberated I^{131} determined.

The simple I^{131} label was slightly unstable in water and very strikingly unstable in duodenal juice. By boiling the duodenal juice, its bond-splitting potency was reduced.

The I^{131} Cl label was stable in water and gastric juice. A minor degree of instability in faeces was detected. In duodenal juice, instability of the bond first became apparent after 24 hours' incubation. No free I^{131} appeared when the duodenal juice was boiled.

The duodenal juice from an achlorhydric patient affected the bond less than did the juice from duodenal ulcer patients.

These results provide one possible explanation for the reported unreliability of the radiotriolein test.

This study was generously supported by the Tuberculosis Research Fund of the University of Sheffield. We wish to express our gratitude to the following: Professor A. W. Kay for constant advice and encouragement in the laboratory work and preparation of the manuscript, Professor S. R. Elsdon for many helpful suggestions, Mrs. M. Fisher and Mr. B. Watson for advice on radio-isotope techniques, and Mr. Crossley of Unilever Ltd. for supplies of triolein.

REFERENCES

- Balint, J., Pendower, J., and Ramsey, N. W. (1960). The stability of radio-iodinated olive oil. *Clin. Sci.*, **19**, 321-325.
- Beres, P., Wenger, J., and Kirsner, J. B. (1957). The use of I^{131} triolein in the study of absorptive disorders in man. *Gastroenterology*, **32**, 1-16.
- Chears, W. C., McCraw, B. H., Tyor, M. P., and Ruffin, J. M. (1958). The I^{131} labeled triolein absorption test; reproducibility and factors affecting blood levels. *Sth. med. J. (Bgham, Ala.)*, **51**, 433-437.
- Clark, B., and Hübscher, G. (1960). Biosynthesis of glycerides in the mucosa of the small intestine. *Nature (Lond.)*, **185**, 35-37.
- Cooke, W. T., Thomas, G., Mangall, D., and Cross, H. (1953). Observations on the faecal excretion of total solids, nitrogen, sodium, potassium, water, and fat in the steatorrhoea syndrome. *Clin. Sci.*, **12**, 223-234.
- Cox, A. G. (1961). An assessment of the radio triolein test. In the press.
- , and Hinchliffe, Z. (1961). A method for the determination of free I^{131} in radio-iodinated lipids. In the press.
- Dossett, J. A. (1960). Personal communication.
- Kay, A. W. (1953). Effect of large doses of histamine on gastric secretion of HCl. *Brit. med. J.*, **2**, 77-80.
- Lagerlöf, H. O. (1942). Pancreatic function and pancreatic disease. *Acta med. scand.*, Suppl. 128, pp. 55-61.
- Lewis, G. T., and Partin, H. C. (1954). Fecal fat on an essentially fat-free diet. *J. Lab. clin. Med.*, **44**, 91-93.
- Lubran, M., and Pearson, J. D. (1958). A screening test for steatorrhoea using I^{131} labelled triolein. *J. clin. Path.*, **11**, 165-169.
- McClure, C. W., Huntsinger, M. E., and Fernald, A. T. (1934). The fatty acids of human duodenal bile. *Amer. J. Physiol.*, **107**, 1-12.
- Pimparkar, B. D., Tulsy, E. G., Kalsner, M. H., and Bockus H. L. (1960). Correlation of radioactive and chemical faecal fat in different malabsorption syndromes. *Brit. med. J.*, **2**, 894-900.
- Sammons, H. G., Vaughan, D. J., and Frazer, A. C. (1956). Synthesis of long-chain fats by bacteria isolated from human faeces. *Nature (Lond.)*, **177**, 237.
- Sidgwick, N. V. (1950). *The Chemical Elements and Their Compounds*. Vol. 2, p. 1153. Clarendon Press, Oxford.
- Siurala, M., Lamberg, B.-A., Turula, K., and Härkönen, P. (1959). The estimation of intestinal fat absorption using I^{131} labelled olive oil. *Ann. Med. exp. Fenn.*, **37**, 424-432.
- Vaughan Jones, R. (1960). *Brit. med. J.*, **2**, 1236-1237.
- Veall, N., and Vetter, H. (1958). *Radioisotope Techniques in Clinical Research and Diagnosis*, p. 344. Butterworth, London.