- Jackson, K. & Entenman, C. (1957). *Amer. J. Physiol.* **189**, 315.
- Kaplan, H. S. (1959). *Cancer Res.* **19**, 791.
- Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
- Kirby, K. S. (1961). *Biochim. biophys. Acta*, **47**, 18.
- Krüger, M. & Salomon, G. (1898). *Hoppe-Seyl. Z.* **24**, 350.
- Lawley, P. D. (1957). *Proc. chem. Soc., Lond.*, p. 290.
- Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
- Lewis, W. H. (1935). *Science*, **81**, 545.
- Magee, P. N. (1956). *Biochem. J.* **64**, 676.
- Magee, P. N. (1958). *Biochem. J.* **70**, 606.
- Magee, P. N. & Barnes, J. M. (1959). *Acta Un. int. Cancr.* **15**, 187.
- Magee, P. N. & Barnes, J. M. (1962). *J. Path. Bact.* (in the Press).
- Magee, P. N. & Hultin, T. (1962). *Biochem. J.* **83**, 106.
- Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
- Müller, E., Haiss, H. & Rundel, W. (1960). *Chem. Ber.* **93**, 1541.
- Reiner, B. & Zamenhof, S. (1957). *J. biol. Chem.* **228**, 475.
- Rose, F. L. (1958). In *Symposium on the Evaluation of Drug Toxicity*, p. 116. Ed. by Walpole, A. L. & Spinks, A. London: J. and A. Churchill Ltd.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
- Stekol, J. A., Mody, U. & Perry, J. (1960). *J. biol. Chem.* **235**, PC 59.
- Tessman, I. (1959). *Virology*, **9**, 375.
- Timmis, G. M. (1960). *Biochem. Pharmacol.* **4**, 49.
- Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
- Webb, J. M. (1956). *J. biol. Chem.* **221**, 635.
- Weissman, B., Bromberg, P. A. & Gutman, A. B. (1957). *J. biol. Chem.* **224**, 407.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
- Wyngaarden, J. B., Blair, A. E. & Hilley, L. (1958). *J. clin. Invest.* **37**, 579.

Biochem. J. (1962) **83**, 124

Citrate-Promoted Helix Formation in Gelatin

THE VISCOSITY-TIME EFFECT

By A. COURTS*

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 7 September 1961)

The changes which take place when native collagen is converted into the water-soluble protein, gelatin, are generally regarded as irreversible reactions. Veis, Anesey & Cohen (1958) describe the essential aspects as: (1) irreversible melting of the polypeptide helical coils; (2) irreversible severance of inter-peptide-chain bonds; (3) irreversible hydrolysis of peptide linkages. Courts (1958) showed that (3) involved only especially-labile peptide bonds and that (1) is likely to be the final stage, resulting from rupture of hydrogen bonds.

In special instances, however, evidence from the application of widely differing techniques shows that the random-coil structure of gelatin molecules appears partially to re-form a collagen-type configuration. Thus Robinson (1953) prepared gelatin films from a 5% solution by hot (55°) and cold (18°) evaporation. The 'cold form' of gelatin gave the characteristic infrared dichroism of collagen. Veis & Cohen (1960) obtained some unusual heat-precipitated gelatin fibres, which revealed in the electron microscope a banded structure with 610 Å spacings. Working with the heat-denatured soluble

fraction of rat-tail-tendon collagen, Flory & Weaver (1960) have demonstrated that a relatively slow transition from a random-coil to a helical configuration seems likely to account for the increases in viscosity in dilute solutions and in optical-rotation values obtained with this material.

Results similar to those of Flory & Weaver (1960) were obtained in the present work, and show that the reversion behaviour is likely to be a general reaction for gelatin. A number of typical gelatins, with a wide variety of values for weight-average molecular weight and rigidity modulus, have been studied as dilute solutions in citrate and other buffers. The increase in viscosity of these solutions with time is clearly enhanced by aliphatic carboxylic acids. Since molecular reorientation of the gelatin is a plausible explanation of this behaviour, the degree to which a gelatin can demonstrate the viscosity-time effect is viewed in relation to some other physical properties, which are governed by the size and shape of the molecule. To examine a possible relationship with molecular weight, it was necessary to fractionate a series of gelatins into units with considerably enhanced weight-average molecular-weight homogeneity. No such relation-

* Present address: Unilever Food Research Department, Colworth House, Sharnbrook, Bedford.

ship was in fact established. On the other hand, the reversion effect can be related to the rigidity modulus of the gelatin. A preliminary note of the results was given by Courts (1961).

The rate of viscosity increase in all the gelatins examined was linear after 24 hr., and this state was maintained for 5 days and sometimes longer. The rate slope between 1 and 5 days was, therefore, taken to define numerically the change in viscosity with time, in viscosity units/day, for a particular gelatin. This particular rate of viscosity increase is expressed as r .

MATERIALS AND METHODS

Laboratory-prepared gelatins. The method for extracting a series of gelatins from a single batch of collagen (de-mineralized ox bone) followed the procedure given by Courts (1959). The object was to provide materials with rigidity moduli ranging from high to moderate values as the molecular-weight pattern followed the reverse order.

Fractionated gelatins. Certain of the laboratory-prepared gelatins were fractionated by the ethanol procedure of Stainsby (1954). This enabled gelatins which were more homogeneous in molecular weight to be studied. In addition, this procedure eliminates all possibility of the properties described being partly attributable to non-gelatin impurities.

Commercial gelatins. The two main types examined were acid-processed pig-skin and alkali-processed ox-hide gelatins.

Serum albumin. Freeze-dried human serum albumin, of the type used in transfusion work, was examined. The author is grateful to Dr Doreen Birch of the Postgraduate Medical School for this material.

Solvent. The proteins were dissolved in a citrate buffer, pH 3.7, similar to that used as the dispersing agent for soluble collagens (Gallop, 1955; Boedtke & Doty, 1956; Courts & Stainsby, 1959). This was prepared by dissolving 14.7 g. of sodium citrate (dihydrate) and 21 g. of citric acid (monohydrate) (21 g.) in 1 l. of water. Viscosity measurements were made on 0.25% gelatin. Other conditions used are described in the appropriate part of the text.

Viscosity of dilute solutions. Times of flow were measured in Ostwald-type U-tube capillary viscometers in which the value for water at 20° was about 70 sec. Times of flow greater than 100 sec. were generally obtained for the solutions under test. The temperature was controlled at $20.0 \pm 0.1^\circ$. Values, expressed as relative viscosities, were derived as $\eta_{rel.} = \eta_{solution}/\eta_{solvent}$.

Increase of viscosity with time. Solutions were maintained in the viscometers at 20° and flow times measured each day at some fixed time ($\pm \frac{1}{2}$ hr.). The most convenient way of assessing r was from the standardized plot. This is detailed in the Results section.

Viscosity of concentrated solutions and limiting viscosity number. The gelatins used in this study were characterized by the viscosity of 5.65% (w/w) solutions at 40°, according to the B.S. 757:1944. This value can be related to the viscosity in dilute solution (2.5 mg./ml. in M-NaCl) by a standard graph, and the limiting viscosity number calculated from this as in Courts & Stainsby (1958).

Rigidity modulus. The method of Saunders & Ward (1953) was used. Values for the modulus of 5.65% (w/w) gelatin were taken at 10° after maturing for 18 hr. at 10°, and expressed in dynes/cm.².

Dependence on pH. Two solutions of gelatin 246 (see Table 3), both 0.25%, were made in 0.1M-citric acid and in 0.1M-sodium citrate respectively. Sodium hydroxide was added to 20 ml. portions of the acid solution to give the pH range 2.2–7, and to 20 ml. portions of the sodium citrate solution to give the pH range 7.2–10. The solutions were made up to constant volume with water, and the pH was re-measured. The pH adjustment, viscosity measurements and reversion were all carried out at 20°.

Preservative action of citrate. No preservatives were added to the gelatin solutions, so that possible secondary reactions (e.g. cross-linking) were avoided. In all instances so far examined, citrate was a useful preservative against bacterial degradation of gelatins in which the initial contamination was known to be low. Some samples have been stored at 20° for 2 months without showing the viscosity fall associated with gelatin degradation. Citrate solutions of eucollagen have been similarly stored for more than 6 months.

The preservative action of citrate was further demonstrated as follows: (1) solutions (5%) of gelatin in water and in citrate, both at pH 7.8, were incubated at 40° for 24 hr. The initial and final viscosities were measured at 40° in a no. 2 viscometer. Four samples of gelatin gave a viscosity fall in water of 0.18–1.11%/hr.; the corresponding changes in citrate were 0.05–0.13%/hr. (2) The presence of SO₂ (50 p.p.m.) was examined as a preservative with 11 of the gelatins used in this work. No significant changes, compared with those of the same gelatins without added SO₂, could be detected in the final viscosity–time results, although some of the intermediate results were erratic. The addition of SO₂ was, therefore, not continued.

The viscosity–time reaction is reversible so that, after denaturation of the reverted gelatin at 40°, the cycle may be resumed along the same path at 20°. This suggests that the gelatin had been adequately preserved by the presence of citrate during incubation.

RESULTS

Most of the gelatins studied showed an increase of viscosity with time, but substantial differences occurred in the velocity, r , with different gelatins. There were a number of other factors which affected the viscosity–time behaviour.

Effect of gelatin concentration. To avoid secondary complications, 'dilute solution' is defined as one containing less than 0.4% of gelatin and used for viscosity measurements at 20°. Relative viscosity increases are detectable at gelatin concentrations as low as 0.09% (Fig. 1). If the concentration (c) factor is applied to give the limiting specific viscosity, $(\eta_{rel.} - 1)/c$, then, as Table 1 shows, this value is independent of concentration (up to 0.4%) at zero time but then increases with concentration as the viscosity–time effect progresses.

Effect of the anion. A comparison of the change in viscosity with time was made on the same sample

of gelatin dissolved in four solvents at pH 4. The rates were in the decreasing order: 0.15M-citrate, 0.17M-lactate, 0.2M-acetate, water (Fig. 2).

Effect of pH. Stainsby (1952) showed that the viscosity of a gelatin-water sol varied considerably with pH but could become largely independent of pH in 0.1M-sodium chloride. The results for gelatin-citrate (Fig. 3) show a similar non-dependence of viscosity on pH at zero time. With the onset of the viscosity-time effect, however, the rate of change of viscosity is clearly related to pH, being greatest near neutrality.

Fig. 1. Effect of gelatin concentration on the viscosity-time effect. Gelatin concentrations: ○, 0.34%; ●, 0.25%; △, 0.18%; ▲, 0.09%.

Table 1. Dependence of the rate of change of the limiting specific viscosity on gelatin concentration

Gelatin solutions in 0.15M-citrate buffer, pH 3.7, were matured for the indicated times at 20° (sample no. 141).

Concn. of gelatin (%)	Limiting specific viscosity			
	Day 0	Day 1	Day 2	Day 8
0.09	0.9	1.7	2.0	2.8
0.17	0.9	1.9	2.3	3.3
0.34	0.7	2.8	4.4	7.6

Numerical assessment of r . All of the gelatins studied gave a pattern similar to that shown in Figs. 1 and 2. Three stages were apparent: (1) a steep rise during the first day; (2) a steady increase from 1 to 5 days; (3) a falling off from the steady velocity after 5 days in some instances. Part of this deceleration may be due to the onset of bacterial

Fig. 2. Effect of different solvents at pH 4 on the viscosity-time effect of gelatin (0.25%). ●, Water; △, 0.2M-acetate; ○, 0.17M-lactate; ▲, 0.15M-citrate.

Fig. 3. Effect of pH on the change of viscosity of gelatin-citrate at different stages of reversion. A, Zero time; B, 1 day; C, 2 days; D, 4 days.

effects. Stage (2) was considered to be the state of greatest uniformity of reaction and was able to show up to best advantage the rate differences between various gelatins under identical experimental conditions. The slope of the straight line in stage (2) was, therefore, measured to give a numerical assessment to r and expressed in arbitrary viscosity units/day.

Values of r for 15 gelatins are given in Table 2. The gelatins are characterized by the method of preparation and physical properties.

Relationship between change of viscosity and molecular weight. A direct relationship exists between limiting viscosity and number-average molecular weight (Pouradier & Venet, 1952) or the weight-average molecular weight, M_w (Courts & Stainsby, 1958), as determined on relatively homogeneous

Table 2. Rate of viscosity increase, r , for 15 laboratory-prepared gelatins, and the relation to the limiting velocity number and the rigidity modulus, G

The gelatins were prepared from ox bone in three experiments, A, B, C, by alkaline pretreatment and successive neutral extractions. They are described in terms of the liquor number, L, and, for fractionated gelatins, the fraction number, F.

Sample no.	Limiting viscosity number	$10^{-3}G$ (dynes/cm. ²)	r (units/day)
C: L1, F3	51	91	0.66
A: L1, F3	52	88	0.63
A: L2	68	86	0.59
B: L1	45	82	0.37
B: L2	51	78	0.26
A: L3	83	74	0.28
C: L3, F3	62	73	0.26
B: L3	56	69	0.14
B: L4	61	62	0.09
C: L5, F2	92	57	0.06
C: L7, F3	67	55	0.03
B: L5	69	51	0.02
C: L8, F3	60	50	0.02
B: L6	92	49	0.01
B: L7	95	45	0.01

Table 3. Comparisons between measured and calculated rigidity in some commercial gelatins

The calculated rigidity, G , was obtained from the measured r in the equation $G = K.r^\alpha$.

Type and sample no. of gelatin	r (units/day)	$10^{-3}G$	
		Calc.	Measured
Acid-processed pig skin (246)	0.89	92	91
Acid-processed pig skin (149)	0.62	87	84
Acid-processed pig skin (97)	0.12	68	60
Limed ox skin (141)	0.12	68	67
Limed ox skin (127)*	0.20	73	72
Limed ox skin (76)	0.03	55	53

* Sub-fraction 2 of fraction 2.

fractions of gelatin. The absence of conformity of r with the limiting viscosity number (see Table 2) shows that the change of viscosity with time is unrelated to the molecular weight of the gelatin.

Relationship between r and rigidity modulus. The indication of a relationship between r and rigidity modulus, G , is given in Table 2. A direct plot with eight of the laboratory-prepared gelatins had shown that an equation of the type $G = K.r^\alpha$ existed, where K and α are constants determined by the experimental conditions. When $\log G$ was plotted against $\log r$, a straight line resulted, from which the constants $K = 93\,500 (\pm 2\,000)$ and $\alpha = 0.15$ were obtained. The conformity of a number of other gelatins to the general equation is illustrated in Table 3, where G , calculated from r by using these determined values for the constants K and α , is compared with the measured value of G . The probable error in the measurements of G is $\pm 1\%$ and the measurement of K from the graph is accurate to $\pm 2\%$. The agreement between the calculated and determined values of G might, therefore, be expected to fall within 4%. The direct relationship between G and r for the laboratory-prepared and commercial gelatins is given in Fig. 4.

Serum albumin. Solutions of serum albumin in 0.15M-citrate were set up at 20° for viscosity measurements under the following conditions: (a) 0.3%, pH 3.7; (b) 1.0%, pH 7.2. No change of viscosity with time was detected in either experiment over 4 days.

Fig. 4. Relationship between the rate of viscosity increase, r , and the rigidity modulus, G , for various gelatins.

DISCUSSION

The increase of viscosity with time, which certain gelatins exhibit in dilute solution, is clearly induced by buffered aliphatic carboxylic acids, of which citrate is particularly efficient. The comparatively small rise in viscosity of gelatin-water sols, which may occur when maintained under conditions at which gelation is unlikely to occur, has been regarded as due to molecular aggregation. These aggregates would be a prerequisite for the continuous gel network.

The magnitude of the viscosity-time effect of gelatin in citrate cannot be accounted for entirely in terms of molecular aggregation, and significant alterations to the shape of the molecule appear more likely. The reaction may be regarded as one of intramolecular-hydrogen-bond interference by citrate, so that randomly coiled gelatin molecules can reorientate into a more rigid structure with some collagen-type order. The globular protein, serum albumin, gave no viscosity-time effect under similar conditions.

The reversible transformation of random coil into helix, which Flory & Weaver (1960) demonstrated with denatured soluble collagen from rat-tail tendon, is therefore a general reaction for gelatin. Differences encountered in the degree of reorientation are likely to depend on factors such as molecular complexity and not molecular weight. Thus the multi-chain molecules proposed by Courts & Stainsby (1958) for certain gelatins would be restricted in taking up new alignments both helical and side-to-side, whereas non-branching gelatin molecules could show high rates of viscosity increase. As Table 2 shows, gelatin fractions with high limiting viscosity numbers of 92-95 [corresponding to M_w approx. 4×10^5 (Courts & Stainsby, 1958)] give only moderate r values, whereas the highest r values are obtained from gelatins with much smaller M_w values of approx. 2×10^5 .

It follows from the direct relationship between the viscosity-time effect and the rigidity modulus of the gelatins that both are governed, to some extent, by the same factors. The rigidity of a gelatin gel depends, therefore, upon the degree of helical structure into which the molecules can become orientated. Similar conclusions were reached by Todd (1961) after the formulation of a direct relationship between rigidity modulus and optical rotation. Two demonstrations of single-chain gelatin molecules leading to a high-rigidity-modulus gel are, therefore, of some interest. First, this may be seen from Bowes, Elliott & Moss (1955), whose soluble calf-skin-collagen preparations were transformed into a gelatin with an abnormally high modulus of rigidity. Citrate-soluble collagens are generally regarded as triple-strand forms de-

naturing into single strands (Gallop, 1955). Secondly, an assessment of the multi-chain effect in a series of four fractions of a gelatin (Courts & Stainsby, 1958) can be given by the ratio of M_w to number-average chain weight, C_n . For $M_w/C_n = 6.0, 2.4, 1.1$ and 1.0 , the corresponding rigidity moduli for the fractions (Courts, 1959) are 63 000, 68 000, 71 000 and 75 000 respectively. In this series the optimum rigidity occurs for the fraction in which $M_w = C_n$, i.e. the condition most likely to correspond to single-chain molecules.

The derived equation, $G = K.r^\alpha$, is probably of general application for gelatins where G is greater than 50 000 dynes/cm.² at 10°. Smaller values of G give values of r too small to be significant under the experimental conditions used. It is possible in these instances to use modified conditions such as higher gelatin concentration, higher pH or lower temperature, but any modification will alter the values of K and α .

Further evidence for triple-chain helical configuration is now being sought in reverted gelatins by thermal-denaturation studies, by fibre formation together with electron microscopy and by rate changes in the optical rotation of solutions in citrate.

SUMMARY

1. Citrate buffer solutions have the property of promoting viscosity increases in a number of gelatins when maintained at 20° for several days, leading to values of relative viscosity several times that of the original.

2. The behaviour with 0.15M-citrate is dependent on the pH; the maximum change in viscosity occurs near pH 7.

3. The magnitude of the viscosity-time effect is unrelated to the molecular weight of the gelatin, above certain limits.

4. The magnitude of the viscosity-time effect is directly related to the rigidity modulus of the gelatin. The two quantities, rigidity at 10°, G , and the rate of viscosity change, r , may be connected by $G = K.r^\alpha$.

5. It is suggested that the viscosity changes induced by citrate are due to reorientation of gelatin molecules to a collagen-type structure and that unbranched chains will revert in this way more readily than branched molecules.

This paper is published by permission of the Director of Research and Council of The British Gelatine and Glue Research Association.

REFERENCES

- Boedtker, H. & Doty, P. (1956). *J. Amer. chem. Soc.* **78**, 4267.
- Bowes, J. H., Elliott, R. G. & Moss, J. A. (1955). *Biochem. J.* **61**, 143.

- Courts, A. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 145. Ed. by Stainsby, G. London: Pergamon Press.
- Courts, A. (1959). *Biochem. J.* **73**, 596.
- Courts, A. (1961). *Biochem. J.* **79**, 28f.
- Courts, A. & Stainsby, G. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press.
- Courts, A. & Stainsby, G. (1959). *Nature, Lond.*, **183**, 440.
- Flory, P. J. & Weaver, E. S. (1960). *J. Amer. chem. Soc.* **84**, 4518.
- Gallop, P. M. (1955). *Arch. Biochem. Biophys.* **54**, 486.
- Pouradier, J. & Venet, M. (1952). *J. Chim. phys.* **49**, 85.
- Robinson, C. (1953). In *Nature and Structure of Collagen*, p. 96. London: Butterworths Scientific Publications.
- Saunders, P. R. & Ward, A. G. (1953). *Proc. 2nd int. Congr. Rheology, Oxford*, p. 284.
- Stainsby, G. (1952). *Nature, Lond.*, **169**, 662.
- Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.
- Todd, A. (1961). *Nature, Lond.*, **191**, 567.
- Veis, A., Anesey, J. & Cohen, J. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 155. Ed. by Stainsby, G. London: Pergamon Press.
- Veis, A. & Cohen, J. (1960). *Nature, Lond.*, **186**, 721.

Biochem. J. (1962) **83**, 129

The Digestive Release of Amino Acids and their Concentrations in the Portal Plasma of Rats after Protein Feeding

BY ADA GOLDBERG AND K. GUGGENHEIM

Laboratory of Nutrition, Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(Received 21 August 1961)

Attempts to evaluate the nutritional value of proteins have shown that discrepancies sometimes occur between methods based on the amino acid content of the protein and methods involving physiological criteria such as growth performance of animals which have been given the protein. The presence of an amino acid in a protein does not necessarily determine its nutritive value. We therefore attempted to correlate the biological availability of the most limiting amino acids of certain food proteins with their concentrations in the respective proteins. Concentrations of lysine and methionine in plasma from the blood of the vena portae after protein feeding were examined (Guggenheim, Halevy & Friedmann, 1960). The extent and duration of rise of these amino acids in portal plasma was not in complete agreement with the content of the protein sources in these amino acids. The question arose whether this discrepancy is due to different rates of liberation of these amino acids during digestion.

In our previous study (Guggenheim *et al.* 1960) the portal plasma was examined 30, 60, 120 and 180 min. after the protein meal. Maximum concentrations of both lysine and methionine in portal plasma were already observed 30 min. after the meal, indicating rapid digestion and absorption. In the present paper data are presented on the digestive liberation of amino acids and of their concentrations in portal plasma obtained 10 and 20 min. after the test meal.

A further extension of the previous study is the inclusion of tryptophan which, together with lysine and methionine, is one of the most limiting amino acids in cereals, pulses and tubers (Waterlow & Stephen, 1957).

METHODS

The rats used and their diets during the preparatory period have been described by Guggenheim *et al.* (1960). At the end of the preparatory period the rats were starved for 48 hr. This relatively long starvation period was essential for the complete removal of food residues from the intestine. The rats were then given a test meal containing a purified protein with corn starch or fat-extracted soya-bean flour. The test meal consisted of an aqueous suspension of 5% protein and 5% starch, 5 ml./100 g. body wt. being administered by stomach tube. When soya flour containing approximately 50% of protein was used, the suspension contained 10% of the soya flour without starch. Thus each test meal provided 40 mg. of nitrogen derived from the respective protein/100 g. body wt. Controls received a 5% corn-starch suspension without protein. At 10, 20, 30, 60, 120 and 180 min. after the test meal the animals were anaesthetized, the abdomen was opened, and blood withdrawn from the portal vein with a heparinized syringe. The plasma was assayed for lysine and methionine with *Leuconostoc mesenteroides* P-60 and for tryptophan with *Lactobacillus arabinosus*, as described by Barton-Wright (1952). For examination of intestinal contents, clamps were applied at the pyloro-duodenal junction and at the lower end of the ileum. The small intestine was then quickly removed and its contents were obtained by gentle washing of the mucosal surface with water. The intestinal