

A COMPARISON OF THE
PHARMACOLOGICAL PROPERTIES OF
THE GUT OF THE YOUNG ANIMAL
WITH THOSE OF THE ADULT

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by R. J. BYWATER

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GENERAL INTRODUCTION

The transition from foetal to neo-natal existence involves an abrupt change in intestinal function. The relatively passive role of the foetal gut must rapidly become that of a completely adequate absorbing membrane (Moog, 1962) able to cope with all nutritive requirements, and even, in some species, with absorption of intact colostrum antibodies.

Nevertheless, the neo-natal intestine has properties by no means identical with those of the adult, since post-natal development occurs both in morphology and function (Spencer, 1964). This seems particularly true of herbivores.

Various aspects of the digestive system of the calf have been investigated: digestive enzymes were studied by Huber et al (1958) by killing calves at various ages and examining the enzymes present in the gut. This showed that pancreatic lipase, protease and amylase increased during the first post-natal week, intestinal lactase decreased with age, and maltase remained static. The dimensions of the various stomach compartments were studied by Godfrey (1961) who found that the reticulo-rumen was still developing at seventeen weeks, while the abomasum had stopped developing at five weeks. Small intestine transit times were examined by Smith (1964) who found no consistent changes between one and twenty weeks of age, while Deutsch and Smith (1957) showed that antibody absorption had largely ceased by twenty-four hours after birth.

Despite the work of the above authors and others, much of the field remains unexplored. It was decided therefore to examine the changes in motor activity of the intestine, and its responses to drugs, in vitro and in vivo (Section I) and also to examine an aspect of the developing mucosa, namely alkaline phosphatase activity (Section II).

S E C T I O N IOBSERVATIONS ON THE ACTIVITY AND RESPONSE TO DRUGS OF
INTESTINAL SMOOTH MUSCLE FROM CALVES AND ADULTSINTRODUCTION.

Most studies of the physiological and pharmacological responses to drugs of the mammalian intestine have been confined to the small laboratory animals. While the information obtained in this way has been valuable, certain species differences have come to light (e.g. in responses to adrenaline, Burnstock et al, 1963 a) which emphasise the dangers inherent in making inter-species generalisations, particularly with respect to drug responses. So far, little experimental work has been done on the physiological and pharmacological behaviour of the ruminant intestine. Another factor which has attracted scant attention, even in laboratory animals, is the effect of ageing on intestinal physiology and pharmacology. This factor seems of interest, since it has been observed that both calves (Lovell, 1955) and children (Davidson et al, 1956) appear predisposed to abdominal conditions, in particular, diarrhoea.

- (i) The effect of drugs on isolated strips of intestinal musculature.

Since Magnus (1904) described an organ bath in which smooth muscle could survive for long periods, the technique has

been widely used on material from laboratory animals to observe and record the effect of drugs on motility, (review by Kosterlitz and Lees, 1964). This technique has also been used, to a much less extent, on material from other species such as the sheep (Duncan, 1954; Sanford, 1961), the horse (Tanaka and Ohkubo, 1940; Alexander, 1949; Angereau and Dontcheff, 1955) and recently man (Fishlock, 1964; Bucknell and Whitney, 1964; Bennet, 1965; Whitney, 1965; Fishlock et al, 1965; Wright and Shepherd, 1966).

So far as can be ascertained, the only authors attempting to use an in vitro method for bovine material were Simmonet et al (1952) and Rave (1952), and of these, the former author found that strips of bovine duodenum suspended in Tyrode were so inactive as to be useless for comparison with the horse duodenum. Rave, however, set up strips of bovine rumen and duodenum in organ baths containing Ringer's solution, and obtained responses to some common drugs. He observed that stimulation of the duodenal strip was produced by acetylcholine (10^{-8} - 10^{-5}), carbachol (10^{-8} - 10^{-6}), prostigmine (10^{-7}), barium chloride (10^{-5} - 10^{-3}) and histamine (10^{-9} - 10^{-7}). The effect of acetylcholine was shown to be blocked by atropine (10^{-5}) and a relaxation was demonstrated after adrenaline (10^{-7} - 10^{-5}). He concluded that the general pharmacological behaviour of bovine intestinal muscle did not differ markedly from that of the laboratory animals.

It is felt, however, that these observations need to be extended to a wider selection of drugs, to muscle from other parts of the gut, and, in particular, to material obtained from calves in order to determine the effect of age on responses.

(ii) The effect of drugs on the in vivo motility of the gut.

The motility of the gut in the living animal has been studied using a variety of techniques. The simplest of these is perhaps direct observation. This has been used by Dukes and Sampson (1937) in adult sheep, and by Duncan and Phillipson (1950) in foetal sheep. The animals used were anaesthetised, laparotomised and ^{the viscera protected by saline or oil} ~~immersed in liquid~~ at 37°C. This method is necessarily subjective, and even when a filmed record is made, interpretation is difficult. There is also an effect of anaesthesia (Burnstein, 1939).

X-ray observation has the advantage of involving the minimum of interference with the physiological state of the animal, since neither anaesthesia nor surgery need be involved. The method has been used by Alexander and Benzie (1951) in the foal, and by Kidder et al (1961) in the piglet. Interpretation of observations made with this method are, however, both difficult and subjective (Connell, 1961).

Various methods of intraluminal pressure recording have been used. Intraluminal balloons have been available since 1869 (Legros and Onimus) and have been widely used, both in animals (e.g. Templeton and Lawson, 1931; Ravaud, 1955) and man (e.g. Foulk et al, 1954; Bachrach et al, 1954). The advantages of this method lie in its convenience, its modest technical requirements, and the satisfactory records which have been obtained (Bachrach et al, 1954; Code et al, 1952). It has been criticised, however,

on the grounds that the balloon represents a foreign body, and that the pressures registered are inaccurate (Quigley and Brody, 1952; Chaudhary and Truelove, 1961). These authors favour the use of intraluminal open-ended tube recording. This method has been used particularly in studies on man (e.g. Lorber and Shay, 1954; Davidson et al, 1956; Barbero et al, 1958) and is said to give an accurate assessment of intraluminal pressures. This is also true of telemetry from pressure sensitive capsules (Connell and Rowlands, 1960).

Biebl loops have been used to record gut motility in the horse (Alexander, 1952) and in the dog (Vaughan Williams and Streeten, 1952). Thiery-vella loops have also been used in the horse (Alexander, 1952) and the dog (Castleton, 1934). These techniques allow records to be made from conscious animals, under

Intestinal motility has been classified by Templeton and Lawson (1931), who used intraluminal balloons to investigate the colonic motility in dogs. They were able to distinguish three types of spontaneous activity. The type I contractions were small, rapid and could be either regular or erratic. The type II contractions consisted of a large rapid contraction followed by a rapid relaxation, although type I contractions could be superimposed. The type III contractions were more complex, consisting of a comparatively slow rise and fall in tonus, with types I and II contractions superimposed.

The existence of these basic types of activity has been

confirmed in the human colon using the open-ended tube technique by Davidson et al (1956) and has been found to also apply to other parts of the gastro-intestinal tract (Posey and Bargaen, 1951).

The effect of drugs on the intestinal motility was recorded by Templeton and Lawson (1932) in the dog, and by numerous authors using human subjects (see Bachrach et al, 1954).

The only extensive investigation of the physiological and pharmacological behaviour of the ruminant intestine appears to be that of Ravaud (1955). This author used sheep which were anaesthetised with chloralose and then laparotomised. Intestinal movements were observed directly, and recorded by balloons introduced through enterotomies at various levels. The 'normal' motility was recorded in each case, and it was found that the duodenum and ileum were very active by comparison with the jejunum. The illustrations of records obtained show the three types of contraction described by Templeton and Lawson (1931).

Ravaud tested the effect on motility of intravenous administration of certain drugs. Acetylcholine was found to inhibit intestinal movements when given in doses greater than 0.05 mg per kg, while doses between 0.005 and 0.01 mg per kg were found to be stimulatory. An inhibitory effect of acetylcholine was described by Duncan (1954) in the rumen, reticulum and abomasum, and this author suggested that the effect was mediated by released adrenaline. Ravaud, however, made a simultaneous record of arterial blood pressure, and observed that doses large enough to cause hypotension also caused intestinal inhibition, while small doses, which were

without effect on blood pressure, caused intestinal stimulation. He therefore ascribed the inhibitory effect to a fall in blood pressure rather than a direct effect on intestinal musculature. The connection between blood flow and motility has been shown by Alexander (1952).

Some parasympathomimetic drugs were tested, ^{on sheep by Ravaud} and it was found that carbachol (carbomylcholine chloride) caused increased peristalsis when injected intravenously in doses as small as 2 micrograms: doses larger than 5 micrograms were found to cause increased activity, but without co-ordination. Esmodil (methyl- β -methylcholine bromide) was found to stimulate the ileum and the duodenum in doses which were ineffective on other regions. Pilocarpine and arecoline were found to be relatively inactive other than in large doses.

Atropine was found by Ravaud to abolish spontaneous activity in doses of 3 mg (intravenously). Adrenaline also abolished spontaneous activity when given in doses of 0.02 mg per kg, but at the same time caused a fall in tone in some areas of the gut.

This study made by Ravaud was carried out on anaesthetised animals, and it has been shown that certain anaesthetics cause at least a temporary inhibition of normal motility (Burnstein, 1939). It was also suggested by Ravaud that his results were probably applicable to the bovine, but this has yet to be confirmed.

In the present investigation, it was decided that some of the drugs tested on strips of muscle in vitro, should also be tested in vivo, using the intraluminal tandem balloon technique on

conscious calves fitted with duodenal cannulae. It was hoped in this way to discover whether any of the direct effects seen in vitro were modified by indirect effects in vivo.

(iii) The variation in behaviour along the gut.

It was realised by Alvarez (1914) that the gut should not be considered a tube with constant properties throughout, and he was able to show, both in isolated tissue (1914) and in the intact animal (1915) that there was a gradient of spontaneous activity in the guinea-pig, cat and dog. These findings were confirmed by Puestow (1931), Castleton (1934) and Douglas and Mann (1939) all of whom used in vivo techniques in the dog, and by Cowie and Lashmet (1929) using in vitro techniques on rabbit tissue. More recent work (Hasselbrach and Thomas, 1961) has added further confirmation, both in vivo and in vitro, using dogs.

Ravaud (1955) in his study of the motility of the gastrointestinal tract of the anaesthetised sheep, noted that the duodenum, ileum and colon all showed greater activity than did the jejunum, and so failed to demonstrate the gradient of activity shown in the other species mentioned above. An in vitro study of bovine intestinal muscle may well demonstrate whether a comparable situation exists in this species, since Alvarez (1915) and Hasselbrach and Thomas (1961) showed that variations of spontaneous activity in vitro paralleled that shown in vivo.

It has also been shown that the action of certain drugs on isolated strips of intestinal muscle may be to some extent

dependent on the site from which the strip was taken. This has been demonstrated for the effect of histamine on cat tissue (Mackay, 1930) for adrenaline on guinea-pig tissue (Munro, 1951) and for 5-HT on human tissue (Fishlock, 1964). It would be of interest to know if these agents, or others, vary in activity on different sections of bovine gut.

(iv) The effect of age on intestinal behaviour.

Gut motility at different ages was recorded by Barbero et al (1958) who used an open tube method to compare the pressure changes in the duodenum of children and adults. They used a system of classification similar to that of Templeton and Lawson (1931) for the recorded movements, and found that the most striking difference was that the overall activity in children was only one-third of that in adults (when expressed as a percentage of total recording time). This may, however, have been partly due to sedation being used in a greater proportion of child subjects than adults.

Starovoitov (1960) found the opposite situation existed in the fistulated stomachs of piglets. He showed that the stomachs were in a constant state of activity for the first thirty days of life, and that 'resting' phases increased with age. Much of the difference between these results is probably a result of differing techniques of investigation, since Davidson et al (1956) using a similar technique to Barbero et al, found no substantial difference between children and adults.

Visual observations of the open abdomen of anaesthetised animals under saline were made by Koschtojantz and Mitropolitanskaja (1935). They found that, in several species, the lower parts of the intestine of the young animal failed to show spontaneous activity, in particular during the suckling period. Unsuccessful attempts were made to delay the onset of motor activity by prolonging the suckling period (Koschtojantz, 1960).

Propulsive motility at different ages has been studied using markers by Stickney et al in the dog (1949), by Castle in the goat (1956), by Koldovsky et al in the rat (1963) and by Smith in the bovine (1964). It was shown in the dog, the goat and the rat, that the rate of propulsion was greater in the adult than in the pre-weaning animal. However, in the dog, Stickney et al noted that when this was expressed as a percentage of the total intestinal length, no difference could then be demonstrated. Smith was unable to demonstrate any consistent difference in small intestine transit time between calves of one and twenty weeks.

Radiography has been used in the foal (Alexander and Benzie, 1951) and in the piglet (Kidder et al, 1961) to follow the rate of passage of a barium meal at various ages. No marked difference in overall rate could be demonstrated over the period of examination (six to seventeen days of age in the piglet and pre- and post-weaning in the foal), although Alexander and Benzie were able to show differences in the time that barium stayed in various compartments in the two age groups. However, it was noted by Kidder et al,

that this method was not very sensitive, and that the period of their examination was short.

In view of the somewhat conflicting and inconclusive evidence provided by the in vivo studies mentioned above, it was considered that an in vitro study, where many of the disturbing influences, such as nervous influences and diet, are excluded, might demonstrate any differences between behaviour of the gut of the calf and the adult. It was decided, therefore, to examine the spontaneous activity, and responses to drugs, of isolated strips of intestinal smooth muscle from calves and adults, and to compare strips removed from different sections of the intestine.

It was also decided that some of the in vitro findings should, if possible, be confirmed in vivo. This was limited to drug responses in the calf, since a cannulated adult animal was not available.

MATERIALS AND METHODS.1. Experiments carried out on isolated strips of intestinal smooth muscle.

Material was obtained from the slaughterhouse as soon as possible after the death of the animal. The delay between death and removal of samples was about fifteen minutes for adults, and five minutes for calves. A short segment of gut was taken from the duodenum, jejunum and colon of each animal, and from the lower ileum of some. The specimen was opened, washed in normal saline, and carried back to the laboratory in Tyrode's solution.

Strips of longitudinal muscle approximately 25 mm x 4 mm were cut. The mucosa was removed from samples where its presence appeared to cause mechanical hindrance. The strips were then suspended in organ baths in which the lower end was attached to a fixed point, and the upper end to a frontal writing lever of 5:1 magnification. The tension on the strip was approximately 1.5 gms, and movements were recorded on a smoked kymograph drum. The total time elapsing between death of the animal and setting up of the preparation was forty-five to sixty minutes.

Three organ baths of 40 mls capacity were immersed in a large bath maintained at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The organ baths contained aerated Tyrode's solution (1910), and each had a separate reservoir and drainage system. Three strips of muscle were observed together, and the records were made on the same kymograph drum, in the same vertical plane.

Drugs:

Most of the drugs used were in the form of salts:-

Acetylcholine chloride (B.D.H.)
Adrenaline acid tartrate (B.D.H.)
Atropine sulphate (T. & H. Smith)
Bradykinin (Sandoz)
1. 1. Dimethyl-4-phenyl piperazinium (D.M.P.P. - Wellcome)
Hexamethonium bromide (May & Baker)
Histamine acid phosphate (B.D.H.)
Hyoscine hydrobromide (B.D.H.)
5-Hydroxytryptamine creatinine sulphate (B.D.H.)
Isoprenaline hydrochloride (Ward Blenkinsop)
Mepyramine maleate (May & Baker)
Methysergide bimaleate (Sandoz)
Nicotine hydrogen tartrate (B.D.H.)
Phenoxybenzamine (Smith Kline & French)
Physostigmine salicylate (T. & H. Smith)
Pronethalol (I.C.I.)
Tetraethylammonium bromide (Koch-Light)
Tetramethylammonium chloride (B.D.H.)

The drugs were dissolved in normal saline made up with deionised water. Dilutions were calculated so that the required amount of a drug could be added to the bath in a volume of not more than 0.5 ml. Concentrations were expressed as the weight of the salt per ml. of bath fluid.

Agonists were left in contact for forty-five seconds, but a longer period was allowed if the maximum contraction had not been attained by this time. Antagonists were left in contact with the tissue for at least ten minutes before subsequent addition of agonist.

2. Experiments carried out by recording intestinal movements in the intact animal.

Balloons, of 2 cm diameter, were made from finger-stall rubber, and tied to the end of polythene tubing (Sterivac cannula, Allen and Hanbury), of 2 mm bore at 0.5 mm wall thickness. Calves were available which had been fitted with duodenal cannulae ~~described~~, and these enabled balloons to be accurately positioned in the lumen of the small intestine.

Tandem balloons were used, placed twelve inches apart, and each having a separate polythene tube. The tubes passed through a perforated stopper which fitted over the end of the duodenal cannula, and were connected to one arm of a water manometer. The other arm of each manometer was connected to a bellows recorder, the movements of which were recorded on a smoked kymograph drum.

The balloons could be inflated through a side-tube which carried a screw clip. The pressure in the balloons was usually between 5 and 10 cm of water, and was not allowed to exceed 15 cm.

The calf was restrained in a holding crate, and the balloons were placed so that the proximal one was situated in the duodenum (six inches from the end of the cannula) with the distal one

twelve inches further away. A period of fifteen minutes was allowed in each case for the animal to settle. By the end of this time, the records obtained were usually steady, with peristaltic waves passing at fairly regular intervals.

Drugs were dissolved in sterile saline, if necessary, and were administered either sub-cutaneously or intravenously via the jugular vein.

Drugs:

Acetylcholine chloride (B.D.H.)

Adrenaline injection B.P. 1:1000 (Boots)

Atropine sulphate injection B.P. (Boots)

Atropine sulphate (T. & H. Smith)

Carbachol B.P. (Boots)

Histamine acid phosphate (B.D.H.)

5-Hydroxytryptamine creatinine sulphate (B.D.H.)

RESULTS.A. IN VITRO RESPONSES OF INTESTINAL SMOOTH MUSCLE.1. Effect of removal of mucous membrane.

It was found that removal of mucous membrane had no demonstrable effect on the subsequent performance of the muscle strip, either in terms of spontaneous activity or in response to drugs.

2. Survival of tissue.(a) Calf material.

It was found that out of a total of twenty longitudinal muscle preparations from duodenum and jejunum, and out of a smaller number from the ileum, ~~approximately~~ one in five was totally unresponsive to the effects of added drugs. By contrast, all twenty of the preparations from the colon were active and responsive.

Occasionally the sensitivity of a preparation increased throughout an experiment but on most occasions, maximum responsiveness was attained within the first hour. Viable preparations usually remained responsive to the effects of drugs for at least five hours, although some of the duodenal and jejunal strips ceased to respond at a time when the corresponding colon preparation was still active.

(b) Adult material.

It was found that, in samples of similar size, the number of inactive preparations was the same as described above for the calf material.

3. Spontaneous activity.

Spontaneous activity normally appeared within thirty minutes of the setting up the preparation, although occasionally the onset appeared to be hastened by the addition of stimulatory drugs such as histamine or 5-HT.

(a) Calf material.

Twenty preparations of duodenum, jejunum and colon were observed. It was found that spontaneous activity occurred in half of the duodenal and jejunal preparations, and in all of the colon preparations. The mean rate of contraction of the active strips was calculated for each site (Table I) and the results were compared using Students t - test. It was found that the rate of contraction of the active jejunal strips was significantly higher than that of either duodenum or colon strips ($P < .01$ in each case). There was no significant difference between the rates of contraction of duodenum and colon strips.

(b) Adult material.

Twelve preparations of duodenum, jejunum and colon were observed, and it was found that a half of the duodenal strips

showed spontaneous activity, while only a quarter of the jejunal strips were active. All the colon strips were active. The mean rate of contraction was calculated (Table I) and it was found that there was no significant difference between the rate of contraction of duodenum and colon. The few active jejunal preparations suggested that, with greater numbers, the rate would be greater than either duodenum or colon.

When the figures obtained for the adult duodenum and colon were compared with the corresponding region in the calf, it was found that the rate of contraction was significantly greater ($P < 0.01$) in both the duodenum and colon of the calf.

4. Actions of Acetylcholine.

Most preparations were contracted by acetylcholine (0.01 - 1.0 $\mu\text{g/ml}$), although there was variation in sensitivity. It was found that strips showing no spontaneous activity could still be sensitive to stimulation by acetylcholine.

The contraction observed was typically rapid in onset, and returned quickly to the baseline after washing of the drug from the bath.

The effect of acetylcholine was potentiated by eserine (1 $\mu\text{g/ml}$ Fig. 1) which also caused increased tone and spontaneous activity.

Atropine (10^{-6} - 10^{-8} , Fig. 2) and hyoscine (10^{-6} , Fig. 3) abolished or reduced the contraction caused by a subsequent dose of acetylcholine. Dose ratios were obtained for the antagonism

between atropine (10^{-8}) and acetylcholine, but it was found that variation between samples was too great to allow analysis. It was noted that atropine (10^{-6} - 10^{-8}) often caused a rise in tone of the preparations.

No difference could be demonstrated between the responses of material from calves and adults, or between the responses of material from various parts of the small and large intestine.

5. Actions of Histamine.

Histamine (0.1 - 0.5 $\mu\text{g}/\text{ml}$) produced two distinct effects on material from either age group and from any of the sites tested. The first and most usual, was a simple contraction (Fig. 2). This differed from the contraction caused by acetylcholine in that contraction and relaxation were both slower. Marked tachyphylaxis was seen if doses were repeated at two minute intervals.

The second effect of histamine was biphasic (Fig. 4), consisting of a short initial relaxation, followed by a contraction similar to that described above. Both types of response were normally abolished by mepyramine (10^{-6}) although on one occasion the relaxant phase appeared resistant to this antagonist. Neither type of contraction was consistently affected by atropine (10^{-6}), hyoscine (10^{-6}), or hexamethonium (10^{-6}).

Duodenal strips were usually less responsive to histamine than were strips from other sites.

6. Actions of 5-HT.

5-HT (0.1 - 1.0 $\mu\text{g/ml}$) produced a rather prolonged contraction when tested on strips of muscle from the small intestine of either age group. The contraction was unaffected by atropine (10^{-6}) or hexamethonium (3×10^{-6}), but was abolished by methysergide (1.0 $\mu\text{g/ml}$, Fig. 5).

The effect of 5-HT on strips of muscle from the colon was less clear. Adult tissue appeared to respond to 1 $\mu\text{g/ml}$ with a weak contraction. Calf material responded either with a contraction, or with a relaxation followed by a contraction (Fig. 6).

7. Actions of ganglion stimulants.

Agents tested were nicotine (1 - 10 $\mu\text{g/ml}$), dimethylphenylpiperazinium (D.M.P.P. 5 - 30 $\mu\text{g/ml}$) and tetramethylammonium (T.M.A. 5 - 30 $\mu\text{g/ml}$). Responses were rather unreliable, since tissues which contracted to acetylcholine were occasionally quite resistant to the effects of the above drugs.

Contractions caused by D.M.P.P. (20 $\mu\text{g/ml}$) and T.M.A. (5 $\mu\text{g/ml}$, Fig. 7) were unaffected by prior addition of up to 100 $\mu\text{g/ml}$ of hexamethonium or by tetraethylammonium (50 $\mu\text{g/ml}$). This was demonstrated on tissue from calves and from adult cattle, and in tissues from various parts of the small and large intestines.

Atropine (10^{-6}) and hyoscine (10^{-6}) were shown to block the effect of T.M.A., while having no effect on response to D.M.P.P. The latter was also found to be unaffected by prior addition of

mepyramine (0.5 $\mu\text{g/ml}$) or methysergide (0.5 $\mu\text{g/ml}$).

Nicotine (1 - 10 $\mu\text{g/ml}$) was inactive in most preparations on which it was tested. On one colon preparation, however, it caused stimulation followed by relaxation, leaving the preparation refractory to a further dose.

8. Actions of bradykinin.

Bradykinin (0.2 μg - 1 $\mu\text{g/ml}$) had a biphasic effect in both age groups causing a transient relaxation followed by a sharp contraction. In the colon preparation, the contraction did not occur until the drug was washed from the bath, while in the preparations of duodenum and jejunum, the contraction occurred before the drug was washed out (Figs. 2 & 8).

Both parts of the biphasic effect were resistant to the effect of atropine (10^{-7}) and mepyramine (10^{-7}) which abolished the effects of acetylcholine (0.1 $\mu\text{g/ml}$) and histamine (0.5 $\mu\text{g/ml}$), respectively (Fig. 2).

9. Actions of sympathomimetic agents.

Adrenaline, noradrenaline and isoprenaline in concentrations of 0.1 - 1.0 $\mu\text{g/ml}$ caused relaxation of longitudinal muscle strips in all preparations tested in both age groups (Fig. 9). Noradrenaline differed from the others in causing a rise in tone which appeared after the drug had been washed from the bath.

Pronethalol (3 $\mu\text{g/ml}$) blocked the effect of isoprenaline (0.5 $\mu\text{g/ml}$) but had no effect on the relaxation caused by

noradrenaline (0.5 $\mu\text{g/ml}$) or adrenaline (0.5 $\mu\text{g/ml}$).

A combination of pronethalol (3 $\mu\text{g/ml}$) and phenoxybenzamine (3 $\mu\text{g/ml}$) was found to abolish the relaxation caused by both adrenaline (0.5 $\mu\text{g/ml}$) and noradrenaline (0.5 $\mu\text{g/ml}$).

B. IN VIVO RESPONSES OF INTESTINAL SMOOTH MUSCLE.

The records obtained demonstrated the classic types of intestinal motility:- Type I: the small, irregular 'base-line' contractions seen in all the illustrations. Type II: the larger rapid waves which moved sequentially from the one balloon to the other. These are shown clearly in Fig. 16. These waves were seen in most recordings, but varied in intensity and frequency. The average frequency was 1 per minute. Type III: these waves represent a rise in tone and are usually associated with one or other of the above types of movement. An example is seen in Fig. 10.

The spontaneous activity appeared to differ in the two animals studied. In almost every record that was made, the calf F showed clearer type II waves, and less marked type I activity than did calf H. There appeared to be no clear correlation between the intestinal activity and acts such as feeding or ruminating.

1. Actions of Acetylcholine.

Intravenous injection of 0.25 mg of acetylcholine produced no discernible effect on intestinal movements, but 1 mg, also

intravenously, caused inhibition of intestinal activity (Fig. 10). The inhibition lasted about ten minutes.

Intravenous injection of a larger dose (5 mg, Fig. 11) caused immediate coughing, followed after two minutes by a rise in tone and rate of movement. This increased activity lasted three minutes, and was followed by a period of ten minutes during which there was inhibition of intestinal activity. At the end of this time, normal activity returned.

Animals pretreated with 10 mg of atropine subcutaneously were unaffected by 5 mg of acetylcholine given intravenously.

2. Actions of Carbachol.

Carbachol (1.5 mg) was injected subcutaneously, and produced visible salivation after one minute. The first intestinal manifestations were seen after four minutes (Fig. 12). These took the form of a type III contraction, with a rise in tone and an increase in the rate of movement. Coughing occurred, and some of the increased movement was probably respiratory in origin.

After the initial increase in activity, there appeared to be a subsequent suppression of movement which persisted for twenty minutes. At the end of this time, small rapid waves appeared, and, although sequential, their co-ordination was poor. After seventy-five minutes, the type II waves were larger and more co-ordinated, occurring at intervals of about two minutes. Defaecation did not occur until about forty minutes after the injection.

The effect of carbachol was prevented or abolished by subcutaneous injection of 10 mg of atropine.

3. Actions of Histamine.

Intravenous injection of 0.1 mg of histamine caused immediate coughing, followed by a period of discomfort, during which the animal kicked at its stomach. This lasted a comparatively short time, and rumination was usually resumed within ten minutes.

The records of intestinal movements showed an increase in activity (Fig. 13). This occurred within three minutes of the injection, and persisted for about ten minutes.

4. Actions of 5-HT.

Intravenous injection of 1 mg of 5-HT caused an immediate increase in tone, together with an increase in size and frequency of type II waves (Fig. 14). This persisted for four minutes, and it was succeeded by a period of about fifteen minutes during which there was inhibition of type II waves, although type I activity appeared unaffected. After this period, normal activity was resumed.

5. Actions of Atropine.

Apart from protecting the animal against the effect of injected carbachol and acetylcholine, atropine exerts a powerful

spasmolytic effect (Fig. 15). This appears within five minutes of a subcutaneous injection of 10 mg of atropine, and is accompanied by the other signs of parasympathetic blockade, the most obvious being the reduced salivary secretion. The intestinal effect lasted for about thirty minutes, after which normal spontaneous activity reappeared. It was noticeable that, even after intestinal activity was resumed, the animal was still protected against the effects of carbachol (1 mg s/c) or acetylcholine (5 mg i/v).

6. Actions of Adrenaline.

Subcutaneous injection of 3 mg of ~~a 1:1000 solution of~~ adrenaline caused a slight decrease in the rate of occurrence of type II waves (Fig. 16). This effect appeared to be quite prolonged.

Intravenous injection of 1 mg of ~~1:1000~~ (Fig. 17) adrenaline caused an immediate cessation of spontaneous activity which lasted for four minutes. At the end of this time, normal activity was resumed.

DISCUSSION.1. IN VITRO RESULTS.(a) Removal of mucous membrane.

There was no evidence in the present experiments that the mucous membrane exerted any inhibitory influence on drug induced responses of bovine intestinal muscle. This was contrary to the findings of Walder (1953) in relation to the human stomach. It was also found in the present work that preparations from which mucosa had been removed appeared to respond normally to acetylcholine and D.M.P.P., suggesting that damage to the intramural nerve plexuses was not sufficient to alter the responses of drugs acting thereon.

(b) Survival of tissue.

The assertion of Simmonet et al (1952) that bovine intestinal muscle was very inactive in Tyrode's solution has been refuted. There was, however, a proportion of inactive preparations, which was greater in the proximal parts of the gut, where it has been shown ^{in other species} that oxygen consumption is highest (Dorman and Steggerda, 1962), and where Alvarez (1914) had found tissues to be most affected by handling. It was also found by Sanford (1961) that the delay between death of the animal and the start of the experiment determined the sensitivity of strips from sheep stomach.

The strips of tissue, even those taken from calves, were thicker than recommended for survival in vitro (Umbriet et al, 1957).

and so it is probable that all the preparations suffered from some degree of anoxia, (Prosad, 1935) and that this would be most marked in the more proximal parts.

(c) Spontaneous activity.

In neither age group was it possible to demonstrate a gradient of spontaneous activity such as has been shown in other species (e.g. Alvarez, 1915; Hasselbrach and Thomas, 1961) between duodenum and colon. It appeared that, in both age groups, the rate of contraction of active jejunal strips was higher than that of duodenal strips (this was statistically verifiable only in the case of the calf). This does not exclude the possibility of a gradient existing between the jejunum and colon, rather than between duodenum and colon, but this could not be shown in the present experiments owing to the relatively limited number of ileum preparations tested.

The low rate of spontaneous contractions noted in the duodenum may reflect (i) rough treatment of animals prior to slaughter (Alvarez, 1914); (ii) delay in transit between slaughterhouse and laboratory (Rave, 1952); and (iii) the high rate of oxygen consumption in intact duodenal strips (Dorman and Steggerda, 1962) leading to hypoxia and lowering of the rate of spontaneous activity (West et al, 1951).

The rate of spontaneous contraction of calf duodenum and colon was significantly higher than that of the corresponding adult strips.

This may also be a result of the poor oxygenation of adult tissue leading to a lowered rate of spontaneous activity (West et al, 1951).

(d) Effect of Acetylcholine.

This drug showed typical muscarinic effects in all preparations tested in both age groups. The contraction was blocked by hyoscine, potentiated by eserine and was unaffected by hexamethonium.

Atropine blocked the contractions due to acetylcholine, but the unsuccessful attempts to obtain repeatable dose ratios suggests that the thickness of the tissue may have a bearing on the degree of block, and that thicker tissues would need longer for equilibrium to be attained.

Anoxia alone was found by Day and Vane (1963) to have little effect as measured by dose ratios on acetylcholine induced contractions of the guinea-pig ileum.

(e) Effect of Histamine.

This drug exerted two different effects:-

(i) A monophasic contraction. This was the response most often seen in preparations from either age group, and differed little from that described in other species. Preparations from different regions responded similarly except for a tendency for duodenal preparations to be insensitive. This has been reported in the cat duodenum (Mackay, 1930).

(ii) A biphasic contraction, seen as an initial small relaxation followed by a larger contraction. This was seen on occasion in all preparations of both age groups, but only sporadically. It was not reported by Rave (1952) in his study of bovine duodenum, although this author did mention a delay of 5-10 seconds after addition of the drug before the contraction occurred. A biphasic effect has been reported in strips of human colon (Fishlock and Parkes, 1963; Bucknell and Whitney, 1964). The latter authors found that storage of the tissues tended to increase the incidence of biphasic responses, and some support for this suggestion has been found in the present experiments.

This result suggests that histamine may be having an effect at two sites, one of which is neurogenic. If storage favours a relaxant effect, this suggests that the contractile one is neurogenic, although hexamethonium had no effect on the contractile response. It has been shown, however (Trendelenburg, 1954) that histamine can stimulate ganglia in a way which is resistant to hexamethonium, and that it has both neurogenic and myogenic effects on the rabbit intestine (Ambache and Lessin, 1955).

It was found ^{here} that both phases of the contraction were usually abolished by mepyramine, but this does not exclude two sites of action.

A biphasic response is not necessarily associated with an effect on two separate mechanisms (Burnstock and Holman, 1966) since it has been shown (Burnstock et al, 1963 b) that hyperpolarisation can lead to a rebound of hypopolarisation sufficient

to trigger action potentials. This does not seem a likely explanation of the effect under discussion, since the most commonly seen effect was a monophasic contraction.

The exact nature of the biphasic response to histamine has not been established, and further elucidation is hindered by the sporadic occurrence of the phenomenon. It is noteworthy that histamine produces relaxation in sheep rumen and abomasal strips (Sanford, 1961) but that this was not found in the single experiment on bovine rumen which was performed by Rave (1952).

(f) Effect of Bradykinin.

A biphasic effect was observed in all the preparations of small intestine which were tested. A short relaxation was typically followed by a stronger contraction. The colon preparations differed in that the contraction occurred only after the drug was washed from the bath.

Neither phase of the contraction was affected by atropine or mepyramine, and the effect of bradykinin is normally accepted as being directly on muscle fibres (Goodman and Gilman, 1965). A biphasic effect has also been observed in the rat colon preparations (Bisset and Lewis, 1962).

(g) Effect of sympathomimetic agents.

The presence of β receptors to catechol amines (Alquist and Levy, 1959) was demonstrated in both age groups by the selective block action of pronethalol on the relaxation induced

by isoprenaline. The effect of noradrenaline was not consistently abolished by phenoxybenzamine, but the effects of both adrenaline and noradrenaline were usually abolished by a mixture of pronethalol and phenoxybenzamine. This suggests that α receptors were not being blocked effectively by phenoxybenzamine, and that pronethalol may have been acting synergistically. The rise in tone seen after washing out noradrenaline from the bath may have been a result of the rebound effect mentioned earlier, or may have been caused by release of active substances.

(h) Effects of 5-HT.

5-HT produced a contraction in all the preparations of small intestine which were tested in both age groups. The calf colon behaved slightly differently by showing an initial relaxation on some occasions.

The response to 5-HT was blocked by methysergide, but was unaffected by atropine or hexamethonium. The effect was probably a direct effect on the muscle, although it has been shown that 5-HT can stimulate ganglia in a way which is not affected by hexamethonium (Gyermeck and Bindler, 1962).

(i) Effect of ganglion stimulants.

D.M.P.P. and T.M.A., where they had an effect at all, gave contraction. In no case was relaxation seen as in the human colon (Bucknell and Whitney, 1964). It was found impossible to block the effect of either of these two drugs by the usual ganglion

blocking agents, even when added in large doses.

The effect of the two drugs could be separated by atropine and hyoscine which blocked T.M.A. in concentrations which had no effect on D.M.P.P. This suggests that T.M.A. was acting indirectly through release of acetylcholine, while D.M.P.P. was possibly acting directly on the muscle.

Nicotine appeared to be inactive on many preparations, but on one occasion gave a stimulation followed by relaxation, leaving the preparation refractory to further doses.

2. IN VIVO RESULTS.

For the present investigation, it was felt that the balloon-water manometer method of recording gut motility was adequate. The balloons used were approximately the same size as those which Hightower (1952) found to give results which correlated well with pressure transducer recordings, and they were also very little larger than the miniature balloons of Atkinson et al (1957) which gave records exactly similar to those obtained using the open tube method.

The method has been criticised (Chaudhary and Truelove, 1961) but has advantages of simplicity and convenience. Moreover, it has been stated by Bachrach et al (1954) that, on the basis of evidence then available, balloon recording appeared to depict the essential characteristics of spontaneous motility.

The classic types of spontaneous motility as observed in the present results, have been associated with propulsive activity (Bachrach et al., 1954):-

The type I contractions are said to be rarely associated with propulsion of gut contents, but are probably important in ensuring contact between the ingesta and the gut wall, at which much digestion occurs (Ugolev, 1965). The type II contractions are often associated with caudal movement of gut contents, and were frequently seen moving sequentially in the present experiments. The type III contractions are very frequently associated with propulsion, but were seen only rarely to occur spontaneously in the present experiments.

Effects of drugs.

Acetylcholine, intravenously, was found to be inhibitory in small doses, while larger doses produced rapid transient stimulation, followed by inhibition. This is opposite to the effect described by Ravaud (1955), but the difference may be in technique, since he used anaesthetised sheep.

The inhibitory effect may have been due to stimulation of the adrenal medulla (Duncan, 1954) or to a cardiovascular effect (Ravaud, 1955). Experiments using sympathetic blocking agents could help decide.

Carbachol, subcutaneously, produced a relatively delayed and inco-ordinated intestinal effect in doses which produced rapid and profuse salivation.

Histamine, intravenously, caused increased activity. There was no sign of the initial inhibition occasionally seen in the in vitro preparations. The effect was opposite to that observed in the sheep rumen by Duncan (1954).

5-HT also gave a period of increased activity, but this was succeeded by a period of inhibition. This corresponds to findings in the guinea-pig (Bülbring and Crema, 1959).

Atropine abolished spontaneous motility and blocked the effect of acetylcholine and carbachol, thus confirming the finding of Ravaut (1955) in sheep, and of numerous other workers in other species. The fact that protection against parasympathomimetics outlasted the inhibition of intestinal activity suggests the presence of a ~~compensation~~ mechanism allowing motility to be resumed in the presence of parasympathetic blockade. This is possibly analogous to the effect of anaesthesia (Burnstein, 1939) which has only a transient inhibitory effect on motility.

Adrenaline, both subcutaneously and intravenously, had the spasmolytic effect which was expected from the reported observations in other species (e.g. Ravaut, 1955), and from the present observations in vitro. It was not considered likely that nor-adrenaline or isoprenaline would respond differently from adrenaline, although this was not confirmed.

3. CONCLUSION.

1. The experiments carried out in vitro and in vivo have shown that, in general, bovine intestinal smooth muscle responds to drugs in a manner similar to that of the laboratory animals and man. Drugs behaving unusually were:- histamine, which occasionally gave a biphasic response, and the ganglion stimulants D.M.P.P. and T.M.A. whose effect was not antagonised by alleged ganglion blockers.

2. Strips of muscle from different parts of the gut behaved similarly, although there was a higher mean rate of contraction in the jejunum (10.27 p.m. for calf, 7.1 for adult) than in either duodenum (6.2 for calf, 3.3 for adult) or colon (5.8 for calf, 3.3 for adult). Colon strips responded to bradykinin with a relaxation followed by a contraction after washing, while strips from other regions gave a biphasic response before washing. Colon strips on two occasions reacted to 5-HT with a biphasic response rather than a simple contraction as seen in strips from the small intestine. Duodenal strips usually showed small contractions in response to doses of histamine which caused strong contractions of strips of jejunum or colon.

3. The responses of the isolated gut to drugs were similar in material from calves and adults, although the rate of spontaneous activity was higher in strips from the calf duodenum and colon than in those from corresponding adult regions.

4. The results obtained in vivo confirmed those obtained in vitro, showing that the direct effect of drugs on intestinal muscle was not overcome by indirect effects. Acetylcholine formed an exception by having an inhibitory effect in vivo possibly through its indirect effects on gut motility.

TABLE 1

Spontaneous activity of isolated longitudinal strips. The figures represent the mean rates of contraction \pm S.E. of the mean.

The figures in brackets signify the number of active preparations.

	DUODENUM	JEJUNUM	COLON
Calf	6.2 \pm 0.6 (9)	10.27 \pm 0.83 (11)	5.8 \pm 0.3 (20)
Adult	3.3 \pm 0.43 (6)	7.1 (3)	3.3 \pm 0.37 (12)

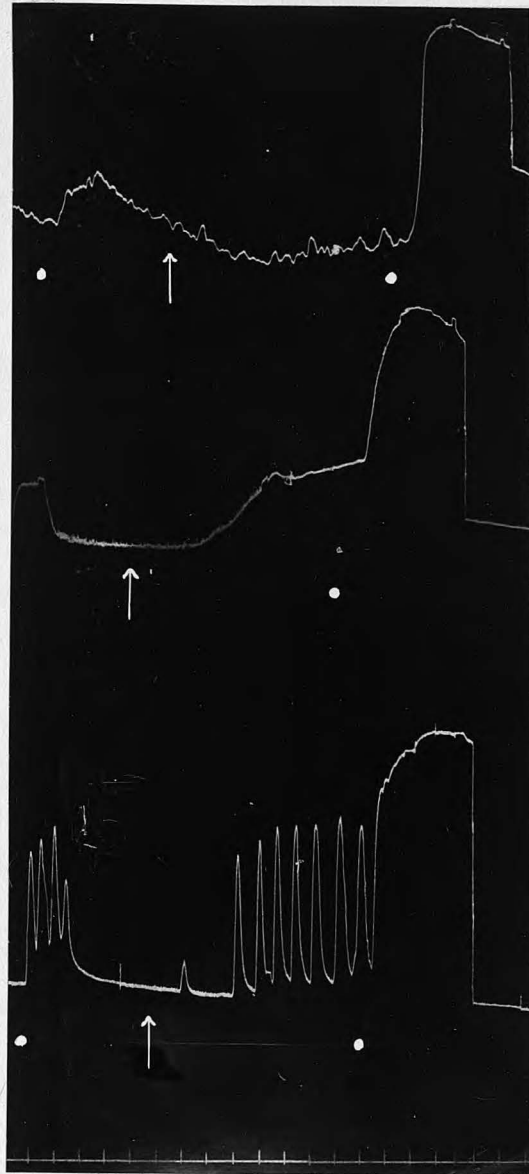


Fig. 1 . Longitudinal strips taken from adult duodenum, jejunum and colon are represented by the upper, middle and lower tracings respectively. The dots signify addition of acetylcholine ($0.5 \mu\text{g/ml}$) and the arrow addition of eserine ($1 \mu\text{g/ml}$). The effect of acetylcholine is potentiated by eserine. Time trace is in minutes.

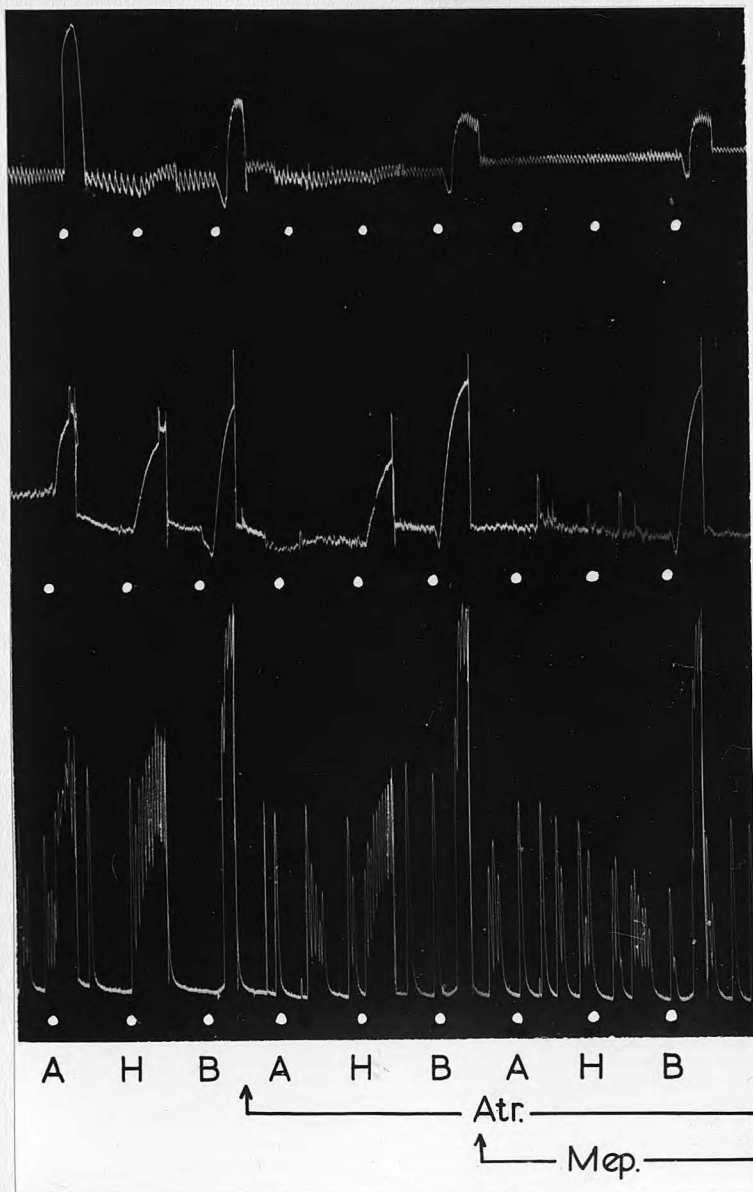


Fig. 2 . Longitudinal strips from adult duodenum, jejunum and colon are represented by the upper, middle and lower tracings respectively. Acetylcholine (A. 0.5 $\mu\text{g}/\text{ml}$), histamine (H. 0.5 $\mu\text{g}/\text{ml}$), bradykinin (B. 0.125 $\mu\text{g}/\text{ml}$), atropine (Atr. 0.1 $\mu\text{g}/\text{ml}$) and mepyramine (Mep. 0.1 $\mu\text{g}/\text{ml}$) were added at the points indicated. Acetylcholine and histamine were abolished by atropine and mepyramine respectively, leaving the effect of bradykinin unaffected.

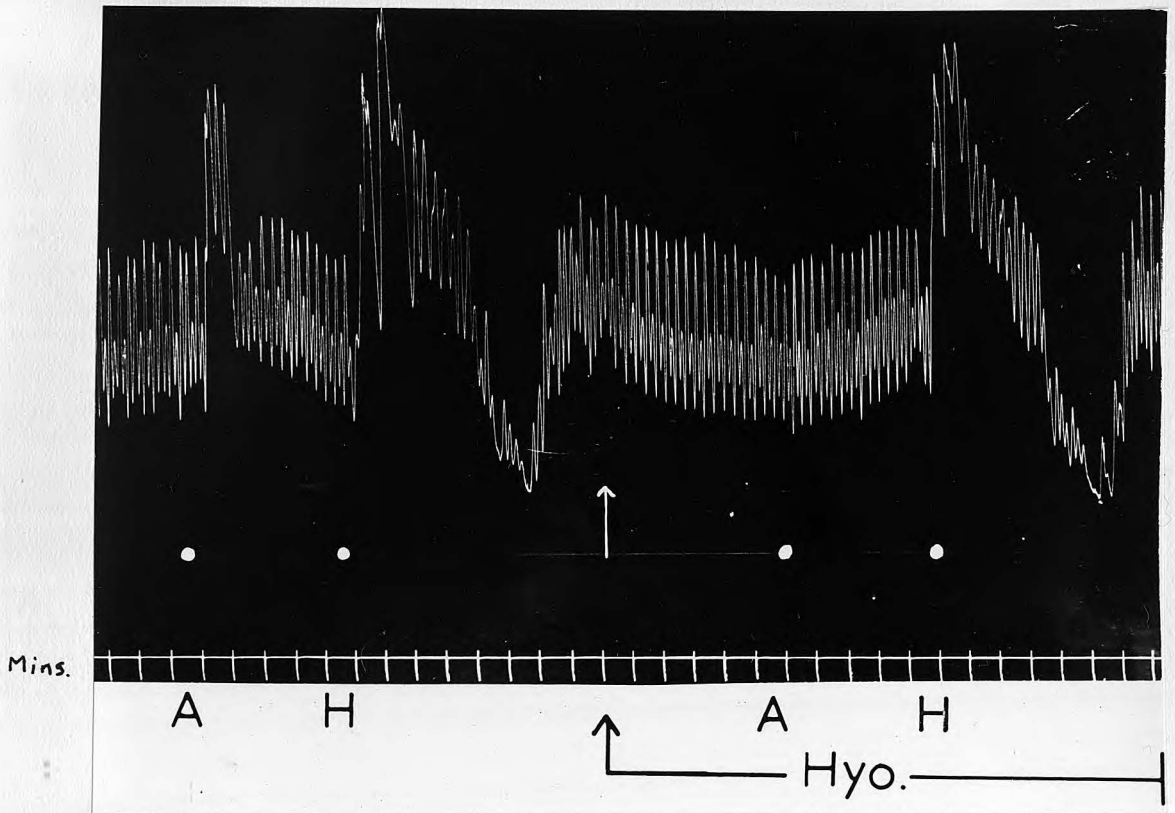


Fig. 3 . Longitudinal strip of calf colon. Acetylcholine (A. 0.1 $\mu\text{g}/\text{ml}$) and histamine (H. 1.0 $\mu\text{g}/\text{ml}$) were added at the points marked.

The contraction due to acetylcholine was blocked by hyoscine (Hyo. 1 $\mu\text{g}/\text{ml}$) but that due to histamine was unaffected.

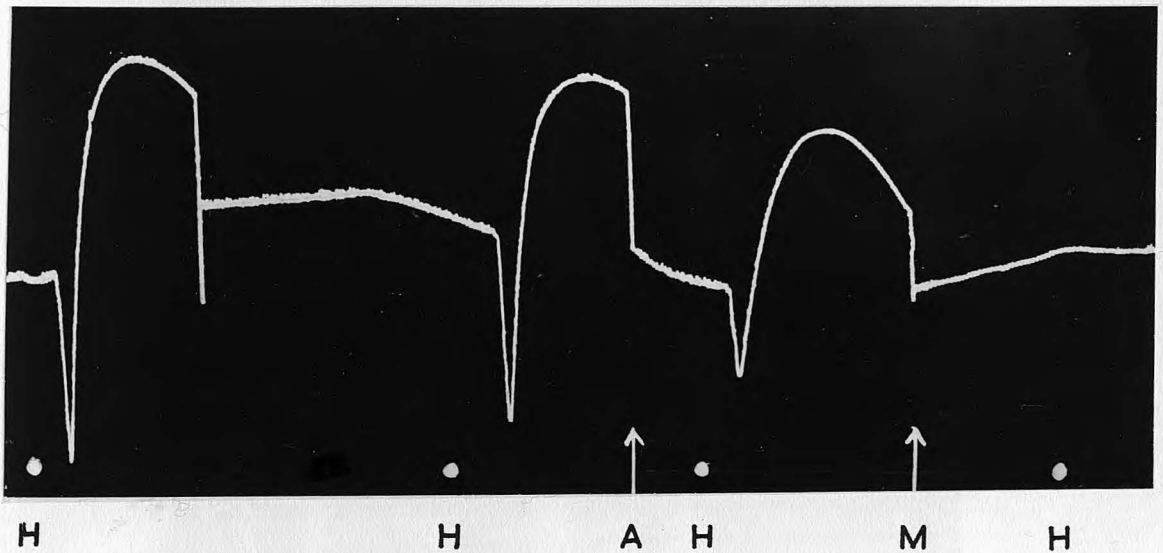


Fig. 4 . Longitudinal strip from adult jejunum. At the points marked by dots, histamine ($1.0 \mu\text{g/ml}$) was added to the bath. Atropine (A. $1.0 \mu\text{g/ml}$) appeared to reduce both the relaxant and contractile phases, although this may have been due to tachyphylaxis. Mepyramine (M. $0.01 \mu\text{g/ml}$) abolished both phases.

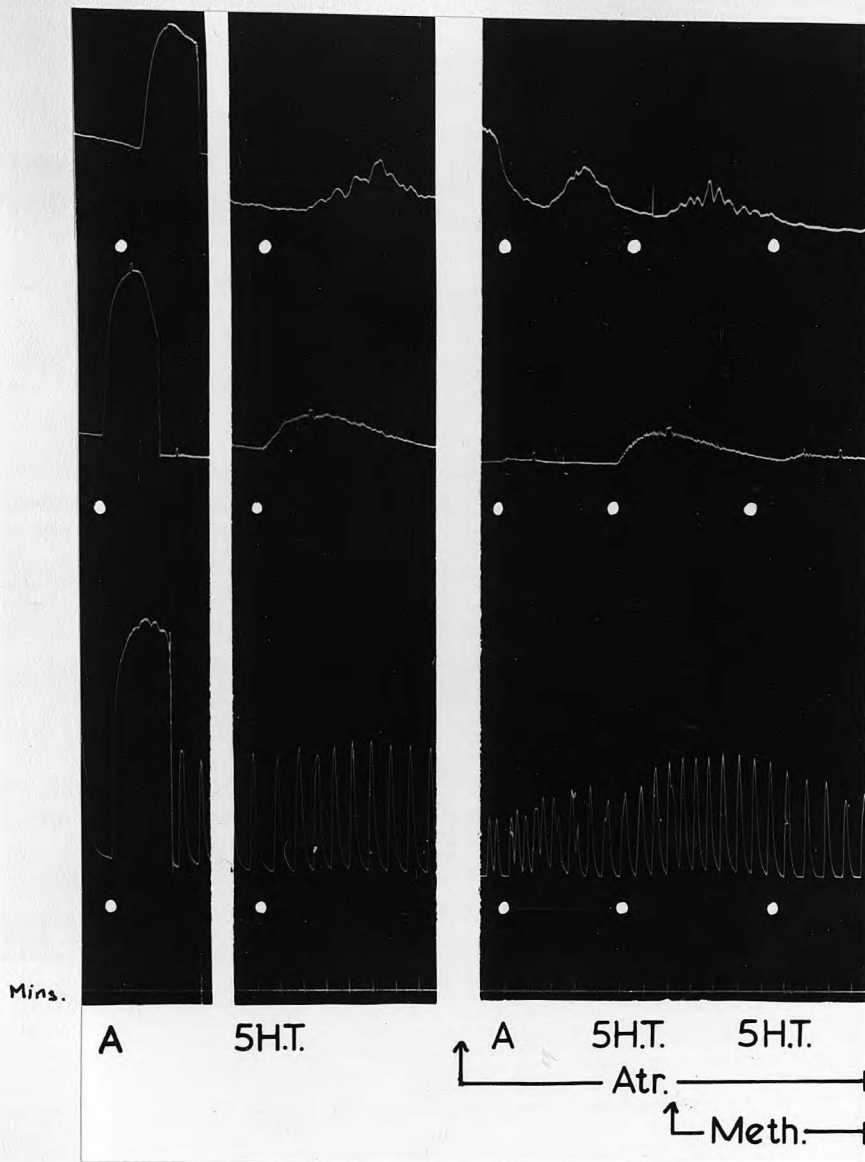


Fig. 5 . Longitudinal strips taken from adult duodenum, jejunum and colon are represented by the upper, middle and lower tracings respectively. Acetylcholine (A. 0.5 $\mu\text{g}/\text{ml}$), 5-hydroxytryptamine (5-H.T. 0.5 $\mu\text{g}/\text{ml}$), atropine (Atr. 0.02 $\mu\text{g}/\text{ml}$) and methysergide (Meth. 1.0 $\mu\text{g}/\text{ml}$) were added at the points marked. This demonstrates the specific blocking action of methysergide against 5-H.T.

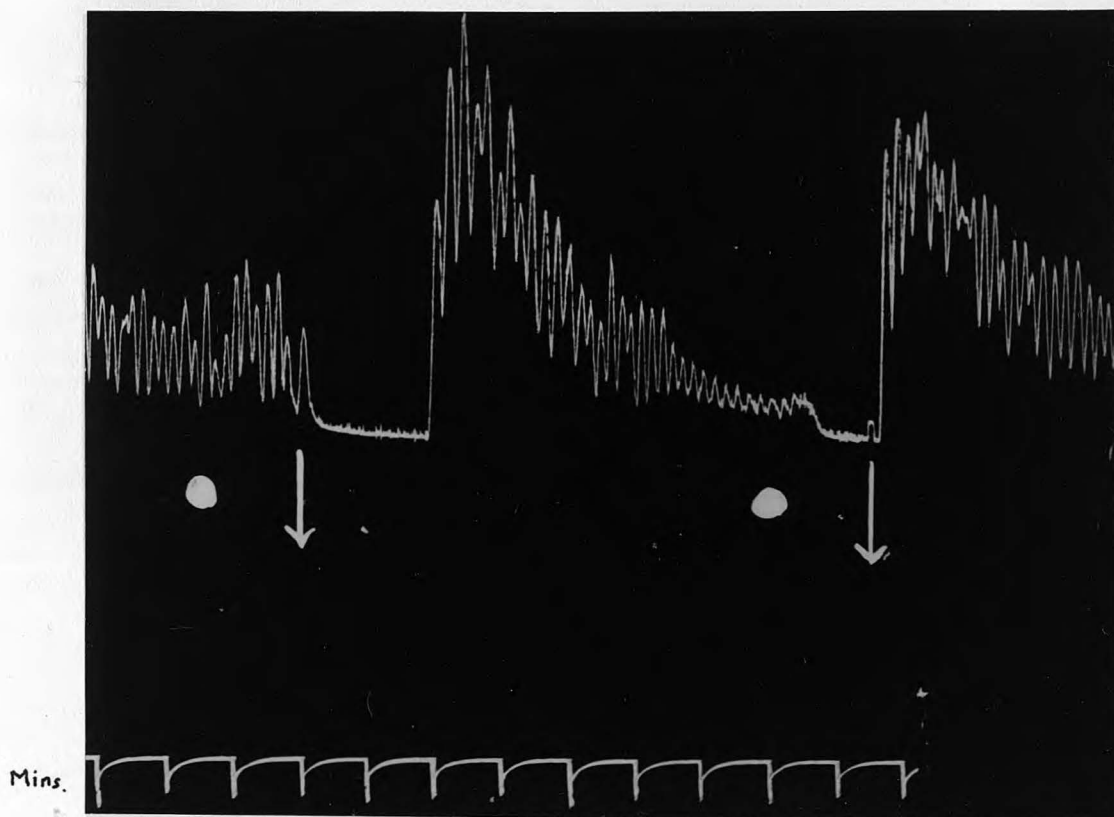


Fig. 6 . Calf colon preparation. 5-Hydroxytryptamine (0.5 $\mu\text{g}/\text{ml}$) was added at the point marked by a dot, and washed out at the arrow. This shows the anomalous response of calf colon to 5-H.T.

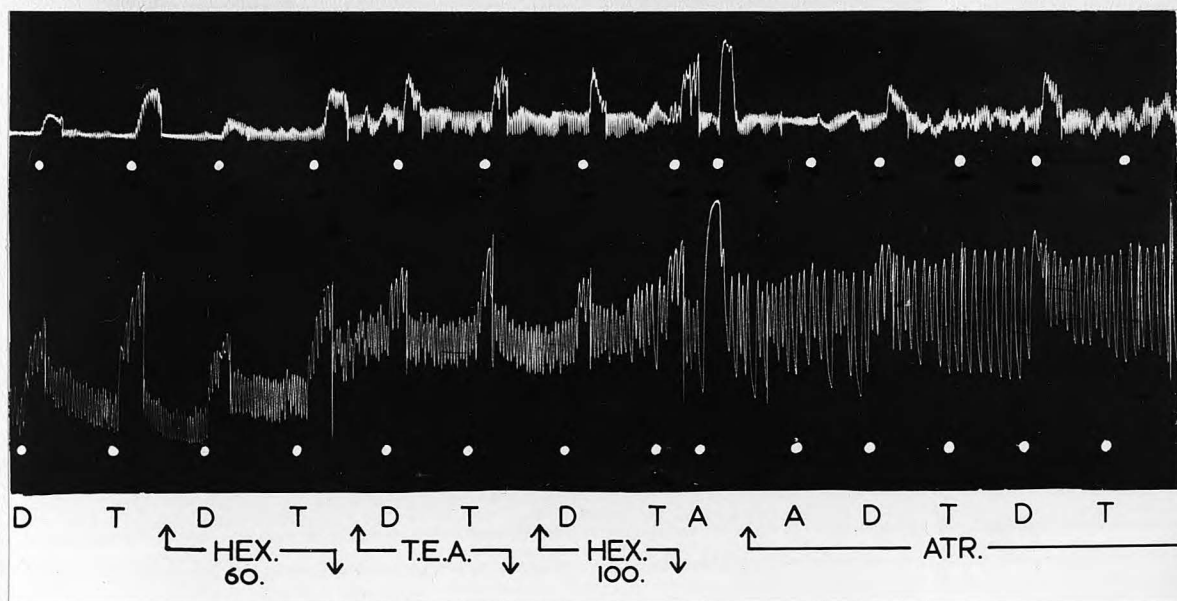


Fig. 7 . Longitudinal strips taken from jejunum and colon are represented by the upper and lower tracings respectively. D.M.P.P. (D. 30 $\mu\text{g}/\text{ml}$), T.M.A. (T. 20 $\mu\text{g}/\text{ml}$), tetraethylammonium (T.E.A. 50 $\mu\text{g}/\text{ml}$), hexamethonium (Hex. 60 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$), acetylcholine (A. 0.5 $\mu\text{g}/\text{ml}$) and atropine (1 $\mu\text{g}/\text{ml}$) were added at the points indicated.

The contractions due to D.M.P.P. and T.M.A. are unaffected by ganglion blockers, but T.M.A. is blocked by atropine.

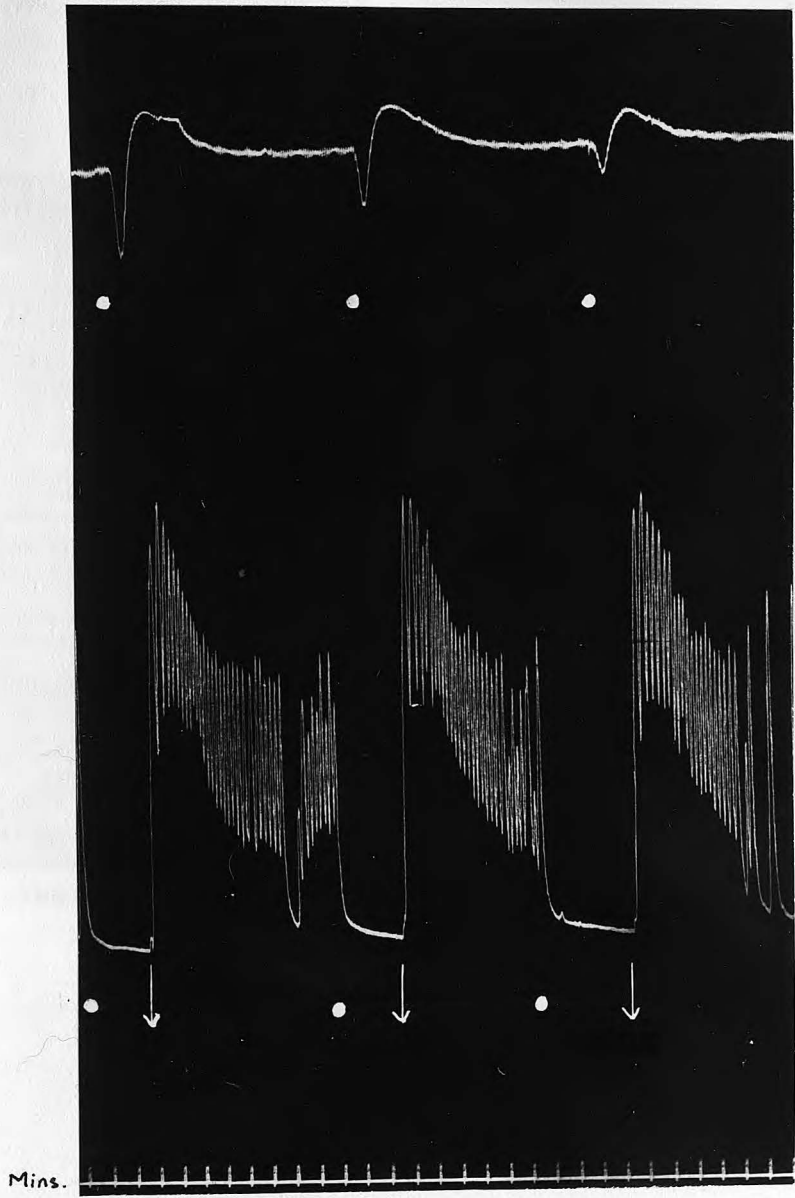


Fig. 8 . Longitudinal strips of muscle taken from the calf jejunum and colon are represented by the upper and lower tracings respectively. The dots signify addition of bradykinin, 0.25 $\mu\text{g}/\text{ml}$ on the first two occasions, 0.125 $\mu\text{g}/\text{ml}$ on the third.

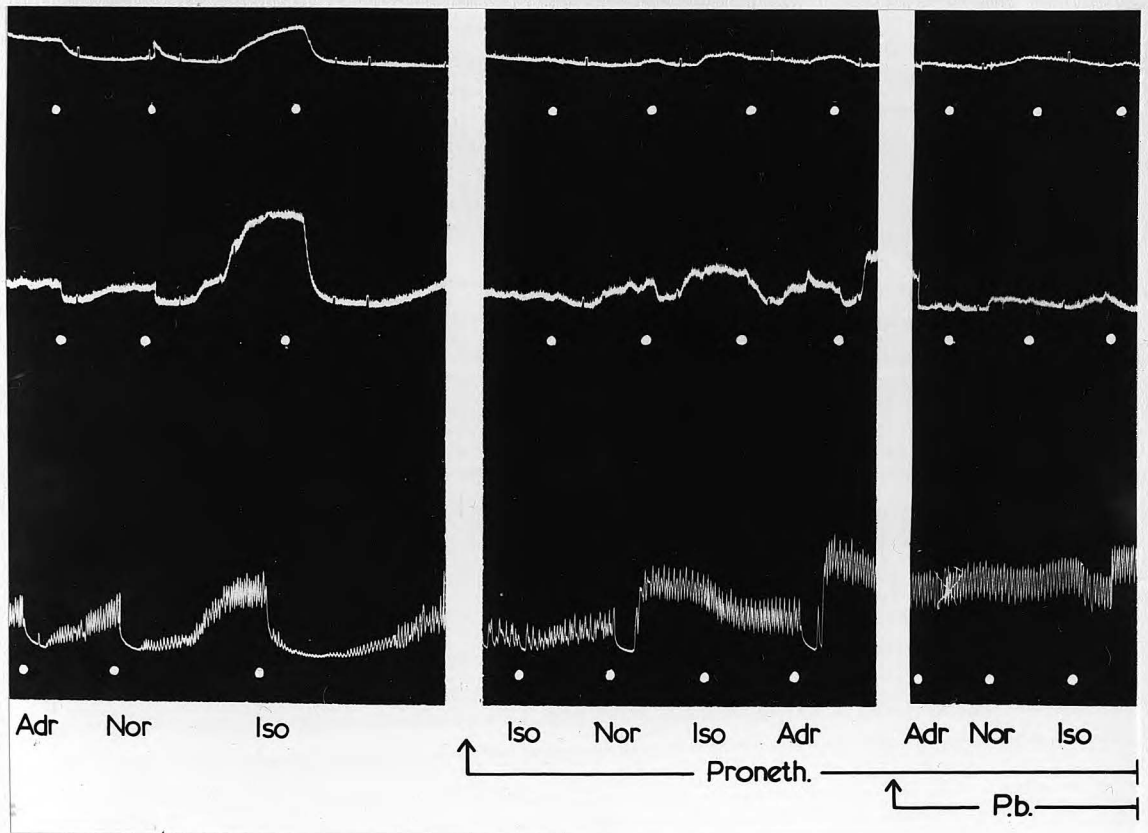


Fig. 9 . Longitudinal strips from calf duodenum, jejunum and colon are represented by upper, middle and lower tracings respectively. Adrenaline (Adr. 0.5 $\mu\text{g}/\text{ml}$), noradrenaline (Nor. 0.5 $\mu\text{g}/\text{ml}$), isoprenaline (Iso. 0.5 $\mu\text{g}/\text{ml}$), pronethalol (Proneth. 3 $\mu\text{g}/\text{ml}$) and phenoxybenzamine (P.b. 3 $\mu\text{g}/\text{ml}$) were added at the points indicated. Isoprenaline is blocked by pronethalol, and adrenaline and noradrenaline are blocked by a mixture of pronethalol and phenoxybenzamine.

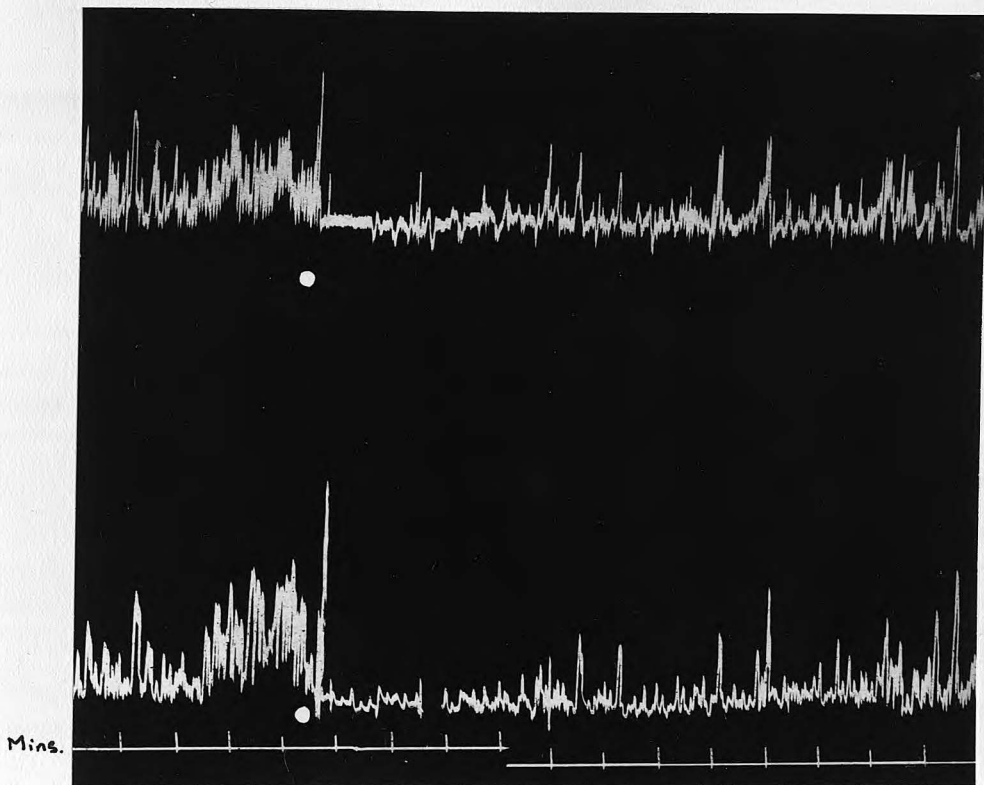


Fig. 10. In vivo record. Upper and lower tracings are from balloons placed at 18 inches and 6 inches respectively from the cannula. Acetylcholine (1 mg) was injected intravenously at the point marked. Time trace is in minutes.

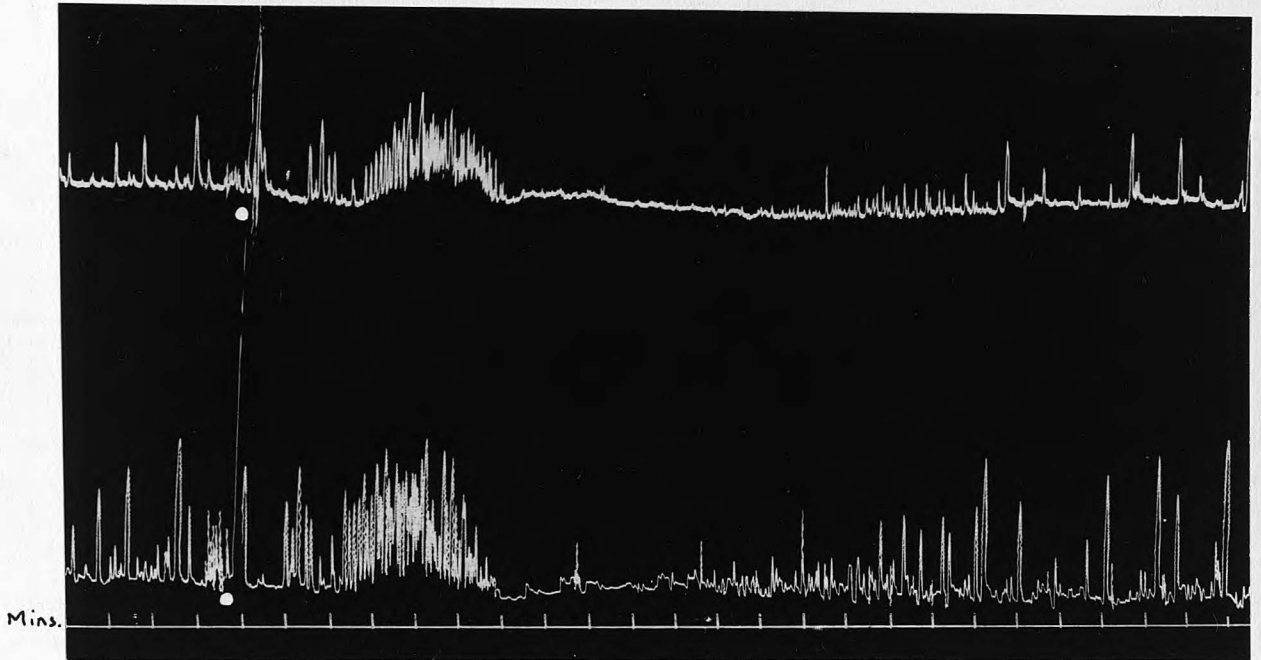


Fig. 11 . In vivo record as Fig. 10 . Acetylcholine (5 mg) was injected intravenously at the point marked. The large excursion immediately after the injection was caused by coughing.

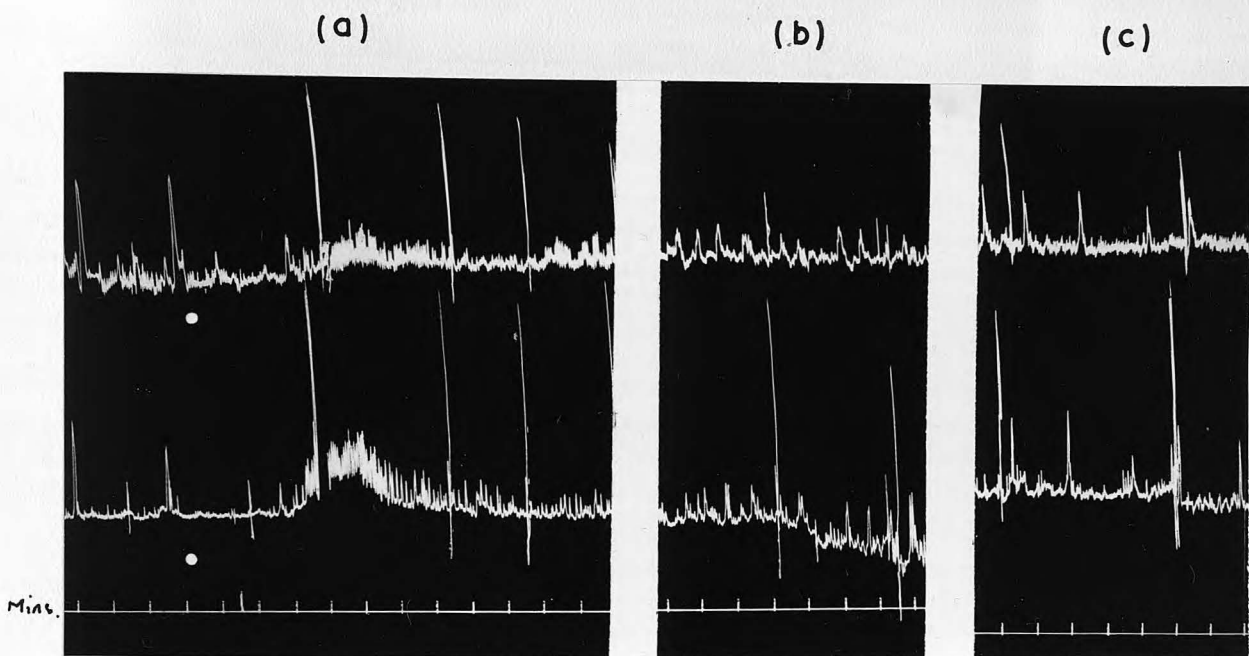


Fig.12. In vivo record as Fig.10. At the point marked on trace (a), carbachol (1 mg) was injected subcutaneously. The trace (b) was taken 20 minutes, and trace (c) 75 minutes after the injection.

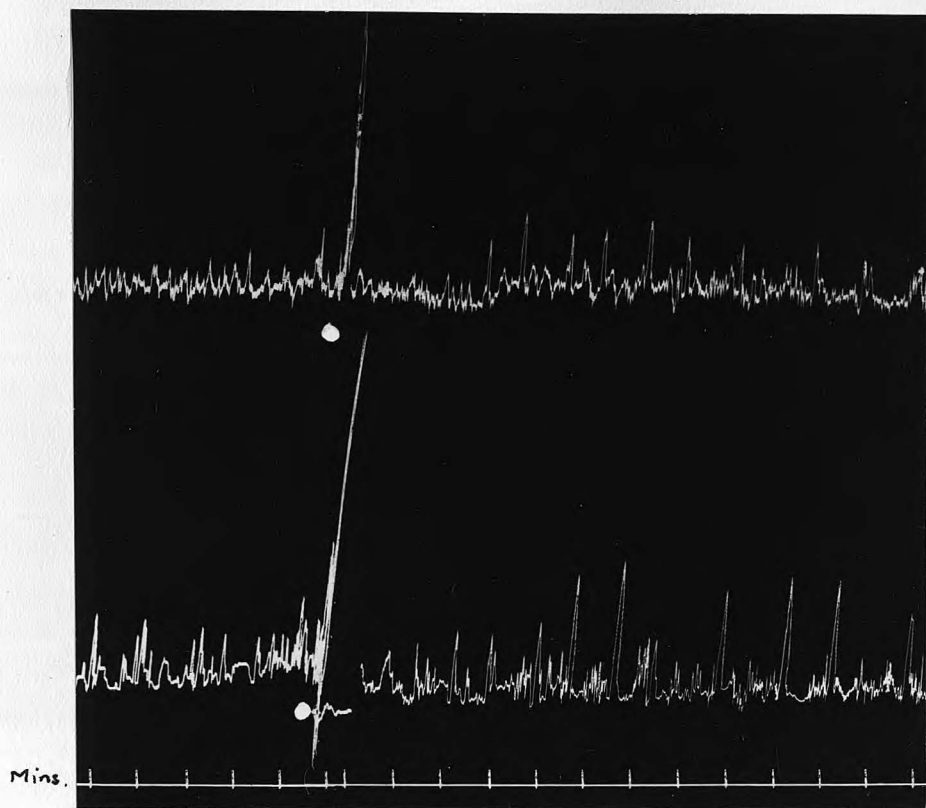


Fig.13 . In vivo record as Fig.10 . Histamine (0.1 mg) was injected intravenously at the point marked. The immediate large excursions were a result of coughing.

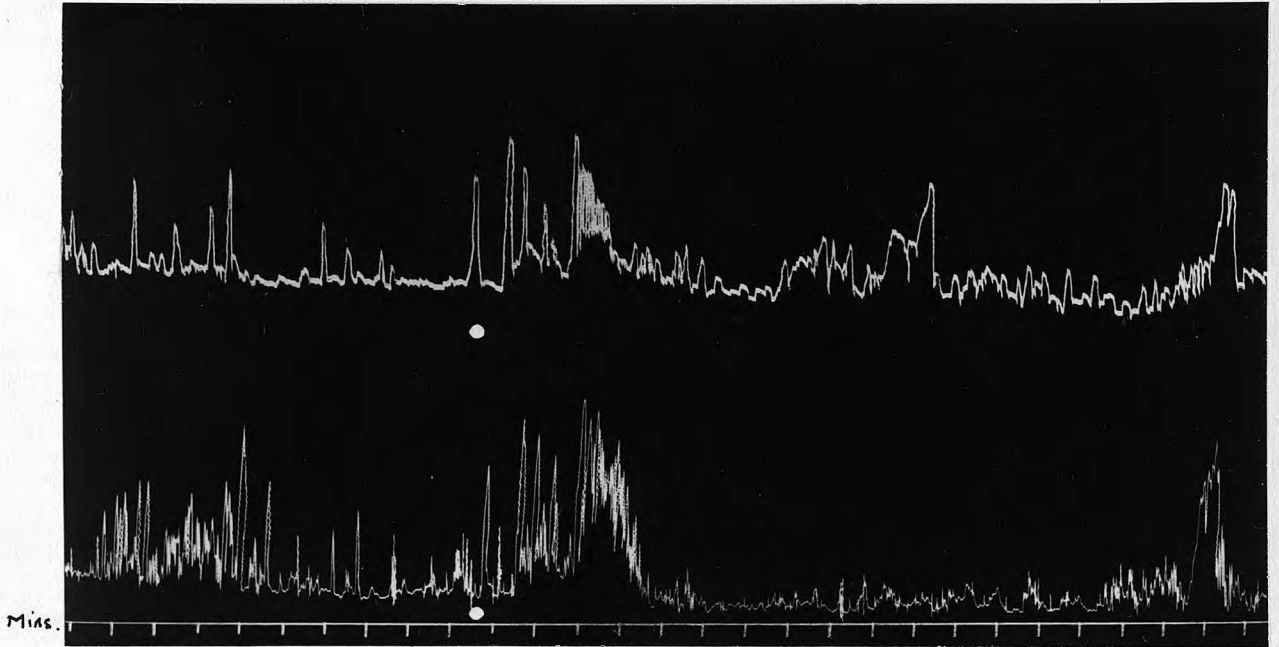


Fig.14 . In vivo record as Fig.10 . 5-H.T. (1 mg)
was injected intravenously at the point marked.

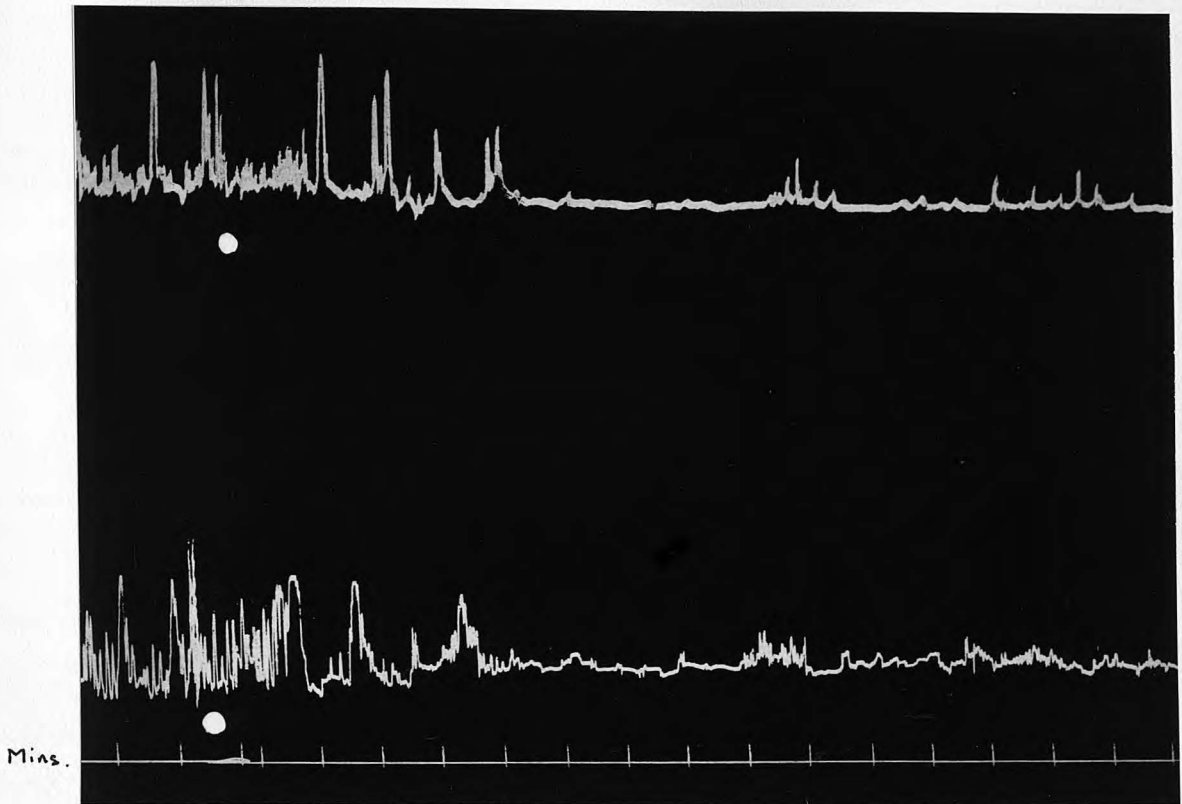


Fig.15 . In vivo record as Fig.10 . Atropine (10 mg)
was injected subcutaneously at the point marked.

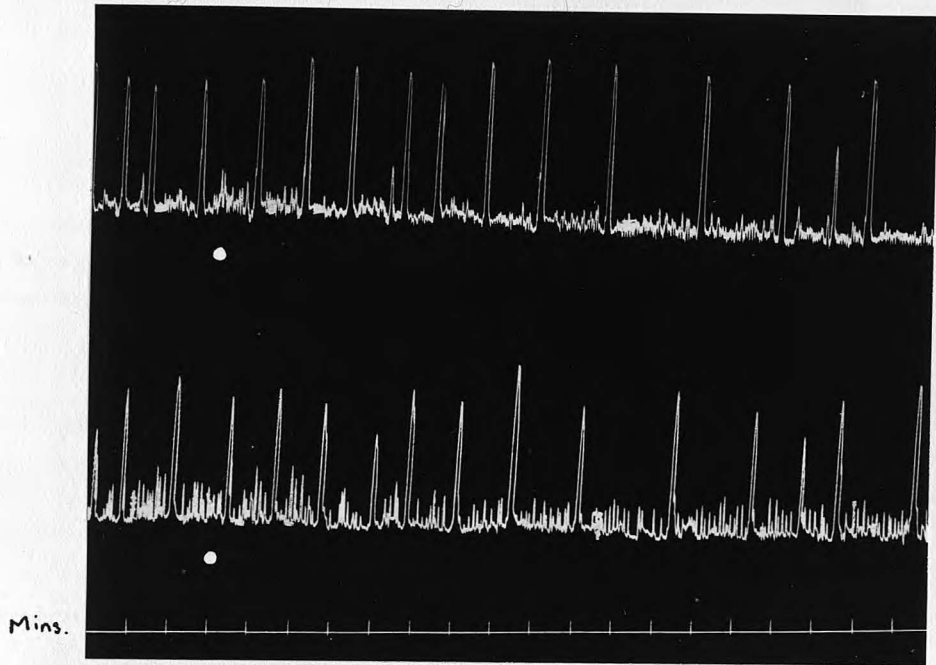


Fig.16 . In vivo record as Fig.10 . Adrenaline (3 mg) was injected subcutaneously at the point marked. This tracing illustrates sequential type II contractions.

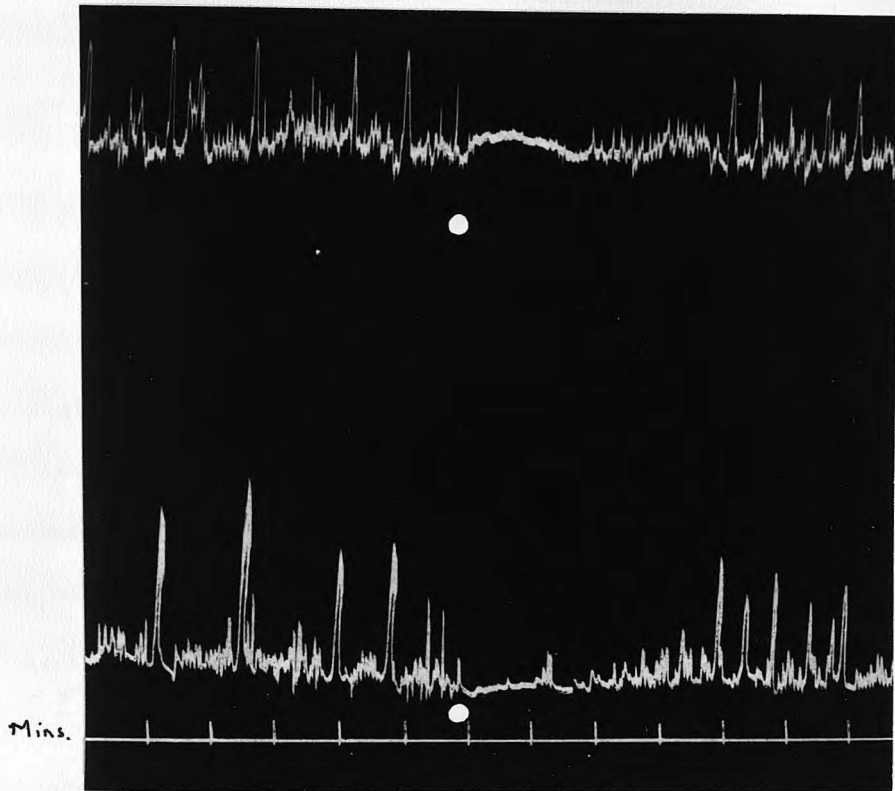


Fig.17 . In vivo record as Fig.10 . Adrenaline
(1 mg) was injected intravenously at the point marked.

S E C T I O N IIALKALINE PHOSPHATASE ACTIVITY IN THE DEVELOPING GUTINTRODUCTION.

'Alkaline phosphatase' has been the subject of extensive investigation ever since reliable methods have been available for its estimation in biological material (e.g. Bodansky, 1933; King and Armstrong, 1934; Bessey, Lowry and Brock, 1946). These methods, among others, have been used to estimate the level of enzyme or enzymes in a wide variety of tissues, species and experimental situations. In spite of this, however, the role of alkaline phosphatase in the organism remains obscure.

(i) Heterogeneity of alkaline phosphatase.

It was evident from an early stage in the investigation of the properties of alkaline phosphatase, that the term was unlikely to apply to a specific enzyme. Bodansky (1937) showed that bile salts inhibited intestinal alkaline phosphatase in a different manner from bone or kidney phosphatase, and this distinction was confirmed by Shlamowitz and Bodansky (1959) using an immunochemical method. The study of the lack of substrate specificity of alkaline phosphatase was later extended (Landau and Shlamowitz, 1961) using various inhibitors and heat inactivation. It was concluded that the activity against a variety of phosphomonoesters was due to an enzyme with broad specificity rather than a multiplicity of enzymes. The distinction between

intestinal and kidney phosphatase remained valid. These authors noted, however, that the chromatographic behaviour of the enzyme could be affected by the mode of preparation, in particular, by the effect of autolysis. The results showed that as autolysis occurred, so the concentration of NaCl needed to elute the enzyme from a D.E.A.E. cellulose column was trebled. However, the relative activity against a variety of phosphomonoesters was unaffected by autolysis.

Although there is a clear distinction between alkaline phosphatase derived from different sources (Bodansky, 1937; Landau and Shlamowitz, 1961; Morton, 1955) the heterogeneity of alkaline phosphatase from intestinal mucosa has yet to be fully established. Moog and Angeletti (1962) compared alkaline phosphatase from the duodenum and jejunum in the mouse, and found, using an immunochemical method, that a difference could be demonstrated (a) between duodenal and jejunal phosphatases and (b) between duodenal phosphatase from a young animal and that from an adult. Moog (1961) had investigated alkaline phosphatase in the mucosa of the developing mouse, using two different substrates, and had concluded that there were at least two enzymes present, and that their relative activity changed with age. A highly purified sample of calf intestinal phosphatase was studied by Engström (1961). This worker found that a high degree of homogeneity was shown in respect of the molecular weight (about 100,000) but that the enzyme was chromatographically and electrophoretically heterogeneous.

The possibility was mentioned, however, that the purification procedure may have been responsible for splitting the enzyme. A similar result was obtained by Moss (1965) who had previously investigated human intestinal alkaline phosphatase using starch-gel electrophoresis (Moss and King, 1962) and later electrophoresis and chromatography (Moss, 1965). In this case, however, the conclusion drawn was that heterogeneity was probably the result of modification of a single enzyme, and that the evidence appeared insufficient to justify the postulation of separate iso-enzymic forms of phosphatase.

However, Behal et al (1965) investigated calf intestinal alkaline phosphatase using column chromatography and starch-gel electrophoresis; he studied the characteristics of the isolated components against a variety of phosphomonoesters, and concluded that the enzyme was in fact heterogeneous, although it was noted that the heat sensitivity of the various components was similar.

Although the evidence for the heterogeneity of calf intestinal alkaline phosphatase is quite extensive, the question of degradation during purification has not been fully answered. This is necessary, especially in view of the work of Schramm and Armbruster (1954) who showed that as autolysis occurred, so the separation of the fractions of alkaline phosphatase increased, and the work of Landau and Shlamowitz (1961) who also noted effects of preparatory procedures on chromatographic behaviour.

Despite the possible heterogeneous nature of this enzyme, the

singular nomenclature 'phosphatase' will be retained for the sake of convenience.

(ii) Regional distribution of alkaline phosphatase in the gut mucosa.

This has been studied using (a) biochemical methods (e.g. Bodansky, 1933) and (b) histochemical methods (e.g. Gomori, 1950).

(a) Biochemical methods.

The biochemical techniques lend themselves to the determination of the regional alkaline phosphatase activity along the length of the gastro-intestinal tract. A gradient of activity from high levels in the duodenum to low levels in the ileum has been demonstrated biochemically in the adult cat and rabbit (Kay, 1928), the adult mouse (Moog, 1961), the adult rat (Fehrer et al, 1956; Triantaphyllopoulos and Tuba, 1959; Koldovsky et al, 1963; Nunn et al, 1963; Hanninen and Hartiala, 1965), and the adult bovine (Kolb and Schulz, 1959).

This gradient is not apparently universal, since in both the horse (Kolb and Wujanz, 1959) and in the pig (Kolb and Schutz, 1961) there appears to be a gradient in the opposite direction, with the highest activity being found in the ileum.

The work done by Kolb and his colleagues is open to criticism in that no statistical analysis was carried out. When this is done, it is in fact found that the results reported for the pig

small intestine (Kolb and Schutz, 1961) are not significant, since too few animals were examined. The figures obtained for horse material Kolb and Wujanz (1959) were not reported in a form which lends itself to analysis.

The studies on the alimentary tract of the cow, horse and pig (Kolb and Schutz, 1961) included stomach and large intestine. It was found that the equine colon and the bovine reticulo/rumen both had high levels of alkaline phosphatase activity in their mucous membrane in contrast to the equine stomach and bovine colon, both of which showed comparatively little activity. This finding is interesting since both the adult equine colon (Alexander, 1963) and the adult bovine rumen (Sutton et al, 1963) are known to be important sites of absorption in the respective species.

(b) Histochemical methods.

These techniques, although they have been used for regional studies in the rat by Lojda et al (1959) and Brodskii (1962) are better suited to a study of the sub-cellular distribution of alkaline phosphatase. It has been clearly demonstrated in laboratory animals that the enzyme is situated in the apical region of columnar epithelial cells (Deane and Dempsey, 1945; Johnson and Kugler, 1953) in association with the striated border (Shnitka, 1960). The exact location within the cell was shown by Clark (1961) who used electron microscopy to show that the enzyme activity was mainly in the cell membrane, and that the high level in the region of the brush-border was at least partly attributable

to the expanse of membrane covering the microvilli.

The histochemical technique was used on the bovine intestine by Renzoni et al (1957) and on bovine fore-stomach by Habel (1963). The former workers found that the distribution in the bovine alimentary tract was similar to that reported for the laboratory species. Habel (1963) confirmed the high level of activity in the adult rumen which had been reported by Kolb and Schulz (1959) and extended his observations to the calf.

(iii) Effect of age on regional distribution of alkaline phosphatase in gut mucosa.

The distribution of alkaline phosphatase along the length of the intestine of the young animal has been studied in the mouse (Moog, 1961) and in the rat (Koldovsky et al, 1963). The situation in the young animal was compared with that in the adult, and it was found in both species that the gradient of enzymic activity which exists along the adult intestine, from high proximal to low distal levels, is absent in the young animal.

A more detailed study of alkaline phosphatase development was made in the chick duodenum before and after hatching (Moog, 1950) in the foetal and young mouse duodenum (Moog, 1951) and in the foetal and young guinea-pig small intestine (Moog and Ortiz, 1960). The post-natal development has been examined in the duodenum of the rat (Halliday, 1959), in the duodenum, jejunum and ileum of the mouse (Moog, 1961) and of the rat and rabbit (Brodskaa, 1962, using a histochemical method); the duodenum of the developing rabbit was also examined by Willenbockel (1961) using a biochemical

method. All the species examined showed fluctuations in alkaline phosphatase activity, but the stage of development at which peaks occurred varied, as did the site in the gut at which these could be demonstrated. The chicken duodenum showed a peak of activity at hatching while the guinea-pig small intestine showed its highest activity ten days before birth. In her original study of the activity of the mouse duodenum, Moog (1951) showed a marked rise in alkaline phosphatase activity at birth, followed by another sharp rise during the third post-natal week (coinciding with weaning). In the later, more extensive study (Moog, 1961) it was discovered that the second rise was largely limited to the duodenum, and involved a marked rise in preference for disodium phenyl phosphate as a substrate rather than sodium β glycerophosphate. Kinetic studies indicated that this was probably due to the appearance of a fresh enzyme, rather than the proliferation of one already there. It was found at the same time that a smaller peak of activity could be demonstrated in the jejunum and ileum, but that this was comparatively insignificant.

The histochemical study of Brodskii (1962) although less quantitative than the biochemical techniques, indicated that in both the rat and rabbit, the duodenum and jejunum showed a fall in activity in the first week, followed by a peak in the second week, and another peak at the end of the fourth week. The ileum did not show any secondary rise: Willenbockel (1961), although using a biochemical technique, obtained results for the developing rabbit duodenum which agreed with those of Brodskii for this organ,

although the former author was able, with his more sensitive technique, to show that the level was rising at birth to an initial peak at two days of age. Willenbockel used sodium β glycerophosphate as a substrate, and so it is possible that peaks of activity against disodium phenyl phosphate may have been missed.

In an attempt to explain the varying enzyme activities, Moog (1962), pointed out that the chicken and guinea-pig, both of which show little post-natal rise, are born in a highly developed state, able to eat a wide variety of food. In comparison, the mouse is born relatively immature. However, the fact that the kitten duodenum reaches its maximum phosphatase activity after only three days (Moog, 1962) indicates that suckling is not necessarily associated with a low level of enzyme activity.

Very little work appears to have been done on the distribution and changes of alkaline phosphatase activity in the digestive tract of the calf. The only biochemical work on this animal was done by Onodera et al (1964) who compared the levels of alkaline phosphatase activity in the rumen mucosa of young calves with that in adults. These workers found that there was a higher level in adult material, and this finding was confirmed, using a histochemical method, by Habel (1963). This result agrees with the work of Sutton et al (1963) who showed that absorption from the rumen of young calves does not occur until after solid food has been taken.

Various physiological mechanisms in the calf have been shown to change and develop with age. These include digestive enzyme

secretion (Huber et al, 1961) and adrenal medullary activity, (Comline and Silver, 1966). This suggests that the calf is born in a state of relative physiological immaturity, and so may be one of those animals showing marked changes in phosphatase activity during development (Moog, 1962).

(iv) Factors other than age which affect alkaline phosphatase activity.

(a) Dietary effects.

Fasting of rats leads to a lowering of plasma alkaline phosphatase (Weil and Russell, 1940; Flock and Bollman, 1948; Tuba and Robinson, 1953). The levels could then be restored by a diet containing high levels of fat (Weil and Russell, 1940; Gould, 1944). The latter of these authors showed that forced feeding of fat elevated plasma alkaline phosphatase levels considerably above normal, and moreover, that this rise was due to the enzyme of intestinal origin.

Intestinal alkaline phosphatase was shown to enter the plasma via lymph after a fat-containing meal in the rat (Flock and Bollman, 1948) and in man (Blomstrand et al, 1965).

Mucosal alkaline phosphatase in rats was found to be lowered during starvation, and raised after a fat or protein-containing meal (Jackson, 1952). This author also found that the minimum fasting levels obtainable in young rats was much higher than in older rats. This might have been expected, since control levels were probably higher in the young animal.

It was noted by Moog (1962) in the mouse and rat that the secondary rise in duodenal alkaline phosphatase activity coincided with weaning time, and suggested a possible association. It was therefore considered to be of particular interest to observe the changes in the calf mucosa at weaning.

(b) Effect of adrenal cortex.

Verne and Herbert (1948) showed that an association existed between the adrenal cortex and the presence of alkaline phosphatase in gut mucosa. This was done histochemically by showing that the enzyme fell after adrenalectomy in the castrated rat, and could be restored by injection of adrenocortical hormones. An even clearer demonstration was given later (Verne and Herbert, 1949) by injecting one half of the foetuses in a rat's uterus with cortisone, and then killing and examining the treated foetuses and the untreated controls, six to twelve hours later. This showed that the injected animals gave a positive Gomori stain in gut mucosa, but the controls did not. Ross and Goldsmith (1955) also used a histochemical method to demonstrate precocious development of alkaline phosphatase in the striated border of gut epithelium in the foetal and neo-natal rat following injection of cortisone in the mother.

Moog and Thomas (1955) tested biochemically the efficacy of various steroid hormones in elevating alkaline phosphatase in the mouse duodenum. It was found that when eleven-oxygenated steroids were used, the rise which normally occurred during the third

post-natal week (Moog, 1951) could be brought forward by up to ten days. These findings were later confirmed and extended (Moog, 1961) when it was found that the rise in activity in the duodenum, either normal or precocious, was due to enzyme with a preference for disodium phenyl phosphate as a substrate. Moreover, exogenous cortisone had no effect on jejunal phosphatase activity, while having a biphasic action on the ileum, causing a lowered, then raised activity.

The effect of cortisone on the development of alkaline phosphatase in the rat duodenum was investigated by Halliday (1959) who confirmed the original findings of Moog and Thomas (1955) in the mouse, and correlated the rise with the cessation of antibody absorption from the gut.

(v) The role of intestinal alkaline phosphatase.

Despite the inconclusive evidence concerning the physiological role of intestinal alkaline phosphatase, there appeared to be three fields of possible importance:-

(a) As a digestive enzyme. Rothstein et al (1953) noted the importance of dietary organic phosphate compounds as a source of phosphate in the body, and so studied the fate of these compounds in the gut. They showed, using radio-active phosphorus, that hydrolysis of phosphomonoesters by phosphatase occurred at the surface of cells. Millar and Crane (1961, a) showed that the intestinal hydrolysis of glucose-1-phosphate had virtually the

same characteristics as the hydrolysis of the disaccharides, sucrose and maltose. They then showed (Millar and Crane, 1961, b) that phosphomonoesterase and disaccharidase activities were both confined to the brush border of the intestinal epithelium, while Holt and Millar (1962) showed that acid phosphatase was not. The conclusion drawn from this series of experiments was that alkaline phosphatase functioned, at least in part, as a digestive enzyme. Ugolev (1965) in his review of membrane digestion, reached the same conclusion.

(b) As a factor in absorption of nutrients. The distribution of alkaline phosphatase in intestinal epithelium, renal tubules and epithelium of secretory organs, suggests that the enzyme may be associated with transport mechanisms (Bitensky et al, 1965).

Further circumstantial evidence has been provided by interspecies comparisons. Kolb and Schutz (1961) compared results obtained in horses, cattle and pigs, and showed that areas known to be sites of active absorption in the respective species were also sites of high alkaline phosphatase activity. More evidence has been given recently by Onodera et al (1964) who showed that the calf rumen, which does not absorb volatile fatty acids, (Sutton et al, 1963) has a low alkaline phosphatase activity, and in the case of a day old calf, this is almost negligible (Habel, 1963).

The theory which postulated phosphorylation as an intermediate

step in glucose absorption (Verzar and McDougal, 1936) explained the presence of alkaline phosphatase at sites of active absorption. Support for this was given when glucose absorption was shown to be accompanied by increased phosphorylation (Beck, 1942) and that rates reflected one another (Hele, 1950). Later it was shown, Fehrer et al (1956), that A.T.P. and alkaline phosphatase were necessary for glucose absorption in vitro.

However, evidence counter to the theory was given by Crane and Krane (1956) who showed that substituted derivatives of glucose which were incapable of undergoing phosphorylation, were nevertheless actively absorbed. More contrary evidence was given by Landau and Wilson (1959) who showed that most of the absorbed glucose did not pass through the cellular pool of glucose-6-phosphate. Finally, Nunn et al (1963) and Koldovsky et al (1963) both showed that the distribution of alkaline phosphatase along the length of the small intestine does not correspond to the distribution of glucose absorptive capacity, and so is not the rate-limiting factor.

Despite this mass of evidence, Crane (1962) did point out that many of the objections to the phosphorylation hypothesis applied only to consideration of the cell as a whole. He suggested that it would still be possible for the phosphorylation hypothesis to apply to the brush border, the place where in fact alkaline phosphatase is found. Nevertheless, Isselbacher and Senior (1964) concluded in their review that the weight of evidence was against the phosphorylation hypothesis for absorption of sugars.

Evidence has been given for a strong association between the 'sodium-pump' and active transport of monosaccharides in the guinea-pig (Riklis and Quastel, 1958), the rat (Csaky and Thale, 1960) and the hamster (Bihler and Crane, 1962). A sodium-dependent form of adenosine triphosphatase (A.T.P.ase) has been demonstrated in tissues such as brain and kidney (Skou, 1962) and it has been suggested (Isselbacher and Senior, 1964) that this may turn out to be an important factor in transport mechanism.

This suggestion implicates alkaline phosphatase if the work of Tosteson et al (1960) is substantiated. They claimed that phosphomonoesterase and A.T.P.ase were functionally indistinguishable, being unable to separate them using D.E.A.E. column chromatography and alcohol fractionation. Evidence in support of this is given by the work of Fehrer et al (1956) who found, using inhibitors, that both A.T.P. and alkaline phosphatase were necessary for in vitro absorption of glucose. Moreover, Nunn et al (1963) after showing that alkaline phosphatase was not the rate-limiting factor in glucose absorption, pointed out that it may still have provided energy by splitting of high energy bonds.

An increase in alkaline phosphatase activity has been associated with absorption of substances other than monosaccharides. These include fat (Tuba and Robinson, 1954) and certain amino acids (Triantaphyllopoulos and Tuba, 1959). This may be taken as indirect evidence that, if alkaline phosphatase is associated with absorption, it does so in a fairly non-specific manner.

(c) As a factor in cessation of antibody absorption.

Halliday (1959) noted that the post-natal rise in duodenal alkaline phosphatase activity in the rat coincided with the cessation of antibody absorption from the colostrum. Injection of cortisone, which was known to cause a precocious rise in phosphatase activity in the mouse (Moog, 1953) was found by Halliday to cause the same effect in the rat and, moreover, that cessation of antibody absorption still coincided with the rise. Halliday suggested, therefore, that a possible connection existed between the two events.

Further suggestive evidence was obtained by Payne and Marsh (1962) who investigated antibody absorption in piglets. They found that there was a seven-fold increase in mucosal alkaline phosphatase activity at the time of absorption blockade, which in the piglet is in the first twenty-four hours after birth. They also found that starvation, which delays the 'closure' to antibodies, also delays the rise in phosphatase activity. Moreover, preliminary studies indicated that cortisone acetate caused a similar effect in the pig to that described for the mouse (Moog, 1953) and rat (Halliday, 1959).

It would be of particular interest to know whether any correlation exists in the calf between antibody absorption and mucosal alkaline phosphatase activity. Moreover, it was suggested by Moog (1962) that investigations of animals with different dietary habits, and in different physiological states might offer



much in understanding of normal absorption in vivo. It was with these points in mind that the following study of alkaline phosphatase activity was undertaken in the developing ruminant.

MATERIALS AND METHODS.

Bovine mucosa was obtained from two sources:-

- (i) the slaughterhouse
- (ii) from the living animal using a biopsy technique.

(i) Material obtained after slaughter.

Alimentary tracts were obtained as soon as possible after the death of the animal. The delay between death and removal of a sample was about five minutes in the case of calves, and fifteen in the case of adult cattle. The gut was separated from its mesentery so that it was possible to identify the site from which each sample was taken. A section of gut wall, with mucosa attached, was removed from the duodenum, upper jejunum, a site midway between the pylorus and the ileo-caecal valve, and from the spiral colon. The pieces of gut wall, about four square centimetres in area, were then washed in saline and carried back to the laboratory in ice-cold distilled water. Material to be used for histochemistry was placed in formalin at 4°C.

In the laboratory, the material was washed with distilled water and dried on filter paper. It was then stored on filter paper in the deep-freeze for up to one week until enzyme determinations were carried out.

Material for histochemistry was fixed in formalin at 4°C for four hours before sections were cut.

(ii) Material obtained by biopsy.

(a) The modified Shiner biopsy capsule.

The technique of mucosal biopsy was first applied to the human stomach by Wood et al (1949) and the same principle was used by Shiner (1956) who described the first practical duodenal biopsy capsule.

This consisted (Fig.18 a) of a flexible tube, 4.8 mm in diameter, coated with plastic material to make it smooth and airtight. The head-piece (A) (Fig.19) is a small tube, 3.9 mm in diameter, at the centre of which is the biopsy aperture, 3.1 mm in diameter. The tube accommodates a tubular knife (B) which is screwed to flexible wire running the whole length of the tube, and ending in a small holder at the tail-piece. The small tube of the head-piece is embedded in a solid weighty nickel ball, and the tail-piece has a lateral exhaust tube (C) to which a syringe and pressure gauge (Fig.18 b) can be attached to apply negative pressure. When the capsule is in position, the pressure draws a knuckle of mucosa into the biopsy aperture, the knife is then operated by a pull on the wire, and the sample is removed.

Since the above instrument was described, other models have followed in which the principle is the same, but which differ mainly in the mode of operation of the knife, which may be spring-loaded and pressure released (Crosbie and Kugler, 1957) or hydraulically operated (Flick et al, 1961). One advantage claimed for these instruments is greater flexibility owing to there being no need for a wire within the tube.

In the present work, however, it was considered that a certain amount of rigidity might be useful, and, moreover that the Shiner model had advantages over the Crosbie-Kugler model in its simplicity, and in the possibility of obtaining multiple samples. The Flick model, although elegant, was both expensive and difficult to obtain.

The instrument used (produced by the Genito-Urinary Manufacturing Company Ltd) was similar to that described by Shiner (1956), the main modifications being in the length of the tube (3 metres rather than the standard 1.28 metres) and in the wire used to operate the knife, since 25 grade piano wire was found to be more flexible, but no less effective, than the stainless steel wire provided with the instrument. The head-piece was embedded in a ball of 10 mm diameter. A moveable plug (D) prevented leakage from the cannula, and a screw-clip (E) maintained the pressure while a sample was being taken.

(b) Use of the biopsy tube in ruminants.

It was realised that the oral approach was unlikely to prove successful in ruminants, and that even if the tube could be passed into the small intestine, there would be considerable difficulty in estimating the position of the capsule at any given time. The alternative was to obtain access to the lumen of the intestine through a permanent cannula placed either in the abomasum or in the upper small intestine. The former approach, made attractive by the relative operative simplicity, and by the

lack of interference with the organ of interest, was tried first. The animal used was a five-day old calf. Despite the successful installation of a relatively large cannula, it was found impossible to pass the tube through the pyloric sphincter, and so this approach was abandoned.

A technique was developed for the cannulation of the duodenum of young calves.

(c) The cannula. (Fig. 20).

This was based on the design of Alexander and Chowdhury (1957) and was constructed from polythene tube (Alkathene, I.C.I.). It consisted of a tube (F) of $\frac{1}{2}$ " internal diameter which carried an elongated, trough-shaped flange (G). The flange lay at an angle to the tube to enable the biopsy tube to negotiate the bend more easily. An elongated, fenestrated washer (H) lay parallel to the flange (G) and was also trough-shaped so that it fitted closely over the flange. Although freely moveable over the tube (F), the washer (H) could be sealed to it by a hot iron. A smaller, flat washer (I) lay outside the washer (H), and the end of the tube (F) could be closed by a water-tight plug (J) (Suba-seal).

(d) The operative technique.

The calves were operated at one to two days of age, having had at least three feeds of colostrum, but nothing on the morning of the operation. Premedication was by promazine 0.75 mg/kg i/m (Sparine, Wyeth) given one hour before operating.

Anaesthesia was induced by halothane (Fluothane, I.C.I.), through an open mask, after which the animal was intubated and maintained under anaesthesia with a mixture of cyclopropane and nitrous oxide. An area on the right flank was clipped, washed and ~~cleaned~~ using cetricimide (Cetavlon, I.C.I.).

An incision was made about four inches behind the last rib, and parallel to it, and an opening made into the peritoneal cavity. The duodenum was located and the site where the cannula was to be placed was separated off using small bowel clamps. The optimal site for placement of the cannula was found to be just posterior to the point of attachment of the duodenum to the liver; about twelve inches from the pylorus. It has been found that if the cannula was placed close to the pylorus, there was (a) possibility of spasm of the pyloric sphincter, leading to complete obstruction, and (b) difficulty in passing the biopsy tube past the liver flexure.

An incision was made into the lumen of the duodenum between the bowel clamps, a sample of mucosa removed with scissors, and the flange (G) of the cannula introduced with the tube (F) pointing cranially. A purse-string suture of 2/0 catgut was then placed in the gut wall around the tube of the cannula, and was tightened while every attempt was made to turn the mucous membrane inwards beneath the suture, this, however, was made difficult by the large size of the cannula in comparison with the size of the gut lumen.

The washer (H) was then pushed down the tube (F) until it lay

snugly over the flange and the gut wall. It was then sealed in position using a hot soldering iron. The gut was not stitched to the fenestrations of this washer, since there was some evidence that this could cause obstruction. The tube (F) was then exteriorised through a stab-wound just above the mid-line and behind the last rib. Particular care was taken to ensure that this wound was not made too large, giving a loose fit, and resulting in leakage. The outer washer (I) was then sealed in position in the same way as (H).

The flank incision was closed using continuous catgut (Grade 2) suture for the peritoneum and muscle layers, and continuous nylon mattress suture for the skin.

Post-operative complications were obstruction and diarrhoea. The former was fatal, but the latter usually responded to treatment with antibiotics and fluid therapy. Skin sutures were removed after ten days, and samples were taken from this time onwards.

The calves were fed as follows:-

1st and 2nd day	-	colostrum	3 feeds of 2 pints (approx)
2nd - 7th day	-	whole milk	3 feeds of 2 pints
7th - 12th day	-	equal parts of whole milk and 'Gold Top' milk substitute (B.O.C.M.)	
12th day to weaning	-	6 pints of 'Gold Top' milk substitute	per day in 2 feeds of 3 pints.

Hay, water and 'Calf-wena' pencils (B.O.C.M.) were freely available from the fifth day onwards. Weaning was carried out

at six weeks (calf H), seven weeks (calf F) and ten weeks (calf B), by reducing the intake of milk substitute by one pint per day.

(e) Sampling technique.

Using the angled cannula described, together with the more flexible wire in the modified biopsy tube, little difficulty was experienced in passing the tube into the lumen of the small intestine. The tube was marked at intervals to enable the position to be accurately determined, although owing to the flexible and elastic nature of the gut, some variation in true position probably occurred.

When the capsule was lying at the required site, a negative pressure of two inches of mercury was applied and the knife operated. Preliminary trials on post-mortem material had indicated that pressures greater than three inches of mercury could lead to perforation of the gut of a week-old calf when the knife was operated. The tube was then withdrawn, and the sample removed from the capsule and transferred to filter paper.

Samples were routinely obtained from six inches, two feet and four feet below the site of the cannula, although it was possible to obtain samples from up to six feet.

The samples obtained were washed in cold distilled water, dried between pieces of filter paper, and weighed on microscope slides. These were then stored in the deep-freeze for up to one week before enzyme determinations were carried out.

After use, the biopsy capsule and knife were washed and stored in surgical spirit. The tube was flushed with water, then with surgical spirit, and finally, air was blown through. The wire was withdrawn as far as possible from the tube, dried, and lubricated with siliconised stopcock grease (Edwards High Vacuum Ltd). The outside of the tube was then washed and cleaned in cetrimide (Cetavlon, I.C.I.),

(iii) Biochemical determination of alkaline phosphatase.

Alkaline phosphatase activity was estimated using the method described by King and Wootton (1964). Phenol is released by the enzyme from disodium phenyl phosphate under defined conditions of time, temperature and pH, and is measured colorimetrically. A coloured compound is formed when phenol reacts with 4-amino antipyrine (Emerson, 1943; Powell and Smith, 1954). The material for analysis was prepared as described by Moog (1950) and magnesium chloride (0.01M) added as an activator (Erdtman, 1928).

The material obtained from the slaughterhouse was removed from the deep-freeze, allowed to thaw, and a sample removed and weighed. Biopsy specimens which had been weighed before freezing were simply allowed to thaw.

The samples from either source were then washed individually with 5 ml of iced distilled water into an all-glass homogeniser (Fisons Scientific Instruments) in which they were ground for two minutes at moderate speed. Some of the earlier slaughterhouse

samples were ground in an M.S.E. metal/glass homogeniser for three minutes.

The tubes of homogenate were allowed to stand in the refrigerator for at least one hour. Dilutions were then made in some cases where the amount of material present was greater than about 5 mg per ml in the case of calves, and 25 mg per ml for adults.

A test and control tube were assigned to each sample, and a standard and blank to each batch. Standard strength = $1 \text{ mg}_{\text{phenol}} / 100 \text{ mls.}$

After incubation ^{for 15 mins} and development of colour using 4-amino antipyrine, the tubes were compared at 510 m μ in an E.E.L. Spectra colorimeter. The enzyme activity was measured in King/Armstrong units (m μ gs of phenol released by one gram of tissue under the conditions of the experiment) and is given by:-

$$\frac{\text{Test - Control}}{\text{Standard - Blank}} \times \frac{100}{1} \times \frac{\text{Vol. of Homogenate} \times \text{Dilution}}{\text{Weight of Tissue}}$$

(iv) Histochemical localisation of alkaline phosphatase.

Material was obtained from the slaughterhouse or by biopsy from the living animal. Fixation was carried out by immersion for four hours in 10% formalin, after which sections were cut. This was done using a freezing microtome, which cut sections of 15-30 μ . The sections were transferred to microscope slides, and allowed to dry on the slide for two hours after which they were stained.

The staining method was Gomori's calcium-cobalt technique as

described by Gurr (1958). The method depends on the liberation of phosphate from sodium β glycerophosphate substrate in the presence of magnesium ions. The phosphate reacts with calcium chloride to form calcium phosphate. This is localised by converting it, first to calcium cobalt, and then to cobalt sulphide. The last product is seen as a fine granular precipitate.

RESULTS.A. Alkaline phosphatase in tissue obtained from the abattoir.

The results of these experiments are shown in Table 2. It appeared that:-

(i) The alkaline phosphatase activity of the intestinal mucosa was at least six times as great as that of outer muscle taken from the same site.

(ii) When the alkaline phosphatase activity per gram of tissue was determined in large samples (150 mg) and compared with that in small samples (20 mg) the activity in ten adult samples was lower in the larger samples. The opposite was found in two samples taken from calves.

(iii) The addition of magnesium ions (0.01M) appeared to increase the amount of phenol released in seven out of ten experiments.

(iv) Storage of tissue in the deep-freeze for up to one week had little significant effect on enzyme activity.

Results obtained by analysis of abattoir material are shown in Table 3. There is considerable variation between individual samples, and analysis using the chi-squared test showed that the distribution in any one column differed significantly from a theoretical normal distribution. This being the case, the results could not be analysed using a parametric technique, and so the Mann-Whitney U-test (Siegel, 1956) was used. This test makes no prior assumption about the distribution of the parent population, and so was well suited to this situation. Significance was attributed at levels of greater than 95% probability ($P < 0.05$).

A comparison was made (Fig. 21) between levels of alkaline phosphatase activity in corresponding regions of the gut of calves and adults, and also between levels in adjacent regions of the same animal.

The comparison between corresponding regions in the two age groups showed that, in comparison with the adult, the calf had a higher level of activity in the duodenum ($P < 0.01$), in the jejunum ($P < 0.001$), midway between pylorus and ileo-caecal valve ($P < 0.05$), and in the distal ileum ($P < 0.001$). There was no significant difference, however, between the levels of activity in the colons of the two age groups ($P > 0.05$).

The comparison between adjacent regions within each individual age group showed, in both cases, that the change in activity between duodenum and jejunum was not significant ($P > 0.05$ in both cases). There was, however, a significant fall in activity between the jejunum and a point midway between pylorus and ileo-caecal valve in both the calf ($P < 0.05$) and adult ($P < 0.01$). This was also found to be the case when the level of activity midway between the pylorus and the ileo-caecal valve was compared with the distal ileum in both the calf ($P < 0.01$) and the adult ($P < 0.05$).

The alkaline phosphatase activity in the distal ileum was compared with that of the colon and it was seen that the age groups differed. In the calf there was a significant fall in activity ($P < 0.01$) while in the adult there was no significant difference ($P > 0.05$).

B. Tissue obtained from the living animal.

Three calves, B, H and F were sampled at intervals during the first eighteen weeks of life, and a fourth calf, I, was sampled over the first four weeks. With the exception of the mucosal sample removed from the duodenum during cannulation, all the other samples were obtained with the biopsy capsule. The biopsy technique gave adequate amounts (6-12 mg) of tissue for alkaline phosphatase estimation, although as little as 1 mg could give a meaningful result.

The results obtained were plotted graphically (Figs. 22, 23, 24 & 25). In all four animals there was a rise in alkaline phosphatase activity in the duodenal mucosa in the first two weeks of life, although this was particularly clearly shown by calf F. It is noticeable, however, that the levels reached in calf B were considerably lower than in the other three calves. This rise could not be demonstrated at the lower levels which were sampled (2 feet and 4 feet from the cannula) since the initial samples could not be obtained within the first two weeks. After two weeks of age, there was a fall in activity, in each animal, and at all three sites.

At about three-and-a-half months of age, a secondary rise in alkaline phosphatase activity was seen in both duodenal and jejunal samples, but more clearly in the latter. This appeared to be of short duration.

Weaning took place at six to eight weeks in calves H and F, and at ten weeks in calf B. The rise in activity seen in some

sites was unlikely to have been significant, since it was not seen in others.

Castration, which was carried out on calf F at eleven weeks of age, appeared to have no effect on alkaline phosphatase levels.

C. Histochemistry.

The results of a limited number of experiments carried out on tissue from various parts of the intestinal tract of calves and adults confirmed the expected distribution of alkaline phosphatase. This was found to be mainly situated along the luminal border of the columnar epithelial cells as had been described by Renzoni et al (1957). The distribution was the same in calf and adult material, although the former as expected, gave a more intense staining reaction.

DISCUSSION.1. Variability of results obtained.

Variation was encountered in the alkaline phosphatase activity at a particular site in the gut in different animals and in the same animal on different occasions.

Variation between individual animals had been reported by Kolb and Schulz (1959) who examined adult animals using a similar technique to that used in the present investigation. In both cases, material was obtained from the slaughterhouse, and there were probably considerable differences in the treatment of animals prior to slaughter. In particular, it has been shown in rats that fasting can lead to lowered levels of mucosal alkaline phosphatase activity (Weil and Russell, 1940; Flock and Bollman, 1948; Tuba and Robinson, 1953), although it might be expected that the large capacity of the adult rumen might offset some of the effects of fasting. However, in the present experiments it appeared that the calves, despite their under-developed rumens (Godfrey, 1961) showed no more individual variation than did the adults.

Variation was found between consecutive samples of mucosa taken from the same site at intervals of two to seven days. This occurred in spite of care taken to minimise any dietary changes (Hough and Freeman, 1942) and to remove samples at the same time of day to avoid diurnal variation (Tuba and Robinson, 1953).

The expression of enzyme activity in terms of tissue wet weight may have led to some inaccuracy, although it has been stated (Sereni and Principi, 1965) that this may give results of greater physiological significance than if expressed in terms of units per gram of protein or nitrogen. In the present investigation the biopsy sample size was too small to allow analysis other than in terms of wet weight.

Another possible source of error may have resulted from the superficial distribution of alkaline phosphatase along the luminal surface of the mucosa, leading to low results in larger samples which contained more inactive material. However, no correlation could be seen between size of biopsy sample and the resulting activity per gram.

It has been shown (Tuba and Robinson, 1953) that mucosal alkaline phosphatase responds quite rapidly to a change in diet. These workers found in rats that a peak of phosphatase activity occurred eight hours after feeding of fat. With an enzyme which can adapt so rapidly, it is, perhaps, not surprising that variations were seen between samples taken at intervals of two days.

2. Results obtained from examination of slaughterhouse material.

(a) Regional distribution of alkaline phosphatase in adult and calf.

The results from adult material confirm the general findings of Kolb and Schulz (1959). These authors examined nine adult cattle, and found a rise in activity between duodenum and

jejunum, and a fall along the rest of the alimentary tract, but they did not analyse their results statistically. Applying the Mann-Whitney U-test (Siegel, 1956) to their results, it was found that the only change attaining statistical significance ($P < 0.05$) was that between upper and middle jejunum. However, it appeared that greater numbers of animals should give more definite results. The present results using larger numbers of animals still fail to attribute significance to the rise in activity between duodenum and jejunum, but do show a significant fall along the rest of the small intestine.

This gradient of activity is similar to that described by Moog (1961) in the adult mouse, and Koldovsky et al (1963) in the adult rat. It is exactly opposite, however, to that described by Kolb and Wujanz (1958) in the adult horse, and Kolb and Schutz (1961) in the adult pig; these workers produced results which suggest, although this is not statistically verifiable, that the gradient in these animals rises along the gut from duodenum to ileum. In view of the high levels in the adult bovine rumen and in the adult equine colon (Kolb and Schutz, 1961) both of which are known to be sites of active absorption, and also the other circumstantial evidence, it does not seem unreasonable to suggest that the gradients in alkaline phosphatase activity represent gradients in absorptive capacity. There is no real evidence to indicate the substrate which is involved, although this may involve fat or the volatile fatty acids, since there is a clear indication of a link between fat feeding and mucosal levels of alkaline phosphatase

in the rat (Weil and Russell, 1940; Hough and Freeman, 1942; Flock and Bollman, 1948) and in man (Blomstrand et al, 1965).

The distribution of the enzyme in the alimentary tract of the calf is quite different from that described by Moog (1961) in the young mouse, and Koldovsky et al (1963) in the young rat, since in both of these species the young animal has the same level of phosphatase activity throughout the intestine. In the calf, the fall is, if anything, more abrupt than in the adult (Fig.21). The gradient in the calf shows no suggestion of a rise from duodenum to jejunum as is seen in the adult, although in neither case is the change statistically significant.

All the other changes along the calf intestine are significant, and this includes the fall in activity between distal ileum and colon, which is quite insignificant in the adult.

(b) Comparison of the levels of alkaline phosphatase activity at corresponding sites in calf and adult intestine.

It was found that, with the exception of the colon, there was a significantly higher level of alkaline phosphatase activity in each site in the calf gut that was compared with the corresponding region in the adult (Fig.21).

These results are in keeping with the observations which have been made on serum alkaline phosphatase levels in cattle (Allcroft and Folley, 1941; Kwiatkowski, 1964) and in cattle, sheep and camels (Vertar and Swaton, 1965). These investigations showed a higher level of activity in the serum of the young animal, and it

was therefore to be expected that there should be a higher level of activity in the intestinal mucosa, since Madsen and Tuba (1952) showed that the greater part of the serum phosphatase in the rat is derived from the intestinal mucosa. There is also evidence for a higher level of mucosal alkaline phosphatase activity in the young of other species such as the guinea-pig (Moog and Ortiz, 1960) and man (Fomina, 1956), so that in this respect, the findings are similar in the calf.

In the absence of a clear role for alkaline phosphatase, the interpretation of the above findings is necessarily difficult. It does seem, however, that the higher levels in the young animal are representative of a difference in the absorptive apparatus in the two age groups. This may simply be a matter of diet, or may involve a more fundamental difference.

3. Results obtained from biopsy material.

The technique of intestinal mucosal biopsy has been shown to be applicable to ruminants. It is a potentially valuable research tool in the investigation of normal and pathological intestinal mucosa of farm animals, and tissue obtained in this way may be subjected to biochemical and histological examination.

The results obtained from duodenal biopsies in all four animals showed a rise in activity during the period of cessation of antibody absorption which, in the calf, is twenty-four to forty-eight hours after birth (Deutsch and Smith, 1957). The level continues to rise for some days after this time, but in general the findings

agree with those of Halliday (1959) in the rat, and Payne and Marsh (1962) in the pig, which suggested a possible connection between the two events.

The results obtained from the jejunum were less complete in that the samples could not be obtained before about two weeks of age. They confirm, however, that a fall occurs after the age of two weeks. This fall coincides with the time when the animal is taking an increasing amount of solid food, and so may be a reflection of dietary changes. This is not supported, however, by the relatively insignificant effect which weaning appeared to cause in all the animals examined. This suggests that the rise which has been observed at weaning time in the rat (Halliday, 1959) and in the mouse (Moog, 1962) are less likely to be associated with weaning, than with some other phenomenon which occurs at about the same time in these species, in particular, antibody blockade.

The delayed rise in activity which occurred at around three-and-a-half months of age was unexpected, and difficult to interpret. It seems possible that this peak of activity may correspond to that occurring in the rat at one-and-a-half months (Brodskaa, 1961) and in the rabbit at two months (Brodskaa, 1961; Willenbockel, 1961). These correspond to a time approximately half way between birth and puberty in the two species (Brodskaa, 1961) and it is noticeable that the calves were at approximately this same stage when the second peak occurred, and, moreover, that the peak is less clearly shown in the castrated animal (F).

Castration appeared to have little effect on the mucosal

alkaline phosphatase activity. This is in contrast with the findings of Tuba et al (1949) who showed in rats, a 30% fall in serum alkaline phosphatase activity in the eight weeks following castration. They also showed, however, that this could be attributed to lowered food intake.

Another factor which must be considered in the interpretation of results using the method described, is that changes in enzyme activity against disodium phenyl phosphate as a substrate, may not be paralleled by those against other substrates. This has been shown to be the case in the mouse, where Moog (1961) showed that the rise in the alkaline phosphatase activity in the weanling mouse is more marked with disodium phenyl phosphate than with β -glycerophosphate as a substrate. This is possibly a reflection of the heterogeneity of 'alkaline phosphatase', and this point is an added complication in interpretation.

4. Conclusion.

Despite reservations concerning possible heterogeneity of alkaline phosphatase, and the lack of a clear role for the enzyme which may be a result of heterogeneity, it is nevertheless possible to draw certain conclusions from these present experiments.

1. Mucosal biopsy is a practical and potentially valuable technique for intestinal investigation in the bovine.
2. The high levels of alkaline phosphatase which have been shown to exist in the young calf probably indicate that the

absorptive apparatus differs from that found in the adult, and that this difference may be a result of different diets.

3. The first few days of life in the calf are associated with considerable changes in enzyme activity, and so, by inference, with absorptive processes. The connection between this enzyme and cessation of antibody absorption was supported by the present results.

TABLE 2

(i) (ii) (iii) (iv)

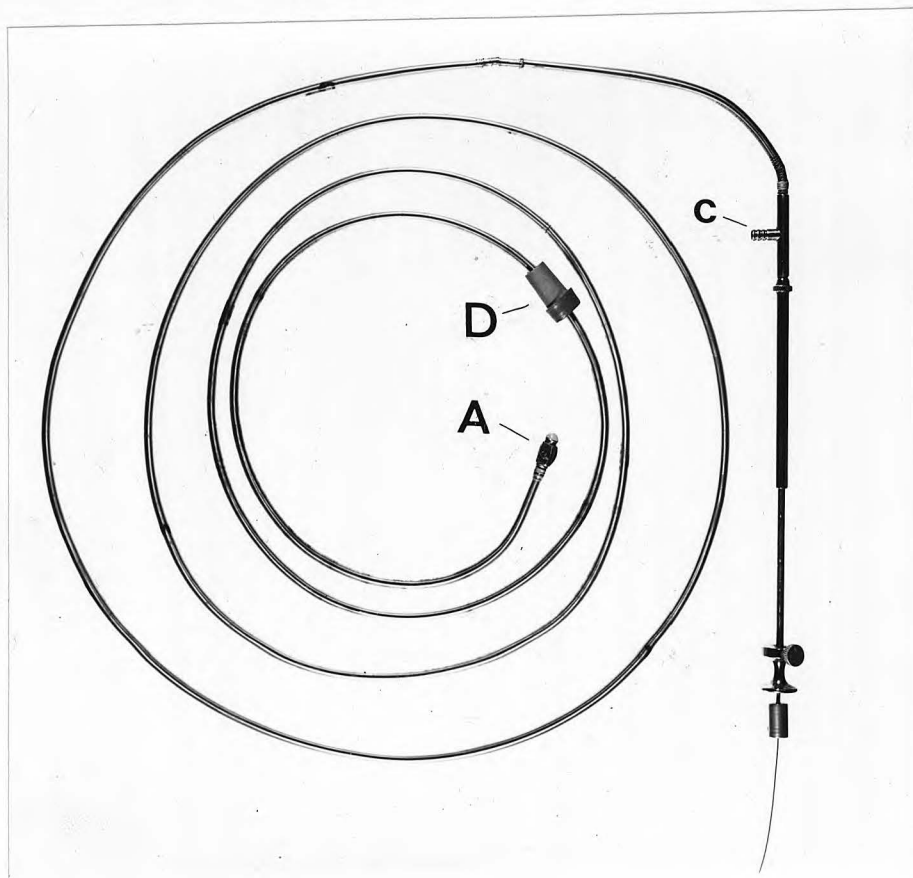
Phosphatase Activity in outer muscle and in mucosa		Effect of size of sample on Phosphatase Activity		Effect of Addition of Mg + ions (0.01M)		Effect of Storage for 7 days deep frozen	
MUCOSA	OUTER MUSCLE	SMALL	LARGE	WITH MG IONS	WITHOUT MG IONS	BEFORE	AFTER
(Adult)		(Adult)		(Adult)		(Adult)	
3.4	0.3	3.4	2.7	0.4	0	7.1	6.1
4.6	0.2	4.6	3.0	0.1	0.2	0	1.7
11.1	0.3	11.1	6.0	0.2	0.1	0	1.8
6.1	0.1	6.1	4.8	(Calf)		11.0	11.1
1.7	0.2	1.7	1.2	238	178	(Calf)	
1.8	0.3	1.8	1.1	120	104	109	123
6.2	0	6.2	2.9	106	84	143	148
13.1	0	13.1	1.7	123	100	177	175
11.1	0	7.1	6.5	245	205	51	48
7.1	0	11.1	0	132	163		
		(Calf)		137	121		
		50.1	109.0				
		140.0	177.0				

TABLE 3

Alkaline phosphatase activity in King Armstrong units per gram of
mucosa determined in post-mortem material

DUODENUM		UPPER JEJUNUM		MID-PYLORUS ILEOCAECAI VALVE		DISTAL ILEUM		PROXIMAL SPIRAL COLON	
Calf	Adult	Calf	Adult	Calf	Adult	Calf	Adult	Calf	Adult
75.4	3.4	147.7	7.0	115.7	0	23.2	11.6	1.5	1.5
116.6	3.5	144.3	24.6	151.6	20.1	0	0.2	0.2	0.7
53.8	3.5	191.9	93.9	29.0	5.9	25.9	2.5	0	1.8
221.3	0	51.9	48.6	11.5	3.5	12.9	0.4	0	0
61.6	40.1	18.4	34.4	63.0	6.3	7.9	1.1	5.2	1.2
28.7	14.5	123.4	8.7	12.2	11.6	3.9	6.2	4.2	0
12.0	1.9	39.0	26.4	98.3	10.3	11.9	0.9	0	0
139.2	8.7	50.0	19.7	11.5	2.0	9.4	0.5	14.7	1.3
26.4	2.5	90.1	1.3	31.4	8.4	16.2	0	2.9	0.9
40.0	8.1	158.5	3.5	1.8	2.7	3.4	2.4	3.0	0.8
120.2	21.6	58.3	38.8	10.6	11.6	8.6	0.5	1.9	2.9
211.0	63.2	73.7	37.2	40.3	4.4	23.1	1.2	3.0	7.8
364.1	11.8	8.7	17.8	33.6	9.8	11.9	1.2	10.7	3.9
88.5	22.3	80.6	22.4	100.0	7.6	6.0	19.4	1.3	0
	16.5	119.5			2.7	15.5	1.6	1.0	0
	14.5				33.3			9.8	0

(a)



(b)

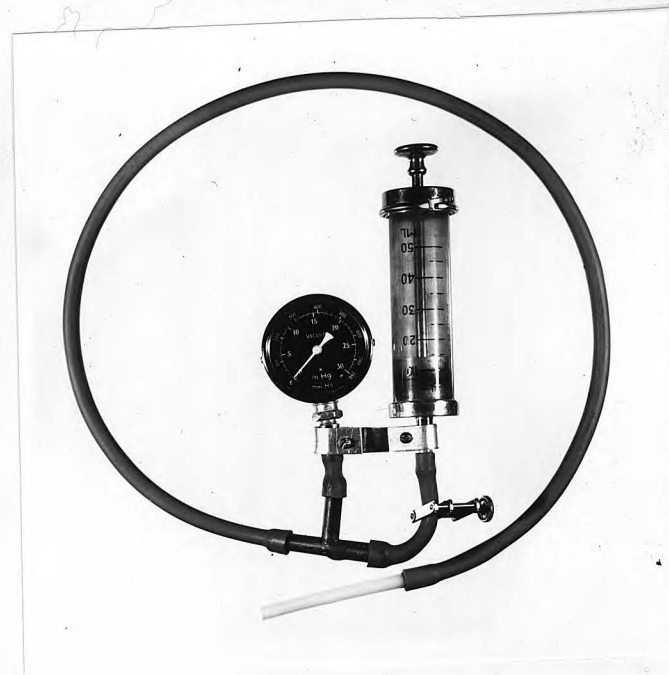


Fig.18 . (a) Modified Shiner biopsy capsule and tube.
(b) Syringe and vacuum gauge.

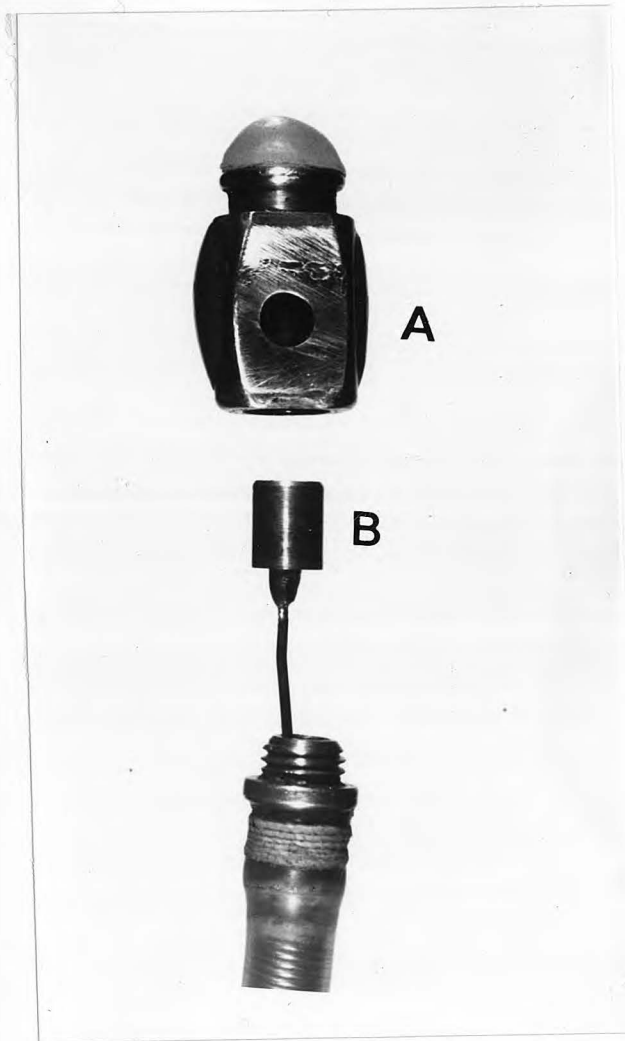


Fig.19 . Biopsy capsule dismantled to show knife cylinder and operating wire.

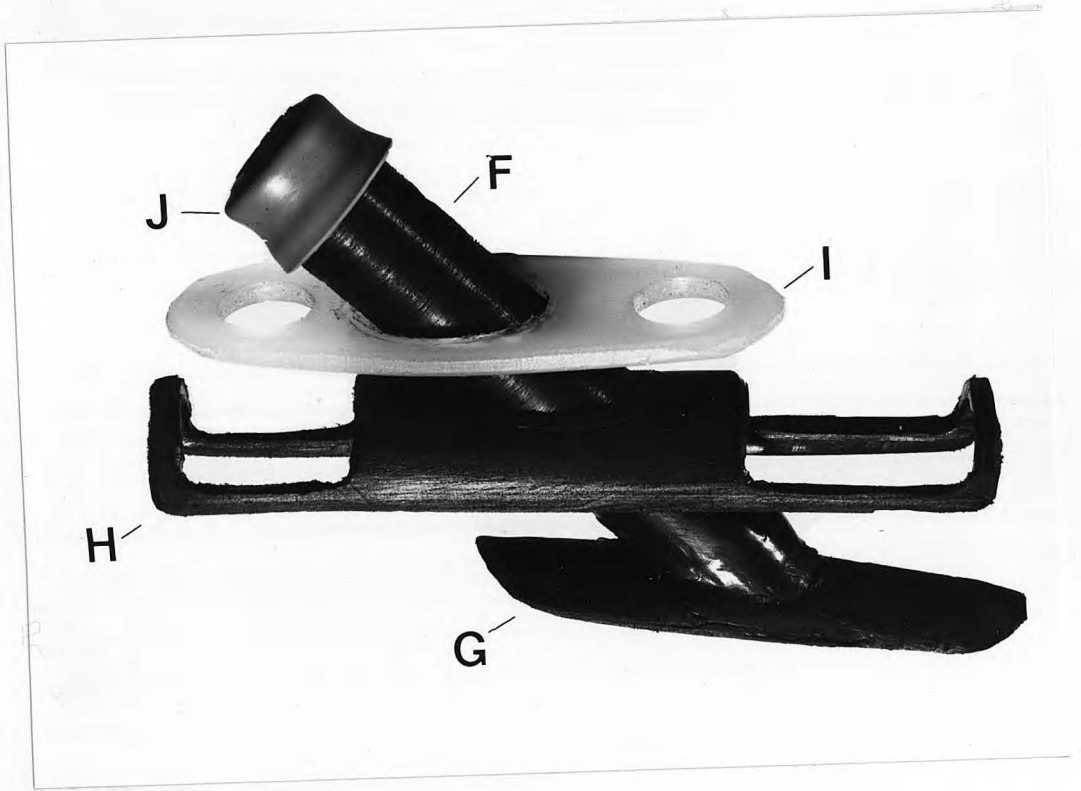


Fig.20. Polythene cannula.

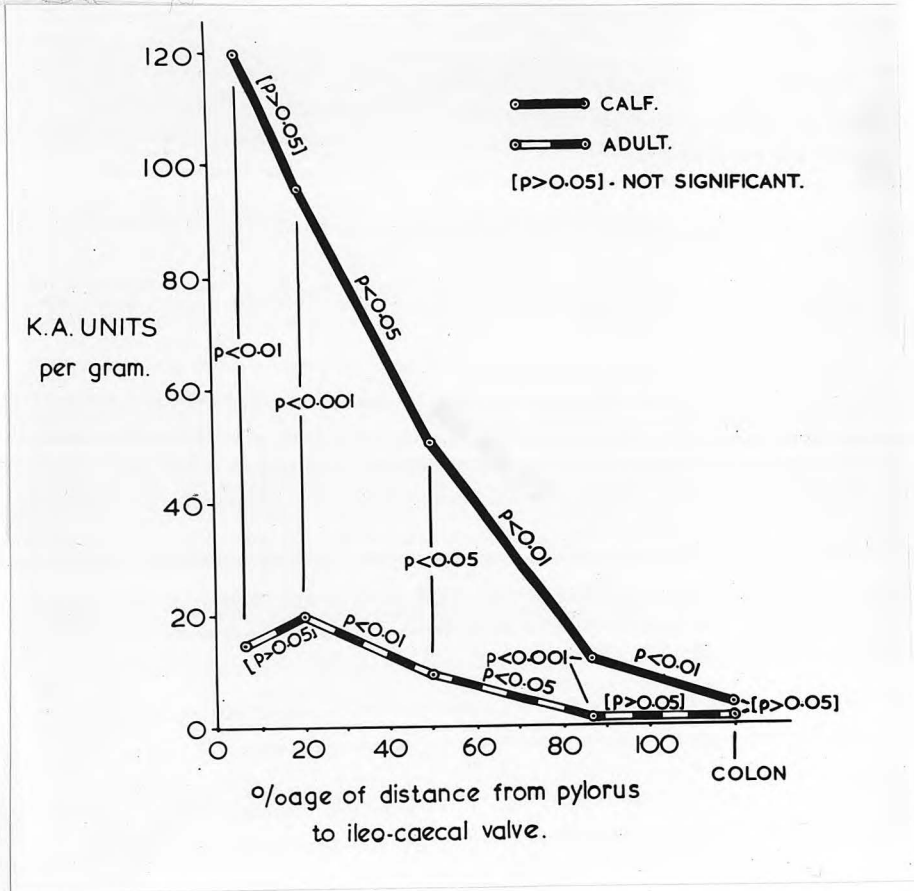
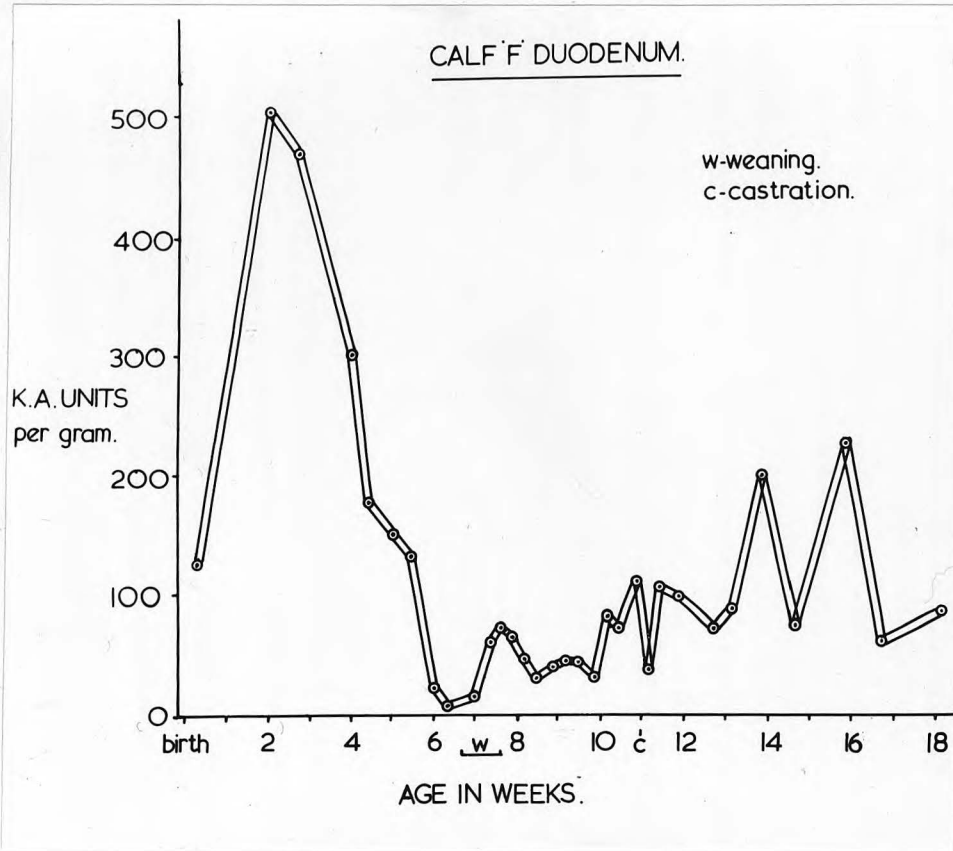


Fig. 21. Mucosal alkaline phosphatase activity determined in material from slaughtered calves and adult cattle. Each point represents the mean of at least fourteen determinations.

(a)



(b)

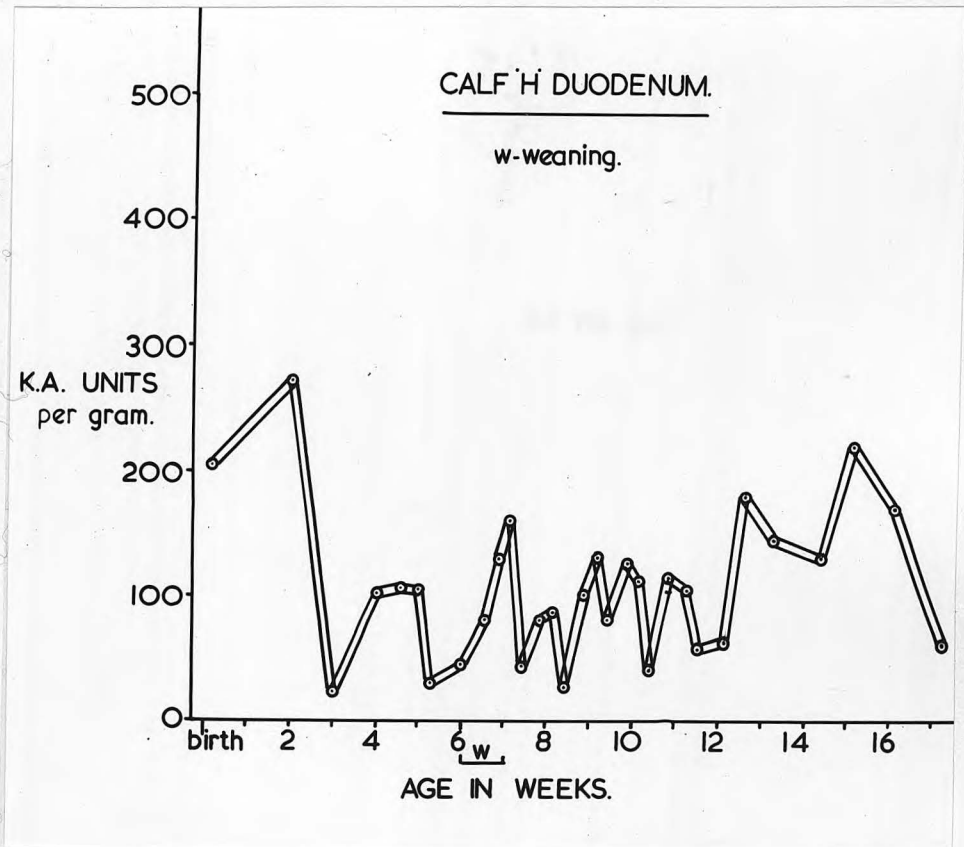


Fig.22. Mucosal alkaline phosphatase determined in biopsy samples from the duodenum of calf F (graph a) and calf H (graph b). Each point represents the result of an estimation carried out on a single biopsy specimen.

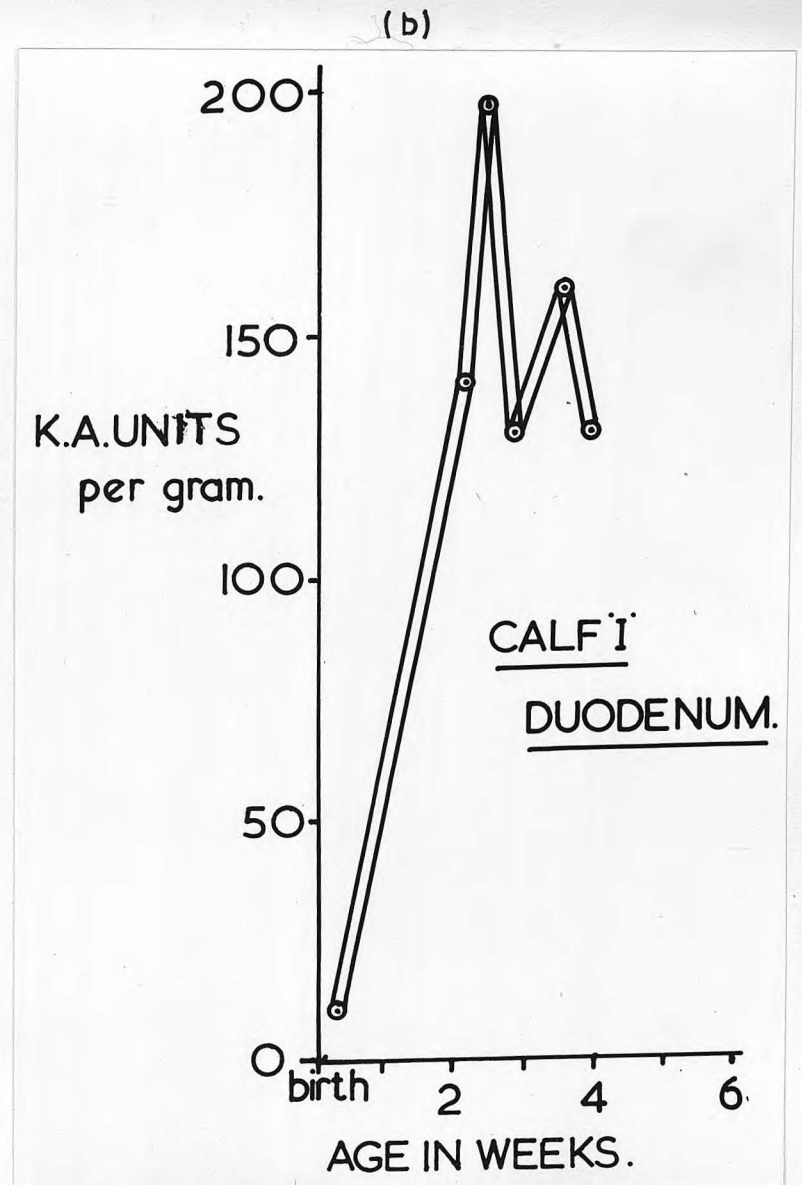
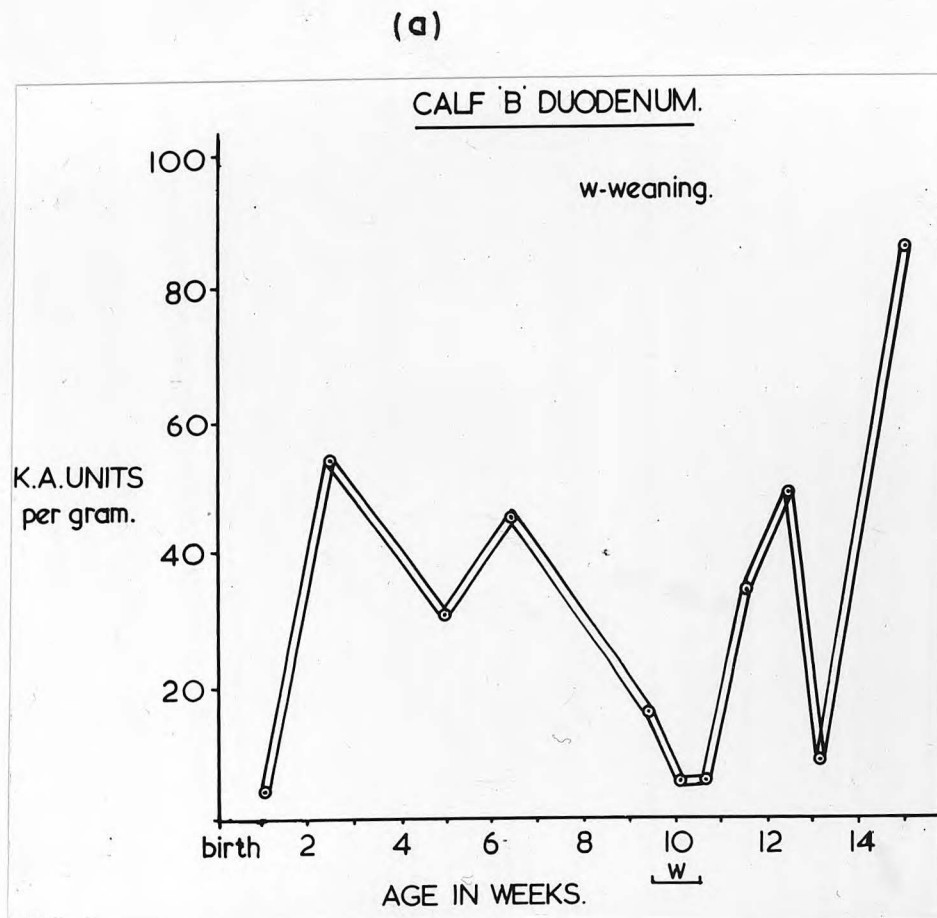
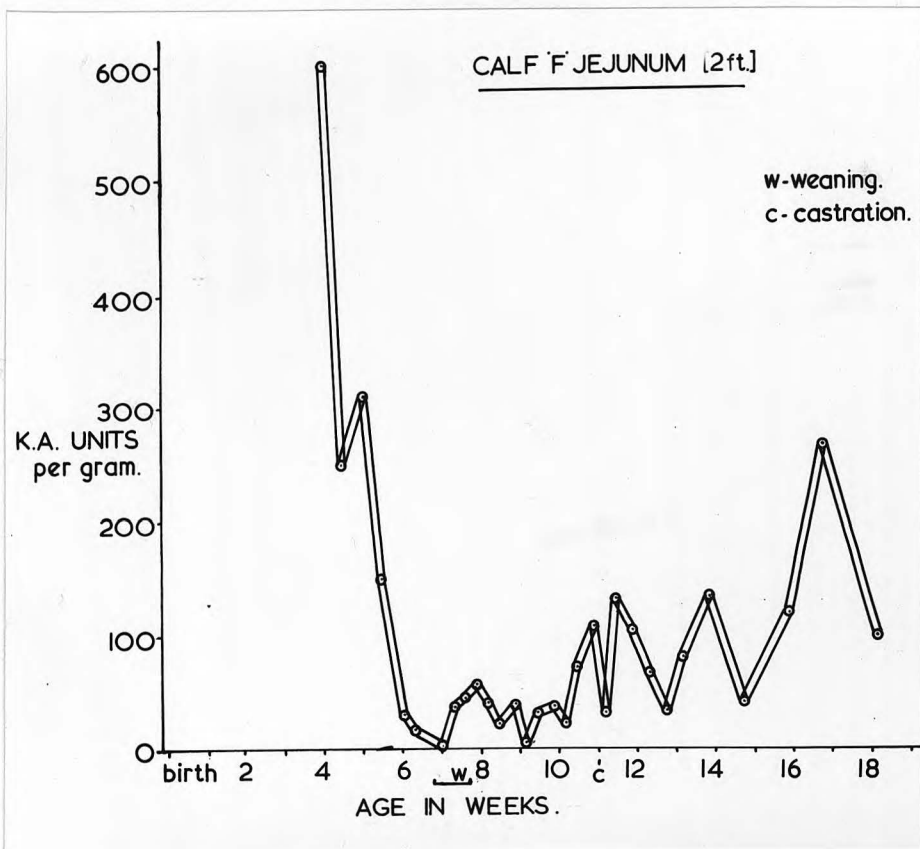


Fig.23. As Fig.22. Biopsy samples taken from the duodenum of calf B (graph a) and calf I (graph b).

(a)



(b)

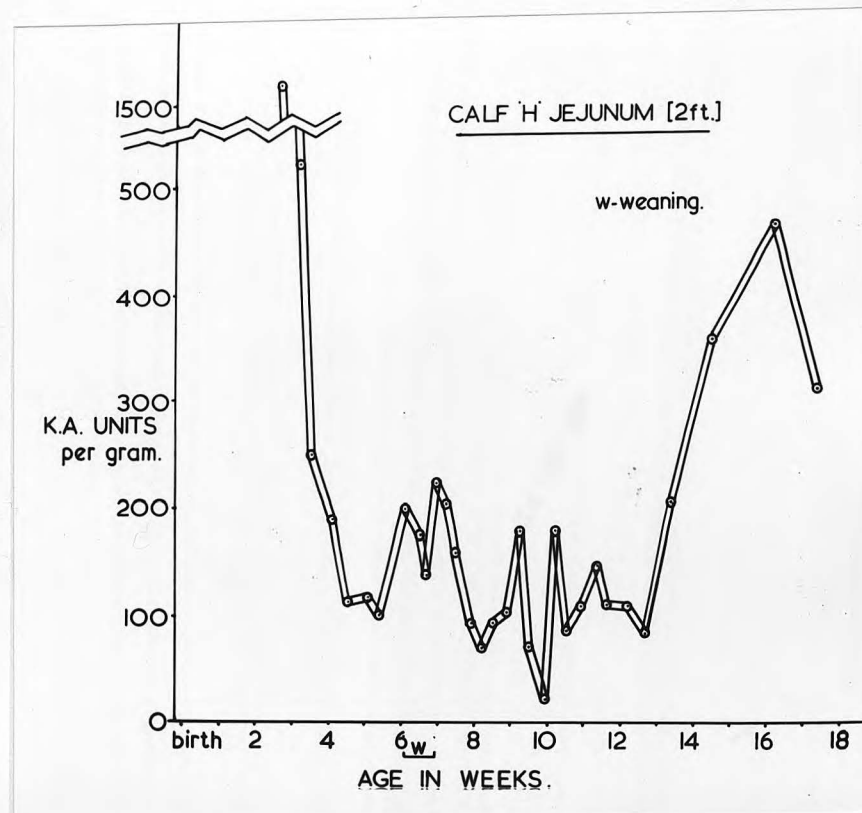
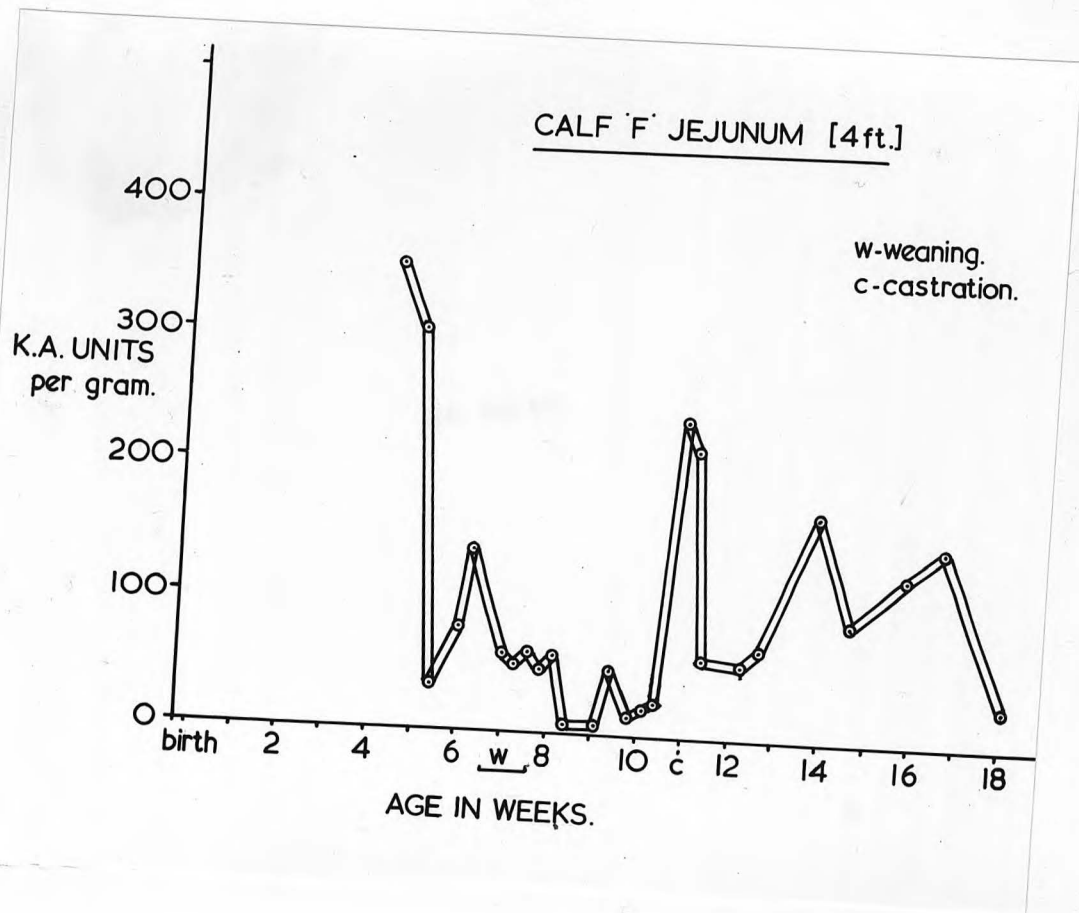


Fig.24. Mucosal alkaline phosphatase activity determined in biopsy samples taken from a site 2 ft from the cannula in calf F (graph a) and calf H (graph b). Each point represents a single biopsy sample.

(a)



(b)

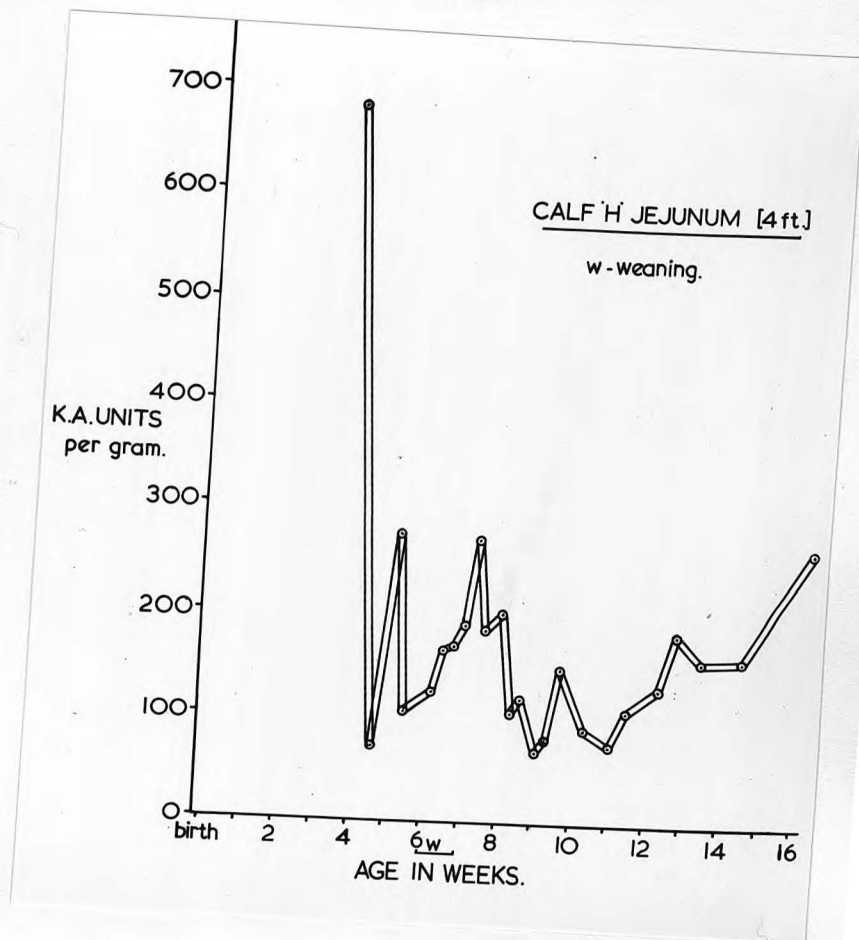


Fig.25. As Fig.24. Biopsy samples taken from a site 4 ft from cannula in calf F (graph a) and calf H (graph b).

S U M M A R Y

1. Isolated strips of intestinal plain muscle from calves showed a greater rate of spontaneous activity than those from adults.
2. Responses to drugs in vitro were similar in muscle strips taken from calf and adult intestine, and were generally similar to the responses seen in material from laboratory animals.
3. Drugs behaving unusually were: histamine, which in some preparations gave a biphasic response; bradykinin, which gave a biphasic response in all preparations; and certain drugs which are alleged to stimulate ganglia, which were not blocked by those which are said to block ganglia.
4. Responses to drugs were similar in strips taken from different regions of the gut, although the colon responded atypically to bradykinin and 5-HT.
5. The responses observed in vivo confirmed those seen in vitro, with the exception of acetylcholine, which was inhibitory in small doses when tested in vivo.

6. Alkaline phosphatase activity was higher in the small intestinal mucosa of the week-old calf than in corresponding regions of the adult. The levels in the colon of the two age groups were not significantly different.

7. Mucosal biopsies showed that alkaline phosphatase activity in calf intestinal mucosa increased during the first two weeks of life, and then declined. A second rise was seen at three-and-a-half months of age.

8. It was suggested that the changes in alkaline phosphatase activity imply changes in absorptive and digestive mechanisms during post-natal development in the calf.

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