(cf. Needham, 1942) suggested that the primary evocator, upon which the differentiation of the embryo depends, is a steroid substance of some kind. They also proposed that this substance was present in a masked condition in the tissues, the active steroid presumably being liberated at the right time and place to cause differentiation. It is tempting to suggest that β -glucuronidase acting on a steroid glucuronide could provide the actual mechanism of this change. It is, of course, dangerous to assume that the measured activity of the enzyme in the crude extracts bears any relation to the activity in the intact embryos. Possibly, β -glucuronidase is best considered as analogous to enzymes such as cathepsin. Such enzymes, showing high activity in tissue extracts, may not be very active in the living tissues. In this connexion it may be noted that de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have demonstrated that in certain tissues cathepsin and β -glucuronidase are associated in one type of cytoplasmic particle, which releases active enzymes after the tissues are homogenized.

SUMMARY

1. The β -glucuronidase activity of extracts from various stages of *Xenopus laevis* embryos has been measured.

2. The only significant change detected was a fall in the activity of the enzyme in the extracts of the neural plates.

3. These results do not support the idea that β -glucuronidase is associated with the proliferation of cells.

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The Phosphoesterase of Pea Plants (*Pisum sativum* L.)

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A number of polyphosphatase activities present in extracts of pea plants (*Pisum sativum* L.) are associated with an unspecified phosphoesterase (Pierpoint, 1957). This enzyme liberates orthophosphate from trimetaphosphate, tripolyphosphate, commercial 'hexametaphosphate' and a number of organic phosphates, although not from tetrametaphosphate. Chromatography of the enzyme on an ion-exchange resin resolves it into two fractions which have similar enzymic activities.

Two problems arising out of this work were the heterogeneity of the enzyme, and the association of the phosphomonoesterase activity with the diesterase activity against trimetaphosphate. The present paper presents evidence that the heterogeneity of the enzyme is not due to the 'purification' procedure to which it is subjected, and confirms the association of the mono- and di-esterase activities. It also provides additional information on the specificity of the enzyme, and the path by which it breaks down polyphosphates.

EXPERIMENTAL

Enzyme preparations. The partially purified preparation of pea phosphatase most generally used was the fraction E of Holden & Pirie (1955a). More purified preparations were obtained from this by gradient-elution chromatography on a column of Dowex 50 (Pierpoint, 1957).

Enzyme determinations. Ribonuclease activity was measured against yeast nucleic acid by the method of Holden & Pirie (1955a). All other phosphatase activities were measured under the conditions previously described (Pierpoint, 1957).

Phosphatase activities were generally expressed as units which were defined as that activity which would liberate 31 mg. of P as orthophosphate/l. of solution, when incubated for 1 hr. at 37° under the standard conditions. Since the liberation of orthophosphate did not vary in a linear manner with amount of enzyme present (Pierpoint, 1957), the assays were performed with various amounts of enzyme, and the amount containing 1 unit of activity was measured graphically. The deviation from linearity was far greater with trimetaphosphate than for the other phosphates, and the activity against this compound was more accurately measured by a smaller unit. Consequently, the enzyme activities measured in the experiments summarized in Tables 1-3 were measured in 'half-units' corresponding to the liberation of 15.5 mg. of orthophosphate/l. under the standard conditions.

Chemical determinations. Orthophosphate and total phosphate were both estimated in the manner described by Holden & Pirie (1955c).

Chemicals. Yeast nucleic acid was prepared by the method of Holden & Pirie (1955b). The pyrophosphate was a mineral sample, given by Dr G. Brown which on analysis by ion-exchange chromatography was found to be at least 97% calcium pyrophosphate. The mixtures of 2'- and 3'-adenylic acids, and one of 2'- and 3'-uridylic acids, were given by Dr R. Markham. Guanidinium tetraphosphate was given by Dr O. T. Quimby.

Calcium phosphate was a 3-year-old specimen that had been made by the method of Singer & Kearney (1950). All the other compounds used, which were not obtained commercially, have been previously described (Pierpoint, 1957).

Chromatography. Chromatographic separation of polyphosphates was carried out on a column (14 cm. $\times$ 2.5 cm.) of Dowex 1 (100-200 mesh; $\times$ 8 cross-linkage) in the manner described by Peters & Rieman (1956). The eluents used in this method are (1) 0.25 M-KCl, (2) 0.4 M-KCl, (3) 0.5 M-KCl, all buffered to pH 5 with 0.005 M-acetate buffer and (4) 3 N-HCl, which is allowed to stand overnight in contact with the resin in order to hydrolyse any firmly absorbed polyphosphates. The automatic fraction collector used collected effluent samples of 10.1 ± 1.5 ml.

Paper chromatography of nucleotides was carried out in the propan-2-ol-aq. NH_3 soln. solvent of Markham & Smith (1952a), on strips of Whatman 3 MM filter paper. Purine- and pyrimidine-containing compounds were located on the chromatograms by ultraviolet-light photography (Markham & Smith, 1949, 1951).

Electrophoresis. Protein electrophoresis was carried out on paper strips in a manner similar to that described by Woods & Gillespie (1952). The strips of Whatman 3 MM paper (57 cm. $\times$ 2.5 cm.) were wetted with the appropriate buffer of ionic strength 0.05, and samples of the enzyme

fraction E, containing 2-6 units of glycerophosphatase in 20-60 $\mu\text{l.}$, applied to their midpoints. They were then laid between strips of glass (30 cm. $\times$ 4 cm.) supported on water-cooled blocks of metal so that the ends of the strips hung down into the buffer contained in plastic-electrode vessels. After equilibration for 1 hr., a voltage of 140 v was applied from a constant-voltage power pack.

When electrophoresis was finished the papers were removed from the apparatus and allowed to dry. They were lightly sprayed with an 8% (w/v) solution of either glycerophosphate or trimetaphosphate in 0.1 M-citrate buffer, pH 6, and stood at room temperature for 2 hr. The position of the two activities could then be detected by spraying with a solution of 1.7% (w/v) ammonium molybdate in 2 N- H_2SO_4 , followed by freshly prepared stannous chloride solution (0.2%).

Electrophoresis on starch gel was carried out in a similar manner. Potato starch was mixed to a thick paste with the appropriate buffer and spread evenly in Perspex troughs (31 cm. $\times$ 3 cm. $\times$ 0.2 cm. deep) which were supported on the water-cooled metal blocks. Contact between the electrode vessels and the gel was made by firmly clamped pieces of 3 MM filter paper. After the enzyme had been applied, the gel was covered with a sheet of glass and left to equilibrate for 1 hr. before the voltage was switched on.

When the electrophoresis was finished, the starch gel was allowed to dry and then divided into successive 1 cm. strips. Each strip was suspended in 5 ml. of water, and the resulting solution divided into two portions for trimetaphosphatase and glycerophosphatase estimations. The conditions of these estimations were the standard ones except that the concentration of citrate was 0.05 M, the concentration of substrate was 0.6 mg. of P/ml. and the incubation time was 5 hr.

RESULTS

Association of glycerophosphatase and trimetaphosphatase

The ratio of the two phosphatase activities was measured in a number of different pea extracts and, as Table 1 shows, was constant within the limits of accuracy of the estimations. This ratio was not appreciably altered by absorption of protein from a solution on to calcium phosphate gel (Table 2), nor by heat denaturation at different pH values (Table 3). These results suggest that the two enzyme activities are associated with the same protein, as also do those results obtained when the enzyme acted on mixtures of the two substrates. At a concentration of glycerophosphate (0.6 mg. of P/ml.) which is just sufficient to produce maximal enzyme activity, the presence of trimetaphosphate does not affect the rate of liberation of orthophosphate (Table 4).

Additional evidence for the association of the two activities comes from their behaviour on electrophoresis. When electrophoresis was carried out on 3MM filter paper with pH values (< 5.5) at which the activities were stable during the period of the experiment, neither moved from the origin. This is probably due to a binding of proteins with the paper

Table 1. *Ratio of glycerophosphatase to trimetaphosphatase in pea extracts*

Extracts were preparations of fraction E that had been prepared from different batches of peas during the last 2 years. Enzyme activities were measured and expressed as described in the Experimental section.

Extract	Glycerophosphatase (half-units/ml.)	Trimetaphosphatase (half-units/ml.)	Glycerophosphatase Trimetaphosphatase
E1	248	115	2.2
E2	22	10.9	2.0
E3	108	52	2.1
E4	515	233	2.2
E5	126	57	2.2
E6	40.2	19.4	2.1
E7	600	275	2.2

Table 2. *Absorption of enzyme activities on to calcium phosphate gel*

Various amounts of phosphate gel were added to 4 ml. samples of fraction E, containing 200 half-units of glycerophosphatase/ml., and water to 6.5 ml. After standing for 10 min. on ice the gel was removed by centrifuging, and enzyme activities and protein were measured in the supernatant liquids.

Phosphate gel added (mg. dry wt.)	Glycero- phosphatase remaining (%)	Trimeta- phosphatase remaining (%)	Protein remaining (%)
0	100	100	100
1.7	100	96	79
5.3	49	48	50
10.6	11	12	42
21	0	0	38

Table 3. *Heat stabilities of the phosphatase activities*

To 7 ml. samples of fraction E containing 160 half-units of glycerophosphatase/ml. was added 0.5 ml. of 0.2M-citrate buffer, pH either 5.2 or 6.3. The mixtures were incubated at 51° and samples taken at various times for enzyme estimations.

Buffer added (pH)	Time of incubation (min.)	Glycero- phosphatase remaining (%)	Trimeta- phosphatase remaining (%)
6.3	0	100	100
	23	7.5	6.5
	59	0	0
5.2	0	100	100
	23	26	29
	49	20	17
	68	12	8
	97	6	4

(Woods & Gillespie, 1952). With starch as a supporting material, however, both activities migrated equally towards the cathode at pH values between 4.5 and 5.5. Fig. 1 shows a typical result obtained at pH 4.7. No separation of the two enzyme activities was observed, nor was either activity resolved into two fractions.

Action of phosphatase on nucleic acids

When incubated with yeast nucleic acid, unchromatographed preparations of pea phosphatase liberated phosphorus in a form which was soluble in

Fig. 1. Electrophoresis of phosphatase. A sample of fraction E (30 μ l.; 3 units of glycerophosphatase) was applied to a column of starch gel that had been prepared with acetate buffer, pH 4.7, ionic strength 0.05. After electrophoresis had proceeded for 24 hr., the gel was dried and 1 cm. portions were taken for glycerophosphatase (---) and trimetaphosphatase (—) estimations as described in the text.

the uranyl acetate-trichloroacetic acid reagent. This activity was not due solely to contamination with the ribonuclease present in these extracts (Holden & Pirie, 1955a), since when the phosphatase was chromatographed on Dowex 50, those fractions of the eluate which contained the glycerophosphatase also showed activity against nucleic acid (Fig. 2). As ribonucleic acids contain both phosphomonoester and phosphodiester bonds, which are attacked by different enzymes (Markham & Smith, 1954), experiments were performed to decide which of these two was attacked by the phosphatase.

The maximum amount of nucleic acid phosphorus which was liberated as acid-soluble phosphorus in the experiment described in Fig. 2 was 7% of the total present, and of this 95–100% was

Table 4. *Action of pea phosphatase on mixed substrates*

Samples (0.25 ml.) of the chromatographed enzyme, component B, were incubated at 37° in 4 ml. of 0.1M-citrate. Glycerophosphate, when present, was at a concentration of either 0.6 mg. or 1 mg. of P/ml., and trimetaphosphate at a concentration of 0.6 mg. of P/ml. Samples were taken at various times for orthophosphate estimation.

Time (hr.)	Orthophosphate liberated (μ g. of P/ml.)				
	From glycerophosphate		From trimetaphosphate	From trimetaphosphate and glycerophosphate	
	(0.6 mg. of P/ml.)	(1 mg. of P/ml.)		(0.6 mg. of P/ml.)	(1 mg. of P/ml.)
0.5	16	17	10	14	14
1.0	26	30	17	27	30
2.5	64	73	38	66	72

Fig. 2. Association of ribonuclease and glycerophosphatase activities. A preparation of fraction E (30 ml.; 3000 units of glycerophosphatase) was chromatographed, and from the fractions produced samples (0.2 ml.) were taken for the estimation of glycerophosphatase (●) and (0.5 ml.) for the estimation of ribonuclease (○).

orthophosphate. If the pea enzyme had only phosphomonoesterase activity on the nucleic acid, this orthophosphate would have been derived from the terminal phosphomonoester groups. If, however, the phosphatase broke the diester bonds in the nucleic acid, liberating nucleotides, the orthophosphate could also have been derived from the enzymic breakdown of these nucleotides. Direct attempts were therefore made to detect nucleotide or nucleoside formation by fraction 20 (Fig. 2) of the chromatographed enzyme. Portions (0.1 ml.) were incubated at 37° and at various pH values between 4.9 and 5.8, with equal volumes of a solution containing 10 mg. of nucleic acid. Samples (20 μ l.) were taken at intervals up to 3.5 hr. later and spotted on to sheets of filter paper. After chromatographic development overnight in the propan-2-ol-aq. NH_3 soln. solvent (Markham & Smith, 1952a), the sheets were dried and examined in ultraviolet light for nucleotides and nucleosides. Minute quantities of nucleotides were visible in those samples that had

been incubated longest, but they contained less phosphorus (0.2 μ g.) than could be detected by the method used. In these experiments the concentration of the enzyme was about ten times greater than that used for the detection of glycerophosphatase activity, and less than 0.2% of the substrate was liberated as nucleotides and nucleosides. The diesterase activity of the pea phosphatase against nucleic acid is therefore negligible compared with its phosphomonoesterase activity.

The conversion of 7% of the phosphorus of nucleic acid into orthophosphate in the experiment described in Fig. 2 suggests that the mean length of the polynucleotide chains in the sample of nucleic acid used was about 14. This degree of polymerization is similar to that reported for some other preparations of yeast nucleic acid (Markham & Smith, 1952b), but much less than that suggested by osmotic measurements for yeast nucleic acid prepared by the method used in the present work (Holden & Pirie, 1955b). The nature of this disagreement is not clear.

The amount of orthophosphate liberated from nucleic acid by the phosphatase was more than doubled when the nucleic acid was digested with purified leaf ribonuclease (Pierpoint, 1956) before being incubated with the phosphatase. This was presumably due to the increase in the number of phosphomonoester bonds present.

Action of phosphatase on other compounds

In addition to the compounds previously listed (Pierpoint, 1957), the phosphatase liberates orthophosphate from pyrophosphate, guanidinium tetraphosphate, phenyl phosphate, phytic acid, a mixture of the 2'- and 3'-adenylic acids, and a mixture of the 2'- and 3'-uridylic acids. The criteria used in assigning these activities to the phosphatase is that they show the same distribution as glycerophosphatase in fractions derived from the chromatography of the enzyme. Fig. 3 shows the results obtained with mixtures of nucleotides as substrates. The results for the adenylic acids are not as accurate as the others, since a slow precipitation occurred

during the phosphate assay, but both demonstrate the existence of A and B, the two enzyme components.

The ratio of the activity of A to that of B, with glycerophosphate as substrate, is a little less than that with the nucleotides (Fig. 3). It is also a little less than the corresponding ratio for pyrophosphate, guanidinium tetrphosphate, phenyl phosphate and phytic acid. The rate of liberation of orthophosphate from these latter compounds by component B is about 200, 100, 120 and 20% respectively of that from glycerophosphate.

Breakdown of polyphosphates

Samples were taken at various times from solutions in which the phosphatase was breaking down polyphosphates and were analysed chromatographically for accumulation of intermediates. Fig. 4 shows the results obtained during the breakdown of tripolyphosphate by component B of the phosphatase. All the tripolyphosphate was converted into orthophosphate eventually but appreciable amounts of pyrophosphate (14% of total phosphorus) accumulated in the solution after incubation for 2 hr. In the absence of enzyme the tripolyphosphate did not break down.

A similar experiment with trimetaphosphate showed the almost complete conversion of this compound into orthophosphate, but no detectable amounts of tripolyphosphate or pyrophosphate accumulated. No orthophosphate was liberated from tetrametaphosphate on incubation with the enzyme, and the chromatographic behaviour of the tetrametaphosphate was not altered.

Fig. 3. Action of the phosphatase on nucleotides. The fractions were those obtained by the chromatography described in Fig. 2. Activity against glycerophosphate (●) was estimated in 0.23 ml. samples, and that against 2'- and 3'-adenylic acid (Δ) and against 2'- and 3'-uridylic acid (○) in 0.4 ml. samples. Enzyme estimations were carried out as usual, except that the concentration of citrate buffer was 0.09 M throughout, and the concentration of the nucleotides was 0.22 mg. of P/ml.

A number of attempts have been made to reverse the breakdown of polyphosphates. Samples of component B were incubated with large amounts of either orthophosphate or mixtures of ortho-, pyro- and tripolyphosphate, and the solution was analysed chromatographically. Although a number of conditions, such as pH and time of incubation, were varied no synthesis of polyphosphates was detected.

Presence of two enzyme components in crude pea extracts

The extracts from which A and B were previously isolated had been subjected to fractionation with ammonium sulphate and to lengthy dialyses. To examine the possibility that one of the two components was formed during these processes, an enzyme preparation which had not been so treated

Fig. 4. Breakdown of tripolyphosphate by pea phosphatase. Tripolyphosphate (50 mg.) was incubated with component B of the phosphatase (3 ml. of fraction 19, Fig. 2) in 10.5 ml. of citrate buffer (0.035 M, pH 6). Samples (2 ml.) were taken at 0 hr. (○), 2 hr. (■) and 18 hr. (●) and frozen until they could be chromatographed on a Dowex 1 column. Total phosphorus was estimated in 2 ml. samples of the resulting fractions. The three peaks present in the 2 hr. sample represent, from left to right, orthophosphate, pyrophosphate and tripolyphosphate.

was chromatographed. The pea plants were minced and extracted with citrate as described by Holden & Pirie (1955*a*), and the resulting extract was adjusted to pH 4.5 and stood in the refrigerator for 30 min. Chloroplasts and cell debris were then removed by centrifuging for 10 min. at 10 000 g, and the clear-brown supernatant solution was dialysed against cold water for 3 hr. The slight precipitate which formed during this time was removed by further centrifuging, and a sample of the extract chromatographed in the usual way. The distribution of glycerophosphatase in fractions of the eluate showed the presence of A (15 %) and B (85 %) in amounts similar to those previously found in fraction E.

DISCUSSION

It has been shown previously that the heterogeneity of the pea phosphatase was not an artifact of the chromatographic procedure (Pierpoint, 1957), and it has now been shown not to be due to the treatment with ammonium sulphate. However, the possibility of it being an autolytic artifact produced during the disruption of the leaves is by no means ruled out. For another phosphatase, fructose 1:6 diphosphatase, there is evidence that different methods of preparation which allow different degrees of autolysis produce enzymes with different electrophoretic mobilities (Mokrasch & McGilvery, 1956). Other phosphatases which are included among the increasingly large number of proteins that can be resolved by chromatographic or electrophoretic techniques into two or more active components are pancreatic ribonuclease (Martin & Porter, 1951), horse-radish phosphatase (Boman & Westlund, 1956), sweet-potato phosphatase (Ito, Kondo & Watanabe, 1955), rattle-snake-venom phosphodiesterase (Boman & Kaletta, 1956) and yeast phosphomonoesterase (Tsuboi, Wiener & Hudson, 1957).

The pea phosphatase has no significant effect on the diester bonds of either diphenyl phosphate or ribonucleic acid, and would therefore probably be classified as a monoesterase. There is, however, no reasonable doubt that it has diesterase activity against trimetaphosphate. As further metaphosphates become characterized and available, it will be of interest to see how far this activity extends to these compounds. That it will not be general is indicated by the inability of the enzyme to split tetrametaphosphate. An attempt was made to prepare some new polyphosphates by gentle acid hydrolysis of commercial 'hexametaphosphate' (McCullough, Van Wazer & Griffith, 1956) followed by ion-exchange chromatography. However, the only compounds detected in appreciable amounts were ortho-, pyro-, tripoly-, trimeta- and tetrameta-phosphate.

Since few, if any, of the highly purified phosphomonoesterases have been tested against trimetaphosphate, it would be premature to state that the pea enzyme does not fit into any of the classes of phosphatases described by Roche (1950). From the characters that can be directly compared, it seems that the enzyme has most affinities with the class II phosphomonoesterases. It resembles very closely the phosphatases purified from citrus fruit (Axelrod, 1947) and sweet potatoes (Ito *et al.* 1955). These three enzymes all attack pyrophosphate. There have been a number of suggestions that the pyrophosphatase and the glycerophosphatase of some other higher plants are separate entities (Roberts, 1956), but few, if any, are accompanied by convincing evidence for the separations of the two activities.

Pyrophosphate has previously been detected as an intermediate in the breakdown of tripolyphosphate by agents such as alkali (Bell, 1947), yeast extracts (Mattenheimer, 1956; Kornberg, 1956) and extracts of *Aspergillus* (Neuberg, Grauer & Mandl, 1950). Alkali and yeast extracts also accumulate pyrophosphate and tripolyphosphate in the degradation of trimetaphosphate. The non-accumulation of these compounds in the breakdown of trimetaphosphate by the pea enzyme is probably due to the speed with which they are further broken down. Orthophosphate is liberated from pyrophosphate and tripolyphosphate by component B at about 7 and 4.5 times respectively the rate that it is from trimetaphosphate. Even allowing for the fact that the breaking of one bond in trimetaphosphate liberates less orthophosphate on an average than it does from either of the other two compounds, it is clear that the splitting open of the phosphate ring is by far the slowest process in the breakdown of trimetaphosphate.

SUMMARY

1. Evidence from a variety of sources indicates that the glycerophosphatase and trimetaphosphatase activities of pea extracts are associated with the same enzyme.
2. This enzyme also splits the phosphomonoester bonds in nucleic acid, phytic acid, phenyl phosphate, pyrophosphate, tetrakisphosphate, adenylic acid and uridylic acid, in addition to the compounds previously listed.
3. Pyrophosphate is an intermediate in the breakdown of tripolyphosphate by the phosphatase. No intermediates could be detected in the breakdown of trimetaphosphate.
4. The chromatographic heterogeneity of the phosphatase is not caused by the treatment with ammonium sulphate in the course of its purification.

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Calcium and Magnesium Metabolism in Calves

PLASMA LEVELS AND RETENTION IN MILK-FED CALVES

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Sheep and cattle are the only animals known to be subject to a clinical disorder apparently due to magnesium deficiency, when receiving diets not themselves grossly deficient in magnesium. The disorder is characterized by hypomagnesaemia and culminates in extreme cases in convulsions which are often fatal. It occurs in adult sheep and cattle under a variety of conditions, but most frequently shortly after they have started to graze young quickly growing herbage. The subject has been reviewed by Russell (1944) and Blaxter & McGill (1956). Duncan, Huffman & Robinson (1935) first showed that calves, fed basically on milk for an abnormal length of time, also develop hypomagnesaemia. This finding has frequently been confirmed (Wise, Petersen & Gullickson, 1939; Huffman, Conley, Lightfoot & Duncan, 1941; Blaxter & Sharman, 1955). Occasionally hypocalcaemia has also been reported in milk-fed calves (Huffman & Robinson, 1926; Parr, 1957). Two principal reasons have been suggested to explain the development of hypomagnesaemia under these conditions: (a) that it is due to a simple dietary deficiency of magnesium (Blaxter, 1956); (b) that the properties of the milk

diet are such that utilization of magnesium is impaired (Huffman *et al.* 1941).

The present work was undertaken in an attempt to throw further light on this problem.

EXPERIMENTAL

Animals

Two sets of calves were used. One set was kept from 1954 to 1955 (nos. 85, 86 and 87) and the other set from 1955 to 1956 (nos. 1, 3, 4, 5, 6, 7, 8, 11). All were male shorthorns except for no. 4, which was a female shorthorn, and no. 11, which was a male Friesian. All the males except nos. 1 and 11 were castrated at 2-3 weeks of age. The calves were removed from their dams 4 days after birth and subsequently fed twice daily from a bucket. Their diets consisted almost exclusively of whole milk in the amounts shown in Table 1 with daily additions of 0.4 g. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.04 g. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.04 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ from 2 months and double these amounts from 6 months. The amounts of milk fed were restricted so that the calves did not generally grow at the optimum rate. This was done to avoid digestive disorders as far as possible and to obtain a uniform treatment. Many of the calves had diarrhoea for a day or two but some developed persistent digestive disturbances; the latter were usually removed from the experiment if they