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DIPEPTIDASE DEVELOPMENT IN COTYLEDONS OF *CUCURBITA MAXIMA* DURING GERMINATION

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Abstract—The dipeptidase activity of an unpurified soluble extract of the cotyledons of *Cucurbita maxima* Duch. var. Hubbard remained unchanged during the first 2 days of germination and then increased at a rapid rate during the next 3 days. The dipeptidase activity of two of three lots of seeds required the presence of the embryo axis for maximal dipeptidase activity, whereas the third lot was uninfluenced by the embryo axis. This discrepancy was possibly due to genetic differences. In those seeds which required the presence of the embryo axis for maximal dipeptidase activity, the cytokinin benzyladenine could replace the embryonic axis. When the protein synthesis inhibitor cycloheximide was added to the seeds at the beginning of germination, it inhibited dipeptidase activity of the cotyledons from 26 to 55 per cent, depending of the basis of calculation, at 5 days. When the cycloheximide was added to 3-day-old seedling the inhibition of dipeptidase activity in the cotyledons was almost immediate. The relative hydrolysis of L-leucylglycine and glycylglycine were compared after temperature inactivation and purification; the results showed that more than one enzyme was responsible for the dipeptidase activity. The presence of a dialysable dipeptidase inhibitor(s) was demonstrated. Relatively high dipeptidase activity was also found in the roots and shoots.

INTRODUCTION

DURING germination, the storage proteins in the cotyledons or endosperm of seeds are degraded to yield free amino acids and amides. These are utilized for the synthesis of new functional and structural proteins as well as a source of energy for the developing seedling. The state of knowledge of the metabolic pathway(s), the enzymes involved and the control mechanisms of these processes is relatively primitive.

Proteolytic enzyme activity has been found in many seeds and reviewed in previous papers.[1–3] Dipeptidase activity has been reported in germinating seeds of wheat,[4,5] rye,[6] barley[6] and squash.[1,2] The dipeptidase activity of squash cotyledons is probably mainly a function of a specific dipeptidase;[2] however, two aminopeptidases[1] have been isolated from squash cotyledons which have dipeptidase activity. Two other proteolytic enzymes isolated from squash cotyledons do not appear to hydrolyse dipeptides. These are an aminooligopeptidase[7] and a proteinase.[3] Wiley and Ashton[8] found that a substance supplied by the embryo axis was necessary for the hydrolysis of storage proteins in squash cotyledons of germinating seedlings. Several cytokinins are able to replace the embryonic axis in the synthesis of a proteinase[3,9] and isocitrate lyase.[10] Protein synthesis inhibitors were able to

[1] F. M. ASHTON and W. J. DAHMEN. *Phytochem.* **6,** 641 (1967).
[2] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* **6,** 1215 (1967).
[3] D. PENNER and F. M. ASHTON, *Plant Physiol.* **42,** 791 (1967).
[4] L. B. PETT, *Biochem. J.* **29,** 1898 (1935).
[5] J. D. MOUNFIELD, *Biochem. J.* **30,** 549 (1936).
[6] C. ENGEL and J. HEINS, *Biochim. Biophys. Acta* **1,** 190 (1947).
[7] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* **7,** 189 (1968).
[8] L. WILEY and F. M. ASHTON, *Physiol. Plant.* **20,** 688 (1967).
[9] D. PENNER and F. M. ASHTON, *Nature, Lond.* **212,** 935 (1966).
[10] D. PENNER and F. M. ASHTON, *Biochim. Biophys. Acta* **148,** 481 (1967).

negate the formation of these enzymes. The *de novo* synthesis of the specific dipeptidase[2] of squash cotyledons has been observed.[11]

In the present study the regulation of dipeptidase activity development in squash cotyledons during germination was investigated. This included investigations of the influence of the embryonic axis, the cytokinin benzyladenine and the protein synthesis inhibitor cycloheximide.

RESULTS AND DISCUSSION

The assay utilized glycylglycine as the substrate and a formol titration as described in detail in the experimental section. The amount of the crude enzyme preparation used in the assay when it had maximal activity (5 days) was saturated by 0·3 M glycylglycine. The activity with time for the crude enzyme preparation was found to be linear within the time used.

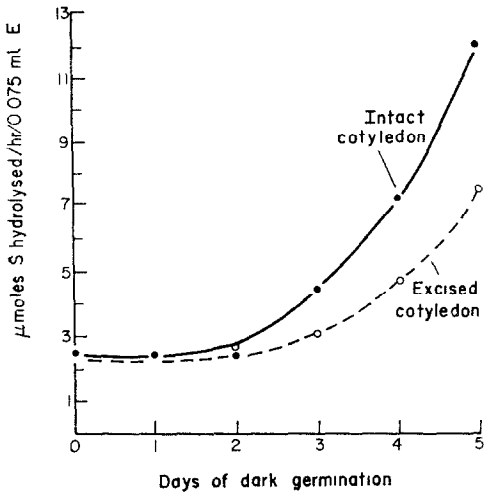

FIG. 1. THE EFFECT OF THE EMBRYO AXIS OF SQUASH SEEDS (1968) ON THE DEVELOPMENT OF DIPEPTIDASE ACTIVITY IN THE COTYLEDONS DURING GERMINATION.
S = glycylglycine, E = crude enzyme preparation.

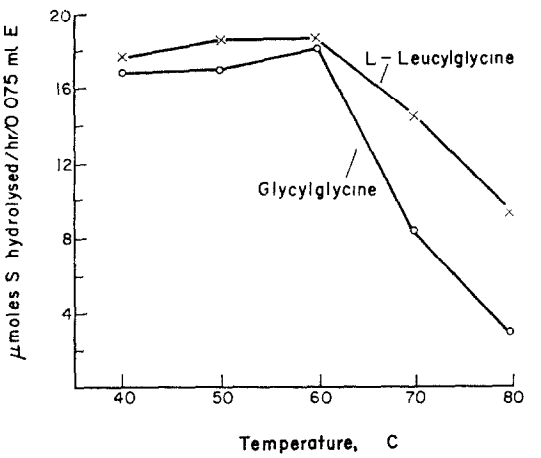

FIG. 2. TEMPERATURE INACTIVATION OF THE CRUDE ENZYME PREPARATION FROM 5-DAY-OLD SQUASH COTYLEDONS USING L-LEUCYLGLYCINE AND GLYCYLGLYCINE AS SUBSTRATES.
S = substrate, E = crude enzyme.

Time-course Studies

The development of the crude dipeptidase activity in the cotyledons of squash was followed by daily assays during germination from 0 to 5 days. During the first 2 days, little if any change in dipeptidase activity occurred. However, after 2 days the development of the enzyme appeared to increase rapidly with time (Fig. 1). The activity was still increasing at 5 days. The initial lag period of 2 days may be due to a period of enzyme synthesis or substrate 'activation'. Lag phases have been described as an indication of protein synthesis,[12] while enzymes showing high activity initially have been interpreted as being activated.[13,14] The lag phase of protein hydrolysis has also been attributed to the initial substrate 'activa-

[11] R. TSAY and F. M. ASHTON, personal communication (1971).
[12] M. J. CRISPEELS and J. E. VARNER, *Plant Physiol.* **42,** 398 (1967).
[13] A. MARCUS and J. VELASCO, *J. Biol. Chem.* **235,** 563 (1960).
[14] A. M. FLINN and B. L. SMITH, *Planta* **75,** 10 (1967).

tion' in germinating seeds by Ghetie.[15] In squash, protein bodies predominate at the early stages of germination; they disappeared after 4–5 days.[16,17] The sudden increase in dipeptidase activity after the second day suggests that the appearance of low molecular peptide fragments may initiate the formation of the enzyme. Furthermore, it has been shown that the specific dipeptidase[2] of squash cotyledons is synthesized *de novo* more rapidly from 72 to 96 hr than from 48 to 72 hr.[11]

The level of activity after day 5 was not followed because the seedling became too etiolated; however, it is not expected to decrease to a low level as the enzyme is also present in shoots and roots (see later). The relative specific activity was quite high in shoots and roots. The presence of dipeptidase activity in non-storage tissues suggest it has some functions other than hydrolysis of storage proteins; it might be involved in the turnover of enzymes which occurs in active metabolic cells.

The quantity of soluble proteins in the cotyledons increased rapidly at almost a linear rate during the initial stages of germination, from 4·4 mg per cotyledon pair in dry seeds which are needed for growth and development of the cotyledons. Squash cotyledons increase in size several fold during germination and become leaf-like in appearance. Marcus and Feeley[18] gave evidence that the soluble enzymes required for protein synthesis were active prior to imbibition. During the first 30 min of imbibition of water by wheat embryo, the protein synthesizing capacity increased but lagged about 10 min behind water uptake.[19] The rise was accompanied by increases in polysome content.

Influence of the Embryonic Axis

The rapid development of dipeptidase activity in squash cotyledons during germination was examined for possible control by the embryo axis. Crude supernatant preparations from intact or excised cotyledons of 0–5-day-old seedlings were assayed for dipeptidase activity. This experiment was performed for each shipment of squash seeds we received. Conflicting results were obtained. The development of dipeptidase was found to be controlled by the axis in 1968 (Fig. 1), suggesting that a stimulus from the embryonic axis was needed for maximal enzyme activity. The excised cotyledons showed approximately 60% dipeptidase activity of intact cotyledons at 5 days. With the 1969 seeds, no axial control could be shown; the development of dipeptidase activity in both excised and intact cotyledons were the same. However, with the 1970 seeds, the dependence of the embryonic axis on maximal dipeptidase activity in squash cotyledons was again shown. This was true whether the data were calculated on the μmole of substrate hydrolysed per hr per unit of enzyme, specific activity, or activity per cotyledon. Other researchers have also found discrepancies such as these. Marcus and Feeley[20] found that isocitrate lyase developed at the same rate in intact and dissected cotyledons of peanut and squash, whereas, Penner and Ashton[10] reported that isocitrate lyase activity was greater in intact cotyledons than in dissected cotyledons of squash. Black and Altschul[21] also found that different samples of cottonseed showed differences in requirements for proximal halves for lipase formation in distal halves. They suspected it arose from differences in conditions of maturation and storage. The same

[15] V. Ghetie, *Rev. Roumaine de Biochem.* **3,** 353 (1966).
[16] J. N. A. Lott, Ph.D. Dissertation. Univ. of Calif., Davis. 242 (1969).
[17] L. Wiley, Ph.D. Dissertation. Univ. of Calif., Davis. 138 (1971).
[18] A. Marcus and J. Feeley, *Proc. Nat. Acad. Sci. U.S.* **51,** 1075 (1964).
[19] A. Marcus, J. Feeley and T. Volcani, *Plant Physiol.* **41,** 1167 (1966).
[20] A. Marcus and J. Feeley, *Biochim. Biophys. Acta* **89,** 170 (1964).
[21] H. S. Black and A. M. Altschul, *Biochem. Biophys. Res. Commun.* **19,** 661 (1965).

suspicion was tested by ageing squash seeds at room temperature for 6 months, but no significant decrease in dipeptidase activity was found in the excised cotyledons. Some morphological differences in the squash seeds suggested possible genetic differences.

The effect of the cytokinin benzyladenine on dipeptidase activity of squash cotyledons with and without the embryo axis was determined (Table 1). The dipeptidase activity of the cotyledons from the intact seedlings showed a slight stimulation at 1 μM benzyladenine but was inhibited at higher concentrations, whereas without the embryo axis no effect was noted at the lower concentrations of benzyladenine but stimulation was observed at 10 μM and at 100 μM maximal activity was achieved. This data suggest that the stimulus from the embryo axis which is required for maximal dipeptidase activity may be a cytokinin. Apparently an excess of cytokinin inhibits dipeptidase activity and amount supplied to the cotyledons from the axis in the intact seedling is almost optimal.

TABLE 1. EFFECT OF BENZYLADENINE ON THE DIPEPTIDASE ACTIVITY OF 5-DAY-OLD SQUASH COTYLEDONS FROM INTACT AND MINUS AXIS SEEDLING

Benzyladenine conc. (μM)	Dipeptidase activity*	
	Intact embryo	Excised cotyledons
0	14·8	8·5
1	19·0	8·9
5	13·8	8·7
10	6·7	11·0
100	5·6	20·0

* μmoles of glycylglycine hydrolysed/hr/0·075 ml crude preparation.

It has been reported that the axial tissue is necessary for maximal proteinase,[3,9] isocitrate lyase[10] and amylase[22] activity of *Cucurbita maxima* cotyledons and that a cytokinin could reproduce, in part or in total, the embryonic axis. It appears that cytokinins may control protein synthesis in general and not be the control mechanism for specific enzymes *per se*, but rather promote the synthesis of many enzymes. Gupta and Maheshwari[23] demonstrated the presence of three distinct cytokinins in seeds of *Cucurbita pepo* Linn.

Distribution of Dipeptidase Activity

Activity in the embryo of 5-day-old seedlings was assayed. The shoots and roots were homogenized and centrifuged. The supernatant showed dipeptidase activity present in both shoots and roots. The μmole of glycylglycine hydrolysed/hr/ml of crude preparation for the shoots and roots was 109 and 164, respectively. The relative specific activities of embryo to cotyledons was 4·4–1. The ubiquity of dipeptidase activity is apparent from its distribution.

Effect of Cycloheximide on the Development of Dipeptidase Activity

Protein synthesis inhibitors have been used to indicate *de novo* enzyme synthesis in germinating seeds. The *de novo* synthesis of the specific dipeptidase[2] of squash cotyledons has been shown.[11] The development of dipeptidase in germinating squash was investigated

[22] D. PENNER, *Weed Sci.* **16**, 519 (1968).
[23] G. R. P. GUPTA and S. C. MAHESHWARI, *Plant Physiol.* **45**, 14 (1970).

by germinating the seeds in culture solutions containing cycloheximide. At 5 days, cycloheximide treated seedlings showed inhibition in growth and development. Crude soluble enzyme preparations of cotyledons were used to determine dipeptidase activity. The results of four experiments are summarized in Table 2. Cycloheximide at 10 μg/ml inhibited the development of dipeptidase activity from 26 to 55 per cent, depending on the method of calculation. Some workers have attributed partial enzyme activity inhibition as an indication of a *de novo* synthesis,[3,9] whereas others have interpreted it as an indication that protein synthesis was not required for enzyme activation.[24]

TABLE 2. DIPEPTIDASE ACTIVITY OF 5-DAY-OLD COTYLEDONS FROM SEEDLINGS WHICH WERE GERMINATED IN 10 μg/ml CYCLOHEXIMIDE

Units	Control	Cycloheximide treated	% Control
1*	18·1	6·2	34
2†	40	22	55
3‡	1192	306	26

* μmoles S hydrolysed/hr/0·075 ml E.
† Specific activity μl 0·1 N KOH/mg protein.
‡ Activity per cotyledon pair.

When cycloheximide (10 μg/ml) was added to 3-day-old squash seedlings and grown for 2 additional days, cycloheximide inhibition of dipeptidase activity was almost immediate (Table 3). Dipeptidase activity in later stages of seedling development appears to require continuous protein synthesis. Partial purification showed that there was increasing inhibition with increasing purity of the dipeptidase (Table 4). This suggests that the specific dipeptidase must be low in activity after treatment with cycloheximide. It also indicates that some other peptidase in the crude system is less affected by the protein synthesis inhibitor. Autolysis of the homogenate of ungerminated squash seeds did not cause any increase in dipeptidase activity suggesting it is not activated.

Enzyme Properties

Animals are known to have numerous dipeptidases that have broad substrate specificities. They have been separated by preparative steps in enzyme purification[25] and

TABLE 3. DIPEPTIDASE ACTIVITY OF 5-DAY-OLD COTYLEDONS FROM SEEDLINGS GROWN FOR THE LAST 2 DAYS IN 10 μg/ml CYCLOHEXIMIDE*

Units†	Control	Cycloheximide treated	% Control
1	17·2	8·2	48
2	39	26	67
3	1337	705	53

* μmoles S hydrolysed/hr/0·075 ml E at 3 days was 5·7.
† See Table 2.

[24] Y. SHAIN and A. M. MEYER, *Physiol. Plant.* **21**, 765 (1968).
[25] A. SCHMITT and G. SIEBERT, *Hoppe Seylers. Z. Physiol. Chem.* **348**, 1009 (1967).

TABLE 4. PARTIAL PURIFICATION OF DIPEPTIDASE ACTIVITY IN 5-DAY-OLD SQUASH SEEDLINGS GERMINATED IN 10 μg/ml CYCLOHEXIMIDE*

Fraction	Control	Cycloheximide treated	% Control
I. Crude	38	24	63
II. Acetone ppt.	395	98	25
III. Sephadex G-50	3408	382	11

* Units are specific activity.

characterized by pH optima differences, differention and metal requirements.[26,27] To test whether squash cotyledons might also have a number of distinct peptidases with broad substrate specificities in the crude preparation, the hydrolysis of L-leucylglycine and glycylglycine were compared after temperature inactivation or purification.

A heat inactivation experiment was conducted with the crude preparation from 40° to 80° (Fig. 2). The activity for the two substrates were similar after the 40°, 50° and 60° treatment, however, after the 70° treatment the hydrolysis of both substrates decreased but L-leucylglycine was hydrolysed more than glycylglycine. After the 80° treatment the hydrolysis of glycylglycine was only about 30 per cent of L-leucylglycine. The heat stability and relative substrate hydrolysis after the 80° treatment are similar to those of a dipeptidase which has been purified and characterized from this species.[2]

The relative hydrolysis of L-leucylglycine and glycylglycine by fractions precipitated by various concentrations of $(NH_4)_2SO_4$ were compared (Table 5). The substrate activity ratios with the crude extract was approximately one to one while all $(NH_4)_2SO_4$ fractions demonstrated a ratio of 2:1 for glycylglycine–L-leucylglycine.

The relative hydrolysis of the two substrates were also compared through the four

TABLE 5. ACTIVITY RATIO OF L-LEUCYLGLYCINE AND GLYCYLGLYCINE OF THE CRUDE PREPARATION AND AMMONIUM SULFATE FRACTIONS

Fractions	Act. ratio (GG/LG)
Crude	0·90
0–25% $(NH_4)_2SO_4$	2·1
25–45% $(NH_4)_2SO_4$	1·7
45–70% $(NH_4)_2SO_4$	2·4

purification steps (Table 6). The crude extract again gave a 1:1 substrate activity ratio, while all other fractions yielded an approximate 2:1 ratio for glycylglycine–L-leucylglycine. The change in ratio to 2:1 suggests that acetone precipitation and $(NH_4)_2SO_4$ precipitation were able to remove an enzyme that favored the hydrolysis of glycylglycine relative to L-leucylglycine. The heat inactivation and purification experiments show that more than one peptidase is present in the crude preparation that will hydrolyse dipeptides.

When the reaction velocity is linearly proportional to enzyme concentration it indicates that the enzyme molecules are acting independently of one another. However, our plot of

[26] L. JOSEFSSON and T. LINDBERG, *Biochim. Biophys. Acta* **105**, 149 (1965).
[27] L. JOSEFSSON and T. LINDBERG, *Acta Chem. Scand.* **21**, 1965 (1967).

TABLE 6. ACTIVITY RATIO OF L-LEUCYLGLYCINE AND GLYCYLGLYCINE DURING PURIFICATION

Fraction	Activity ratios (GG/LG)
I. Crude	0·90
II. Acetone ppt.	1·78
III. Sephadex	2·10
IV. DEAE	1·94

Substrate conc. 0·4 M.

velocity against crude enzyme concentrations curved as the enzyme amount was increased (Fig. 3). The presence of a dissociable inhibitor was suspected. A crude preparation of 5-day squash cotyledons was dialysed overnight against 100 vol. of 0·1 M K phoshate buffer pH 7·6. The relationship between enzyme concentration and activity turned out to be very near linearity for the dialysed crude preparation (Fig. 3). The result indicated strongly there was a reversible inhibitor in the crude enzyme preparation and it was removable by dialysis. It does not seem to be the same inhibitor Ashton and Dahmen[2] suspected to be present in their preparation as they suggested the inhibitor was removed in the DEAE column when Fraction IV (35·6%) showed increased activity over Fraction III (28·3%).

EXPERIMENTAL

Germination Methods

All experiments were conducted with seeds of *Cucurbita maxima* Duch. Chicago Warted Hubbard squash. They were soaked in H_2O for 4 hr and then grown in vermiculite at 27° in a humid dark chamber for 1–5 days. For experiments where the axial influence or the effect of cycloheximide were examined, seed coats

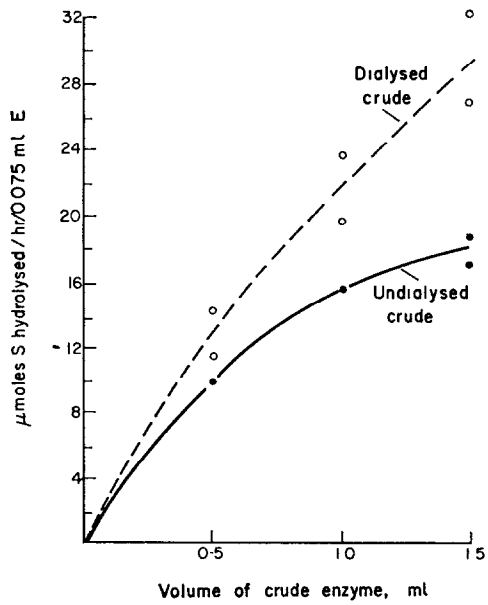

FIG. 3. EFFECT OF INHIBITOR IN ENZYME PREPARATION.
(S = glycylglycine)

were removed and the seeds were germinated in sterile Petri dishes containing 12 ml of 20 ppm streptomycin sulfate culture solution plus cycloheximide for the protein synthesis inhibitor experiments, and benzyladenine at different molarities for hormonal control experiments. Except for time course studies, all other experiments utilized cotyledons from 5 days old squash seedlings.

Assay of Dipeptidase

Protein analysis was by the Lowry method.[28] Total soluble proteins in the cotyledons was determined by assaying the supernatant of the crude extract after centrifugation at 34,800 *g* for 1 hr. The results were comparable to Biuret protein determination assays performed after 10% TCA (trichloroacetic acid) precipitation of the crude supernatant.

The assay procedure for dipeptidase activity was adapted from Ashton and Dahmen.[2] This formol titration method depended on the determination by an automatic titrator of increased acidity due to substrate hydrolysis. The reaction mixture contained 1·5 ml of 0·8 M glycylglycine in 0·1 M phosphate buffer at pH 7·6 and 1·5 ml enzyme preparation to make a total vol. of 3 ml. 1·0 ml was removed immediately and added to 1 ml of 36% HCHO at pH 7·0. This was the 0 time reaction mixture. The mixtures were incubated at 35° for 60 min, and a second sample taken. After standing at room temp. for 1 hr, protein coagulates were removed by centrifugation. The clear supernatant was titrated against 0·1 M KOH to 8·6 at 34°. Activity was most frequently expressed as μmole of dipeptide hydrolysed/hr/0·075 ml enzyme preparation. Crude specific activities and activity per cotyledon pair have also been used and compared. Two titrations were made for each sample. All experiments have been repeated at least once

Purification and Fractionation

(i) *Fractional denaturation by heat.* Aliquots of the crude enzyme preparation were incubated for 10 min at 40°, 50°, 60°, 70° and 80°. The samples were cooled at 0° and coagulated proteins removed at 34,800 *g* in a Servall centrifuge between 0° and 4° for 15 min. The supernatant was assayed for dipeptidase activity. Both glycylglycine and L-leucylglycine were used as substrates and their activities were compared.

(ii) *Fractional precipitation by* $(NH_4)_2SO_4$. The crude supernatant was fractionated by $(NH_4)_2SO_4$ at 0–25%, 24–45%, and 45–70% saturation. The $(NH_4)_2SO_4$ was added in very small quantities at a time while the solution was stirred and kept cold. The protein salted out were pelleted at 34,800 *g* for 15 min and dissolved in 0·1 M K_3PO_4 buffer pH 7·6. This was dialysed overnight in two changes of 100 × vol. 0·1 M phosphate buffer pH 7·6. The enzyme preparation was assayed for dipeptidase activity with glycylglycine or L-leucylglycine.

(iii) *Purification.* Purification procedure followed that used by Ashton and Dahmen.[2] Crude supernatant (Fraction I) was prepared by homogenizing two cotyledon pairs per ml 0·1 M K_3PO_4 buffer pH 7·6 and centrifuging at 34,800 *g* for 1 hr. A 40–80% acetone fractionation of the crude preparation (Fraction II) was dissolved in 0·05 M K_3PO_4 buffer pH 7·6 and passed through Sephadex G-50. The cloudy fraction (Fraction III) was placed on a DEAE cellulose column and eluted with a linear NaCl gradient from 0–0·35 M. The active fractions were concentrated by precipitation with 80% $(NH_4)_2SO_4$. The proteins were pelleted and dissolved in few ml of 0 05 M K_3PO_4 buffer pH 7·6 and dialysed.

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[28] O. N. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).