Phytochemistry, 1968, Vol. 7, pp. 1491 to 1498. Pergamon Press. Printed in England

ACTIVATION OF ENZYMES DURING GERMINATION—TRYPSIN-LIKE ENZYME IN LETTUCE

Y. SHAIN and A. M. MAYER

Botany Department, Hebrew University, Jerusalem, Israel

(*Received* 31 *January* 1968)

Abstract—In lettuce seeds trypsin-like enzyme activity develops during germination, and this process is inhibited by inhibitors of protein synthesis. The enzyme was purified more than 450 fold. It was shown that during germination in the presence of $^{35}SO_4$, no radioactive sulphur was incorporated into the purified enzyme. Incorporation did take place into an acid phosphatase, which was also purified 110 fold. It is concluded that the trypsin-like enzyme does not arise by net protein synthesis. As the enzyme is formed an endogenous trypsin inhibitor disappears, due to enzymic destruction. It is suggested that the disappearance of the inhibitor controls the liberation of the trypsin-like enzyme from a precursor form. Possible mechanisms are discussed. The results are taken as further proof that during germination enzymes arise by liberation from inactive forms.

IN A PREVIOUS paper[1] we suggested that the trypsin-like enzyme found in lettuce seed during germination might arise by a release from a precursor form. Similar suggestions have been made by other authors for other enzyme systems which appear during germination.[2-6] Proteolytic enzymes have not been studied extensively during germination, and the mechanism which controls their formation is as yet unclear. Penner and Ashton[7] have brought evidence that in squash seed proteinase formation is hormonally controlled. In most cases the trigger mechanism for the release is unknown. The most convincing evidence for the activation of enzymes during germination is that of the *R*-enzyme in peas, which is released during germination from a precursor form.[8]

In the following paper we wish to report on the release of the trypsin-like enzyme from a precursor form and we will also bring preliminary results dealing with the possible release mechanism.

RESULTS

Germination of lettuce seeds in chloramphenicol does not prevent the development of trypsin-like enzyme activity.[1] As trypsin-like activity increases, the endogenous trypsin inhibitor present in the seeds disappears (Tables 1 and 2).

The appearance of trypsin-like activity with germination at pH 6·8 was studied using BAEE as the substrate for the assay (Table 2). Hydrolysis of BAEE was linear with time for

[1] Y. SHAIN and A. M. MAYER, *Physiol. Plantarum* **18**, 853 (1965).
[2] A. M. MAYER and A. POLJAKOFF-MAYBER, *Int. Symp. Physiol. Ecol. Bioch. Germin. AVIIa2 Greifswald* (1963).
[3] H. J. PRESLEY and L. FOWDEN, *Phytochem.* **4**, 169 (1965).
[4] A. M. FLINN and D. L. SMITH, *Planta* **75**, 10 (1967).
[5] C. T. GREENWOOD and A. W. MACGREGOR, *J. Inst. Brewing* **71**, 405 (1965).
[6] R. BIANCHETTI and M. P. CORNAGGIA, *Giorn. Bot. Ital.* **72**, 370 (1965).
[7] D. PENNER and F. M. ASHTON, *Plant Physiol.* **42**, 791 (1967).
[8] Y. SHAIN and A. M. MAYER, *Physiol. Plantarum*, in press.

3 hr. Development of activity towards BAEE during germination paralleled that observed previously using pectinase as a substrate. Using the more sensitive assay with BAEE, very slight activity was noted already in the dry seeds, amounting to 12 per cent of that present after 24 hr of germination (Table 2).

All of the trypsin-like activity present in seeds germinated for 72 hr could be recovered from the supernatant fraction after centrifugation for 1 hr at 100,000 *g*.

These results appeared to support our previous suggestion that the trypsin-like enzyme might arise from a precursor form, rather than by *de novo* protein synthesis.

TABLE 1. DISAPPEARANCE OF TRYPSIN INHIBITOR DURING GERMINATION

Length of germination (hr)	Inhibitor activity* in extract (units/1 mg dry seeds)	% decrease from dry seeds
0	2·07	0
6	0·73	65
15	0·30	86
24	0	100

* For method of extraction see Ref. 1.

TABLE 2. DEVELOPMENT OF TRYPSIN-LIKE ENZYME ACTIVITY WITH GERMINATION

Hours germination	Activity/g equiv. seed* wt. (units)	Specific activity U/mg protein
0	7·5	0·05
24	60·0	0·5
48	257·0	1·82
72	333·0	4·60

* BAEE as substrate; 100,000 × *g* supernatant as enzyme source. 1 unit = 1 μmole BAEE hydrolysed/hr.

In order to be able to determine whether the trypsin-like enzyme is formed by a process of liberation it is necessary to purify it. It should then be possible to demonstrate that, if seeds are germinated in the presence of a suitable radioactive label, none of the label is incorporated into the purified enzyme, while it is present in other marker fractions.

Ways were therefore sought to purify the trypsin-like enzyme. In view of the high content of phenolic compounds in lettuce seeds, which interfere in purification procedures, ways of preventing browning reactions were sought. Following Loomis and Bataille[9] we attempted the use of Polyclar AT (a solid polyvinyl pyrrolidone) in the presence and absence of ascorbic acid. Best results were obtained using 1·5 g Polyclar/g dry weight of seeds. This resulted in a significant rise of both total and specific activity recovered in the extracts of the seeds. Such extracts could be purified to some extent by ammonium sulphate fractionation. 48 per cent of activity with a 5·6 fold purification could be recovered in a fraction precipitating between

[9] W. D. LOOMIS and J. BATAILLE, *Phytochem.* **5**, 423 (1966).

45 and 65 per cent saturation with ammonium sulphate. The bulk of the residual activity was spread in the fraction above and below this percentage saturation, with little or no purification. Attempts to purify the purified fraction on ion exchange columns of DEAE or TEAE cellulose were unsuccessful.

Further purification of the trypsin-like enzyme after $(NH_4)_2SO_4$ fractionation and dialysis for 24 hr was accomplished on a Sephadex G-75 column (Fig. 1).

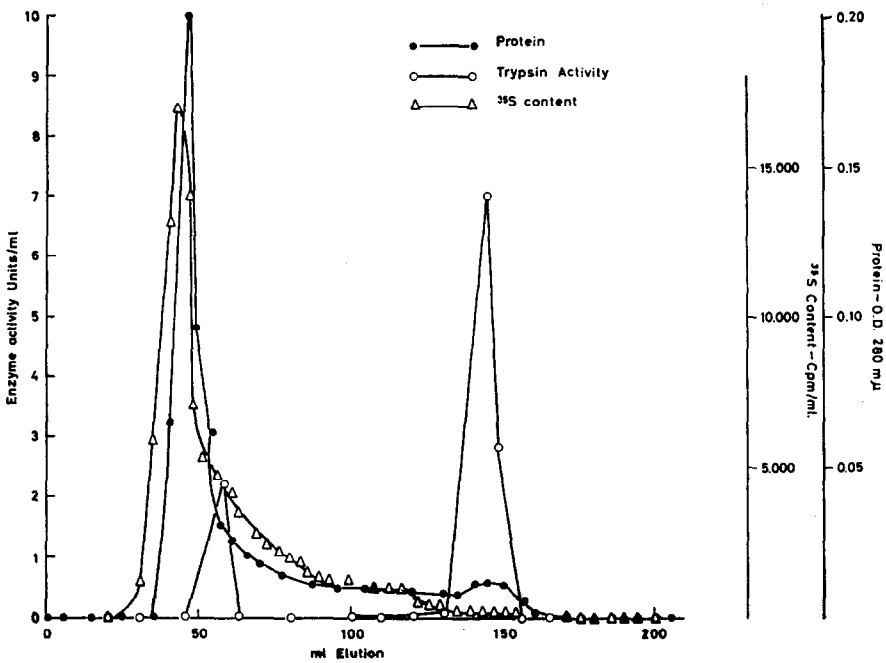

FIG. 1. PURIFICATION OF TRYPSIN-LIKE ENZYME FROM GERMINATING LETTUCE AND INCORPORATION OF ^{35}S INTO THE VARIOUS PROTEIN FRACTIONS

●——● Protein
○——○ Trypsin activity
△——△ ^{35}S content

(Fractions eluted from a Sephadex G-75 column. Dimensions: 90 × 1·5 cm; eluted with McIlvaine buffer 0·02 M, pH 6·8; flow rate 30 ml/hr.)

When 4 ml enzyme with a specific activity of 12·4 units/mg protein were applied, the first peak eluted had a specific activity of 20 units/mg protein. The second peak eluted, containing most of the activity, has a specific activity of 2333 units/mg protein. Its molecular weight, as estimated from calibration of the column, was between 3000–4000. Sometimes higher molecular weight fractions were obtained but their purity was very low.

The seeds were now germinated in the presence of radioactive sulphate—$^{35}SO_4$—for 72 hr and the amount of label incorporated into the TCA precipitable protein in the supernatant fraction of the seed homogenates was determined (Table 3).

The lettuce seeds incorporated 2 per cent of the radioactive label supplied into the soluble protein fraction. Germination of the seeds for 72 hr in 5×10^{-3} M K_2SO_4 did not inhibit the development of the trypsin-like enzyme activity; in fact it was slightly higher than in the control.

TABLE 3. INCORPORATION OF ^{35}S INTO THE SOLUBLE PROTEIN FRACTION OF 72-hr GERMINATED LETTUCE SEEDS

Amount of $^{35}SO_4$ in germination medium*		^{35}S incorporated into TCA ppt.	
μc	CPM $\times 10^{-6}$	CPM/g original dry weight ($\times 10^{-6}$)	% of ^{35}S incorporated
25	60	1·2	2·0
100	240	5·0	2·1

* 1 g seeds germinated in 7·5 ml 5×10^{-3} M K_2SO_4 which contains various amounts of $^{35}SO_4$.

The incorporation of ^{35}S into the trypsin-like enzyme was measured. Two lots of 1 g lettuce seeds were germinated in the presence of 7·5 ml 5×10^{-3} M K_2SO_4 containing 50 μc $^{35}SO_4$. After 72 hr the seeds were ground and the trypsin-like enzyme purified. 2·5 ml of the 40–65 per cent ammonium sulphate fraction, after dialysis, containing a total of $1{\cdot}28 \times 10^6$ cpm, were applied to the Sephadex G-75 column. 3·3 ml fractions were collected and the amount of ^{35}S in each fraction determined (Fig. 1). Although ^{35}S was incorporated in significant amounts into other fractions, the incorporation into the purified trypsin-like enzyme was almost nil and was of the order of 50–100 cpm/ml above background (depending on the exact fraction).

It can be argued that there is no incorporation of ^{35}S because the trypsin-like enzyme does not contain any sulphur groups. Using the DTNB method for the determination of SH groups a value of 0·46 μmole/ml of the purified trypsin-like enzyme fraction was measured. Since the concentration of protein in this fraction was 2 μg/ml and assuming that its molecular weight is about 4000, this corresponds to a value of about one SH group per molecule of partially purified trypsin-like enzyme. If the development of the trypsin-like activity during germination were due to *de novo* synthesis, using the above labelling technique, the incorporation of 0·46 μmole sulphate should be accompanied by a radioactive sulphate content of approximately 10,000–20,000 cpm into 1 ml of purified enzyme preparation. This would be easily detected by the techniques used. In order to show that when an enzyme is synthesized ^{35}S from $^{35}SO_4$ is incorporated, a suitable marker was sought. When lettuce seeds were germinated for 72 hr in the presence of 3 mg/ml chloramphenicol, more than 75 per cent of the normal development of acid phosphatase activity was inhibited. Acid phosphatase was therefore chosen as a marker enzyme. The enzyme was partially purified by ammonium sulphate fractionation after germinating the seeds for 72 hr in water. Two-thirds of the activity with a 4·5 fold purification was recovered in the fraction precipitating between 33–66 per cent saturation of ammonium sulphate. This fraction (2·5 ml) was then dialysed for 4 hr against 1 l. Tris-maleate buffer 0·04 M, pH 6·0, containing $1{\cdot}5 \times 10^{-3}$ M $MgSO_4$. 1·6 ml of this dialysate was further purified on a Sephadex G-100 column (Fig. 2).

Before purification the enzyme preparation had a specific activity of 2·5 units/mg protein. After ammonium sulphate fractionation and separation on the Sephadex column, the specific activity of the purified enzyme was 275·0. Its molecular weight as estimated from calibration of the column was 80,000–90,000.

The development of the acid phosphatase activity during germination was not affected

at all when seeds were germinated in the presence of 5×10^{-3} M K_2SO_4. The incorporation of ^{35}S into the acid phosphatase preparation was examined. Two lots of 1 g lettuce seeds were germinated for 72 hr in the presence of 7·5 ml 5×10^{-3} K_2SO_4 containing 50 μc $^{35}SO_4$. 1·6 ml of the 33–66 per cent ammonium sulphate fraction after dialysis, containing a total of 162,000 cpm, were applied to the Sephadex G-100 column and eluted as described above, and the amount of ^{35}S in each fraction determined (Fig. 2). It can be seen that there was considerable incorporation of ^{35}S into the fraction which contains the purified acid phosphatase.

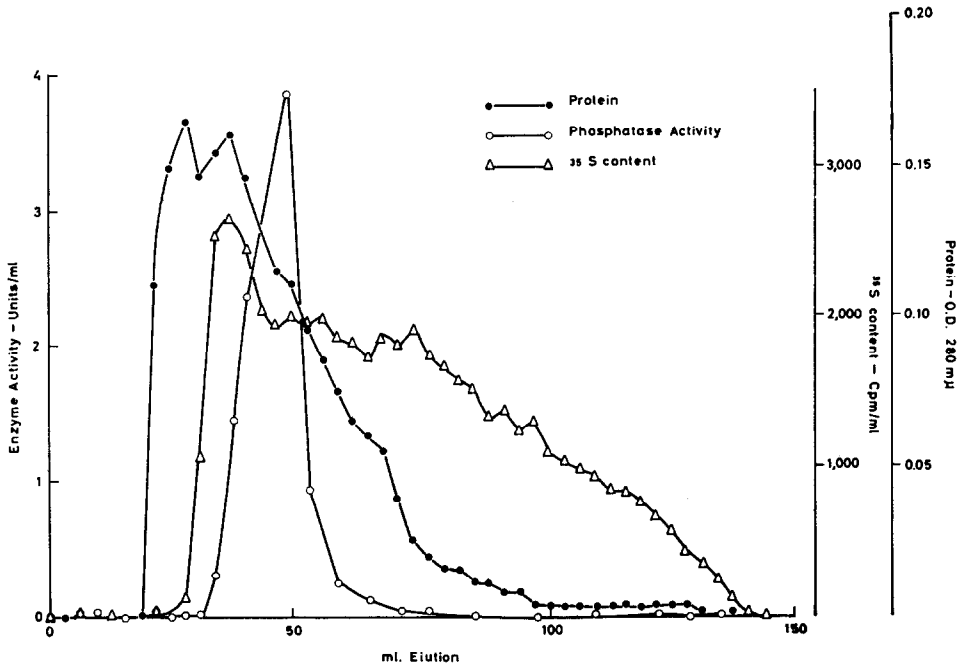

FIG. 2. PURIFICATION OF ACID PHOSPHATASE FROM GERMINATING LETTUCE AND INCORPORATION OF ^{35}S INTO THE VARIOUS PROTEIN FRACTIONS

●——● Protein
○——○ Phosphatase activity
△——△ ^{35}S content

(Fractions eluted from a Sephadex G-100 column. Dimensions: 75 × 1·5 cm, eluted with Tris-maleate buffer 0·04 M, pH 6·0, containing $1{\cdot}5 \times 10^{-3}$ $MgSO_4$; flow rate 11 ml/hr.)

From the data presented it appears that the appearance of the trypsin-like enzyme occurs only when the trypsin inhibitor disappears. It was therefore decided to investigate the mechanism of its disappearance. Since the dry seeds contain a proteolytic enzyme with a pH optimum at 6·8, it seemed possible that it is responsible for the destruction of the inhibitor as suggested earlier.[1] Extracts of dry seeds were therefore incubated with a commercial soybean trypsin inhibitor at 37°, layered under toluene. The residual proteolytic enzyme activity at pH 6·8 and the residual inhibitor activity were determined (Table 4).

The same experiment was repeated using different amounts of a partially purified preparation of endogenous trypsin inhibitor (Table 5).

These results bear out the conclusion that the destruction of the endogenous trypsin

inhibitor is enzymic and, concomitantly with its destruction, the trypsin-like enzyme is released from some bound form.

TABLE 4. PROTEOLYTIC ACTIVITY AT pH 6·8 AND SOYBEAN TRYPSIN INHIBITOR ACTIVITY REMAINING AFTER INCUBATION OF DRY SEED HOMOGENATES WITH COMMERCIAL SOYBEAN TRYPSIN INHIBITOR

Amount of trypsin inhibitor in incubation mixture* (units)	Proteolytic activity remaining (%)	Trypsin inhibitor activity remaining (units)	% inactivation of trypsin inhibitor
0	100	—	—
95	43	15	87
190	26	10	95

* Mixtures contain homogenates from 250-mg dry seeds. Proteolytic activity was measured using pectinase as the substrate of the 6·5 hr incubation at 37°. A suitable control which contained 190 units of trypsin inhibitor but no seed homogenate showed no decrease in the activity of soybean trypsin inhibitor over the 6·5 hr incubation period.

TABLE 5. EXAMINATION OF PROTEOLYTIC ACTIVITY AT pH 6·8 AND OF ENDOGENOUS TRYPSIN INHIBITOR ACTIVITY REMAINING AFTER INCUBATION OF DRY SEED HOMOGENATES WITH A PREPARATION OF ENDOGENOUS TRYPSIN INHIBITOR

Amount of endogenous trypsin inhibitor originally present in mixtures* (units)	Proteolytic activity remaining (%)	Trypsin inhibitor activity remaining (units)	% inactivation of trypsin inhibitor
0	100	—	—
255	26	75	71
904	9	388	57

* Mixtures contain an homogenate from 250-mg dry seeds. Proteolytic activity measured using pectinase as a substrate after incubation at 37° for 4 hr. A suitable control which contained 340 units of endogenous trypsin inhibitor but no seed extract showed only a 14 per cent loss of activity under these conditions.

DISCUSSION

The purpose of this work was to demonstrate that the trypsin-like enzyme activity, which develops in lettuce seeds during their germination, is not formed by *de novo* protein synthesis. The fact that germination in a solution of an inhibitor of protein synthesis does not prevent formation of the trypsin-like enzyme does not in itself provide the required proof. Even the observation that formation of an acid phosphatase is prevented by the same inhibitor is only supporting evidence for the view proposed here. This type of experiment can be criticized because Neuman[10] observed that Actinomycin D does not penetrate all parts of the seedling equally. More rigorous proof for the occurrence or non-occurrence of enzyme synthesis can be obtained using incorporation studies with a radioactive isotope. We selected $^{35}SO_4$ for this purpose, since the molecule is small and is readily taken up by the seed and because it does not undergo isotope exchange reactions with the other sulphur atoms. Moreover, the

[10] J. NEUMAN, *Physiol. Plantarum* **17**, 363 (1964).

sulphur in protein is in the form of S—S or SH bonds. Therefore the presence of ^{35}S in a protein would be unambiguous proof that the protein did arise due to net synthesis.

The results in Fig. 1 demonstrate clearly that no ^{35}S was incorporated into the highly purified preparation containing the trypsin-like enzyme. From Fig. 2 it is clear that some incorporation did occur into other proteins, for example an acid phosphatase. Furthermore it has been shown that the trypsin-like enzyme contains at least one —SH group per molecule. The incorporation of one sulphur atom per molecule of enzyme would be easily detected by the techniques used in these experiments. The failure to detect incorporation of ^{35}S could be due to the fact that the purified trypsin-like enzyme is diluted 100 fold with contaminating protein. Under these conditions the ^{35}S incorporation would become invalid. However, since the purification is more than 450 fold, so high a degree of contamination is unlikely.

The results on the incorporation thus provide strong evidence for the view that the trypsin-like enzyme does indeed arise from a process other than protein synthesis. Final conclusive proof can only be obtained by isolating and crystallizing the enzyme after incorporation studies. The question thus arises what is the mechanism of its formation.

The endogenous inhibitor present in the seeds disappears during germination (Table 1) and there is a concomitant rise in activity of the trypsin-like enzyme (Table 2). It has been demonstrated previously[1] that the endogenous inhibitor is capable of inhibiting the trypsin-like enzyme. Thus a possible mechanism of its release might be its liberation from a complex with the inhibitor. This could be due to destruction of the inhibitor, by a proteolytic enzyme present in the seed which has been previously demonstrated.[1] During this process the proteolytic enzyme involved is itself destroyed (Tables 4, 5). However, this is not sufficient to account for the whole process of enzyme formation. When the endogenous inhibitor has completely disappeared, after 24 hr, the trypsin-like enzyme has only reached 10 per cent of the activity present after 72 hr of germination. Therefore its subsequent formation must be due to additional processes. We can suggest two possible mechanisms for the subsequent liberation. Liberation might be due to an autocatalytic process involving the trypsin-like enzyme and some kind of trypsinogen form of it. Such processes are well known in animal tissues.[11] Alternatively, the additional proteolytic enzymes, also present in the seed, a carboxypeptidase active at pH 5·6 and the aminopeptidase active at pH 4·8 might be involved in liberating the trypsin-like enzyme from an inactive precursor form. These possibilities require further investigation before their validity can be established.

METHODS

Seeds were germinated, seed fractions prepared, proteolytic activities using pectinase as a substrate determined, trypsin inhibitor extracted and assayed as previously described.[1]

Activity towards benzoyl arginine ethylester (BAEE) obtained from Yeda (Rehovot) was determined according to Hestrin[12] at 37°. Incubation mixtures contained 3 ml McIlvaine buffer 0·1 M, pH 6·8, 1 ml BAEE 0·02 M, 1 ml enzyme. 1 ml aliquots were removed at zero time and after 2 hr and assayed for ester group content. A unit of enzyme is defined as the amount of enzyme which hydrolyses 1 μmole BAEE per hour under the above conditions.

Acid phosphatase was determined using *p*-nitrophenyl phosphate as the substrate.[13]

[11] P. DESNUELLE, in *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBACK), Vol. 4A, p. 119, Academic Press, New York (1960).
[12] S. HESTRIN, *J. Biol. Chem.* **180**, 249 (1949).
[13] O. H. LOWRY, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 4, p. 371, Academic Press, New York (1957).

The assay was carried out at 37° in Tris-maleate buffer 0·1 M, pH 5·2, containing 3×10^{-3} M $MgSO_4$. One unit of activity is defined as the amount of enzyme which releases 1 μmole *p*-nitrophenol/15 min under these conditions.

Fractions were collected in an LKB UltraRac fraction collector equipped with a drop counter. The effluents were monitored at 280 nm with a LKB Unicord II Ultraviolet Absorptiometer connected to an LKB chopper bar recorder.

Sephadex columns were calibrated using crystalline bovine serum albumin, soybean trypsin inhibitor and cytochrome C as markers.[14] The concentration of SH groups was determined with DTNB [5,5-Dithiobis (2 nitrobenzoic acid)] according to Asahi *et al.*[15] and Ellman.[16] Protein was determined according to Lowry *et al.*[17]

The amount of $^{35}SO_4$ was determined in a Packard Tri-Carb Scintillation Spectrometer Model 3002. The scintillation liquid was a mixture of xylene:cellosolve:dioxane prepared according to Bruno and Christian.[18] The optimal gain was 8·1 per cent in a 50–1000 window. A quench correction curve was prepared using 0·1–2·0 ml water and the automatic external standardization. (1·5 per cent gain—300–700 window). On the basis of this curve all readings were corrected for quenching. Incorporation of $^{35}SO_4$ into the soluble protein fraction was determined for 72 hr in different amounts of $^{35}SO_4$. The seeds were ground and the supernatant fraction obtained after centrifugation at $30{,}000 \times g$ for 0·5 hr was collected. 3 ml 20 per cent TCA were added to 3 ml of this supernatant. The mixture was centrifuged at 10,000 *g* for 10 min. The resulting precipitate was suspended in 10 ml 10 per cent TCA and heated at 90° for 10 min. The mixture was cooled and again centrifuged at $10{,}000 \times g$ for 10 min. The resulting precipitate was suspended in 3 ml buffer and 0·5–1·0 ml aliquots were added to a mixture of 15 ml scintillation liquid and 0·8 g Cab-O-Sil. The resulting stable suspension was counted in the Tri-Carb.

$^{35}SO_4$, carrier free, was obtained from the Radiochemical Centre, Amersham.

Soybean trypsin inhibitor type II-S was obtained from Sigma Chemical Co.

Acknowledgements—This work is part of a Ph.D. thesis of Y. S. to be presented to the Hebrew University. It is supported in part by a grant Fg Is 239 of the United States Department of Agriculture to A. Poljakoff-Mayber and A. M. Mayer.

Our thanks are due to General Aniline & Film Corp. for a sample of Polyclar A.T. and to Miss Hanna Meyer for technical assistance.

[14] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).
[15] T. ASAHI, R. S. BANDURSKI and L. G. WILSON, *J. Biol. Chem.* **236**, 1830 (1961).
[16] G. L. ELLMAN, *Arch. Biochem. Biophys.* **82**, 70 (1959).
[17] O. H. LOWRY, N. J. ROSEBROUGH, L. A. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[18] G. A. BRUNO and J. E. CHRISTIAN, *Anal. Chem.* **33**, 1216 (1965).