This number is approximate, but even allowing for error the value lies in the range typical of globular proteins.

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A Water-insoluble Polyanionic Derivative of Trypsin. I. Preparation and Properties*

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A number of water-insoluble polyanionic derivatives of trypsin (IMET) were prepared by coupling the enzyme to a copolymer of maleic anhydride and ethylene, cross-linking with hexamethylenediamine, and hydrolyzing the unreacted maleic anhydride residues. Different IMET preparations with varying bound protein-to-carrier ratios (from 1:20 to 3:1) were obtained. Their esteratic activities per unit weight of bound protein corresponded to 40–70% of that of crystalline trypsin. All of the IMET preparations studied were found to be considerably more stable than trypsin in the alkaline *p*H range (*p*H 7.0–10.7). The polyelectrolyte trypsin derivatives, IMET-2 and IMET-8, retained practically all their enzymatic activity on storage for 3–8 months under 0.1 M phosphate buffer of *p*H 7 at 4°. The IMET preparations could be lyophilized and kept in dry form, at room temperature, without marked loss in activity. IMET samples rich in carrier retained some of their enzymatic activity in 8 M urea. The digestion of the high-molecular-weight substrates casein, hemoglobin, lysozyme, and poly-L-lysine by the various IMET preparations has been investigated. Chymotrypsinogen could be activated to chymotrypsin by IMET-1 and IMET-6 as well as by a water-insoluble polytyrosyl trypsin derivative. Most of the esterase activity toward benzoyl-L-arginine ethyl ester of the various IMET preparations could be inhibited by pancreatic trypsin inhibitor at a 1:1 weight ratio of inhibitor to bound protein. Practically no inhibition of the esteratic activity by soybean-trypsin inhibitor occurred even at a 50:1 weight ratio of inhibitor to bound enzyme protein. The soybean-trypsin inhibitor was found, however, to be an effective inhibitor of the proteolytic activity of the IMET's as tested on casein. The *p*H-activity profiles of an IMET preparation rich in carrier (IMET-1) and of an IMET preparation rich in protein (IMET-6) at different ionic strengths were determined.

The preparation of a water-insoluble carboxymethylcellulose derivative of trypsin was reported by Mitz and Summaria (1961). The water-insoluble preparation obtained was found to be more stable than native trypsin. Epstein and Anfinsen (1962) used a preparation of trypsin bound to carboxymethyl-cellulose in their experiments on the reversible reduction of the disulfide bonds of this enzyme. Water-insoluble polytyrosyl derivatives of trypsin (IPTT)[1] were obtained in this laboratory (Bar-Eli and Katchalski, 1960, 1963) by coupling polytyrosyl trypsin (Glazer *et al.*, 1962) with a water-insoluble polydiazonium salt derived from a copolymer of *p*-amino-DL-phenylalanine and L-leucine. The water-insoluble polytyrosyl trypsin preparations showed an esteratic activity per unit weight of bound protein corresponding to 15–30% of that of crystalline trypsin. Lower enzymatic activities were recorded for water-insoluble trypsin derivatives obtained by coupling unmodified trypsin with the water-insoluble carrier. The water-insoluble enzyme preparations were considerably more stable in the alkaline *p*H range (*p*H 7–9) than either trypsin or polytyrosyl trypsin. A column possessing tryptic activity was prepared from water-insoluble polytyrosyl trypsin.

In this paper a description is given of the preparation and properties of a new type of water-insoluble trypsin derivative in which trypsin is covalently bound to a

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[1] The abbreviations used are: IPTT, water-insoluble polytyrosyl trypsin; IMET, water-insoluble copoly-(maleic acid–ethylene)-trypsin; BAEE, benzoyl-L-arginine ethyl ester; ATEE, acetyl-L-tyrosine ethyl ester.

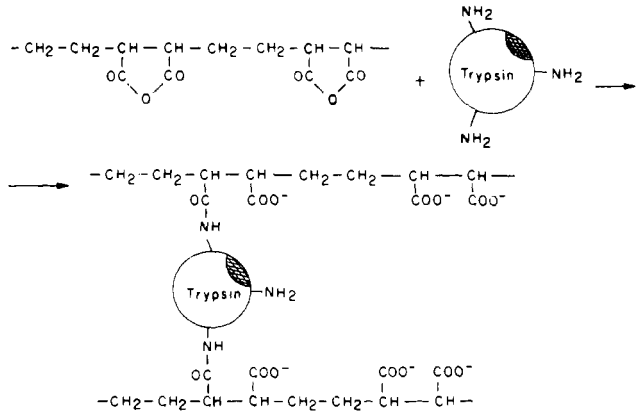

FIG. 1.—Schematic representation of the preparation of a water-insoluble copoly-(maleic acid–ethylene)-trypsin derivative (IMET).

water-insoluble polyelectrolyte gel. Our interest in trypsin derivatives of this type stems from their possible use in the elucidation of the effect of an electrostatic field in the molecular domain of an enzyme on its properties and mode of action.

The water-insoluble trypsin derivatives required were obtained by the covalent binding of trypsin to a copolymer of maleic anhydride and ethylene. In the proposed scheme (see Fig. 1) the ε-amino groups of the lysyl residues of trypsin, known to be nonessential for enzymatic activity (Terminiello *et al.*, 1955; Sri Ram *et al.*, 1954, 1962; Wong and Liener, 1960), were assumed to react with the maleic anhydride residues of the copolymer. Cross-linking of the carrier was effected by the polyfunctional trypsin molecules themselves, as well as by hexamethylenediamine. Hydrolysis of the unreacted maleic anhydride residues yielded the water-insoluble copoly-(maleic acid–ethylene)-trypsin derivatives (IMET) discussed.

By the preparative procedure elaborated it was possible to prepare IMET samples containing up to 80% by weight of bound protein. The esteratic activity of the bound protein was high (40–70%) in practically all the IMET preparations synthesized. IMET suspensions could be dried or lyophilized without loss in activity. The dried IMET powders were found to retain most of their activity for many months, when stored under appropriate conditions. Of interest is the finding that various IMET preparations preserve a considerable amount of enzymic activity in 8 M urea. The digestion of casein, hemoglobin, and lysozyme by IMET preparations of varying protein content was investigated.

The effect of the electrostatic field of the polyelectrolyte carrier on the *p*H-activity curve, and on the Michaelis constant of the bound trypsin, is discussed in the following paper (Goldstein *et al.*, 1964).

EXPERIMENTAL

Materials.—Trypsin (2 × crystallized, salt free, and lyophilized), chymotrypsinogen (crystalline, salt free), lysozyme (2 × crystallized, salt free, LYSF 628), soybean-trypsin inhibitor (3 × crystallized), and pancreatic-trypsin inhibitor (crystallized and lyophilized), were obtained from Worthington Biochemical Corp., Freehold, N.J. Water-insoluble polytyrosyl trypsin (IPTT) was prepared according to Bar-Eli and Katchalski (1963). Casein, Hammersten quality, and hemoglobin, substrate powder HB588, were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), acetyl-L-tyrosine ethyl ester (ATEE), and poly-L-lysine hydrobromide of average degree of polymerization, n = 1000, were obtained from Yeda Research and Development Co., Rehovoth, Israel.

The carrier, a linear copolymer of maleic anhydride and ethylene (molar residual ratio, 1:1), DX-840, lot 949, was a gift from Monsanto Chemical Co., Inorganic Chemical Div., St. Louis, Mo. It is soluble in dimethylformamide and insoluble in dioxane and water. A 1% solution in dimethylformamide at 25° yielded a specific viscosity of η_{sp} = 0.6, corresponding to an average molecular weight of 40,000. The DX-840 preparation used was found to undergo partial hydrolysis as a result of aspiration of moist air. However, over 90% of the hydrolyzed groups could be converted back to the anhydride on heating to 105–110° for 24 hours *in vacuo*. Complete hydrolysis of the maleic anhydride residues, and the formation of a water-soluble maleic acid–ethylene copolymer, could be effected by heating a suspension of DX-840 in water at 95° for 10–15 minutes with stirring. For further information concerning the chemical properties of the resin used and some of its industrial applications, see Monsanto Bulletin No. 1066, entitled "DX-840 Resins."

Water-insoluble Copoly-(maleic Acid–Ethylene)-Trypsin Derivatives (IMET).—Several water-insoluble trypsin derivatives of this kind were prepared (see Table I). The general procedure adopted was as follows: The maleic anhydride–ethylene copolymer (100 mg) was suspended in ice-cooled 0.2 M potassium phosphate buffer, *p*H 7.5 (10 ml), the suspension was homogenized, and the cross-linking agent hexamethylenediamine (1 ml of a 1% solution) was added with stirring. The appropriate amount of trypsin, dissolved in the same buffer (5 ml), was added after 2–3 minutes, and the reaction mixture was left overnight at 4° with magnetic stirring. At the end of this period practically all of the anhydride residues which had not reacted with the protein underwent hydrolysis as indicated by a negative hydroxamic acid test (Hestrin, 1949). The precipitate was centrifuged and washed about 20 times alternately with water and 0.05 M phosphate buffer, *p*H 7.0, or 0.1 M NaCl, until no residual enzymatic activity toward BAEE could be detected in the supernatant after filtration through a Millipore filter, Type HP, of 0.45 μ mean pore size.

Optimal binding of trypsin to the maleic anhydride–ethylene copolymer was found to take place in the *p*H range 6–8. Considerable binding was observed also at *p*H 4–6, however. Because of the high solubility in water of the maleic acid–ethylene copolymer, cross-linking of the carrier via a bifunctional agent, such as hexamethylenediamine, was found necessary. IMET preparations with highest activity were obtained at a molar ratio of maleic anhydride to hexamethylenediamine of 10:1. Larger amounts of cross-linking agent resulted in preparations with considerably lower activities. In preparations containing excess enzyme (such as IMET-6 to -8), the use of hexamethylenediamine could be omitted without significant decrease in yields. With hydrazine as cross-linking agent, water-insoluble trypsin derivatives more dense and compact than those produced with hexamethylenediamine were obtained. To free the various IMET preparations from adsorbed trypsin or water-soluble trypsin-copolymer derivatives, repeated washing with water, phosphate buffer, or aqueous NaCl was found necessary. No active enzyme could be detected in solution after filtration through a Millipore filter of reaction mixtures containing substrate (BAEE) and the exhaustively washed IMET preparations listed in Table I.

TABLE I

COMPOSITION AND ACTIVITY OF WATER-INSOLUBLE COPOLY-(MALEIC ACID–ETHYLENE)-TRYPSIN PREPARATIONS (IMET)[a]

Water-insoluble Trypsin Derivative	Trypsin in Coupling Mixture (mg/100 mg carrier)	Yield of Water-insoluble Enzyme (mg)	Protein Content per 100 mg of Water-insoluble Enzyme (mg)	Esteratic Activity of Bound Protein (%)
IMET-1	10	87	4.6	53
IMET-2	13	100	6.2	60
IMET-3	50	113	16.8	76.5
IMET-4	50	135	15	68.5
IMET-5	50	148	22	43
IMET-6	400	280	73	42
IMET-7	400	184	44	69.5
IMET-8	400	335	70	48.5

[a] The water-insoluble trypsin preparations listed were obtained as described in the text. Coupling mixtures contained, per 100 mg carrier, trypsin in the amounts given in column 2. The yields of water-insoluble enzyme derivatives are given in column 3. The protein content of the various IMET preparations was calculated either from total nitrogen analysis (Kjeldahl), or from the amount of amino acids liberated on acid hydrolysis (see text). Esteratic activity was determined by the *p*H-stat method with benzoyl-L-arginine ethyl ester as substrate.

The anhydride content of the maleic anhydride–ethylene copolymer was found to play a significant role in determining the amount of active, bound enzyme. Best results were obtained with copolymers in which 60–80% of the maleic anhydride groups were unhydrolyzed. The copolymer undergoes spontaneous hydrolysis on exposure to moisture; the polymer samples used were stored, therefore, over sulfuric acid. In all cases it was possible to transform the maleic acid residues of the copolymer into maleic anhydride residues by heating to 100° *in vacuo*.

The maleic anhydride–ethylene copolymer is soluble in dimethylformamide; several attempts were made, therefore, to prepare some water-insoluble trypsin derivatives of the above type, by reacting the copolymer in dimethylformamide with trypsin in 0.1 M phosphate buffer, *p*H 7.5. Preparations with relatively low tryptic activity were obtained.

The physical characteristics of the various water-insoluble trypsin derivatives described here were found to depend on their protein content. IMET preparations of high protein content (IMET-6, -7, -8) are flakelike; whereas preparations of low protein content (IMET-1, -2, -3, -4) are gellike. Suspension in water causes swelling of all the IMET preparations studied; addition of salts (0.05–0.1 M phosphate, *p*H 7–8, or 0.1 M NaCl), on the other hand, leads to contractions and a flakelike appearance.

The data given in Table I show that the protein content of the various IMET preparations increases as the initial amount of trypsin in the reaction mixture is increased. Thus it was possible to obtain IMET preparations with as high a protein-to-carrier ratio as 3:1 (IMET-6), and as low a protein-to-carrier ratio as 1:20 (IMET-1). Most of the experiments to be described below were performed with IMET-1 and IMET-6 because of their widely different carrier-to-protein ratios.

Maleic Anhydride and Maleic Acid Content of the Ethylene–Maleic Anhydride Copolymer.—Different samples of the maleic anhydride–ethylene copolymer were found to contain varying amounts of maleic acid residues owing to hydrolysis of the anhydride residues on contact with moisture. The maleic anhydride and maleic acid contents were determined as follows: The total amount of maleic acid residues + maleic anhydride residues was determined by titration of the copolymer in aqueous solution, 0.5 M in NaCl, after hydrolysis in boiling water for 10–15 minutes, using aqueous 0.1 N NaOH as titrant and phenolphthalein as indicator. Two moles of titrant are obviously consumed per base mole of maleic acid or maleic anhydride. In a parallel experiment, the intact copolymer was dissolved in anhydrous dimethylformamide and the solution was titrated with 0.1 N sodium methoxide in methanol-benzene, using thymol blue as indicator (Patchornik and Ehrlich-Rogozinsky, 1961). One mole of titrant is consumed per base mole of anhydride, whereas two moles of titrant are consumed per base mole of maleic acid. The amount of anhydride and free carboxyl groups initially present in the ethylene–maleic anhydride copolymer was calculated from the two titration values.

Determination of Bound Protein.—The protein content of the IMET preparations containing relatively high amounts of protein (IMET-6, IMET-7, and IMET-8) was calculated from their nitrogen content (Kjeldahl), assuming 14.9% nitrogen for trypsin. The contribution of the bound hexamethylenediamine to the total nitrogen, in these preparations, did not exceed 2%. The protein content of the IMET preparations containing relatively small amounts of protein (IMET-1, IMET-2, IMET-3, and IMET-4) was calculated from their amino acid content after exhaustive acid hydrolysis in 6 N HCl for 48 hours at 115° in sealed tubes. The neutral amino acids liberated were analyzed according to Spackman *et al.* (1958; see also Moore and Stein, 1963). A control analysis of native trypsin yielded the values reported by Neurath (1957).

Determination of Enzymatic Activity by the pH-Stat Method.—Rates of hydrolysis of benzoyl-L-arginine ethyl ester were determined by the *p*H-stat method (Laskowski, 1955) with an automatic titrator Model TTTlc, and titrigraph type SBR2c (Radiometer, Copenhagen). The assay mixture was placed in a jacketed cell maintained at 25°. The activities of trypsin and the various IMET preparations were calculated from the initial rates of hydrolysis.

The enzymatic activities of trypsin and of the different IMET preparations were determined at *p*H 7.6 and 9.5, respectively, using BAEE as substrate. A *p*H of 9.5 was chosen for assaying the IMET preparations since they show maximum esteratic activity in the *p*H range 9.5–10 (see Fig. 4). The reaction mixture (5 ml) was 0.01 M in phosphate and 5.8×10^{-3} M in substrate. NaOH (0.1 N) was used as titrant. Trypsin at a concentration of 2.0–20 μg/ml of reaction mixture gave a specific activity of 35×10^{-6} mole/minute per mg enzyme per ml. The determination of the rate of substrate hydrolysis by the different IMET preparations permitted the calculation of the esteratic activity

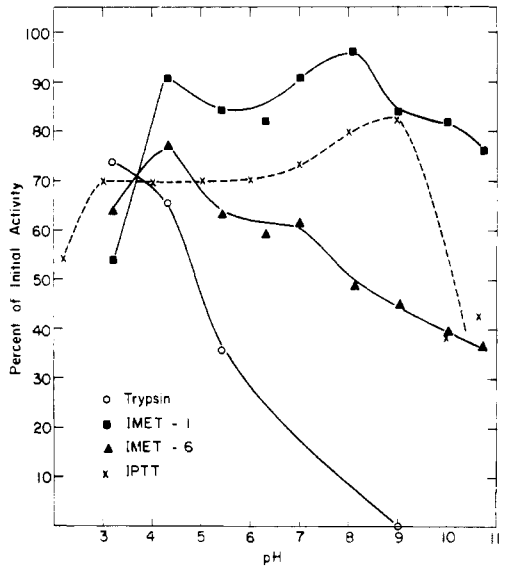

FIG. 2—Effect of pH on the stability of trypsin, IMET-1, IMET-6, and IPTT. The test solutions (0.5 ml) in the appropriate buffer contained trypsin (0.5 mg/ml) or an amount of IMET-1, IMET-6, or IPTT possessing a similar esteratic activity. The mixtures were incubated at 25° for 24 hours, 0.1-ml aliquots were withdrawn, and the esteratic activity was determined by the *p*H-stat method using BAEE as substrate (5 ml of a 5.8 × 10^{-3} M solution). NaOH (0.1 N) was used as titrant (see Experimental). The buffers used to cover the *p*H range investigated were as follows: *p*H range 3.2–6.3, 0.05 M citrate-phosphate buffer; *p*H range 7.0–9.0, 0.05 M Tris buffer; *p*H range 10.0–10.7, 0.05 M carbonate-bicarbonate buffer.

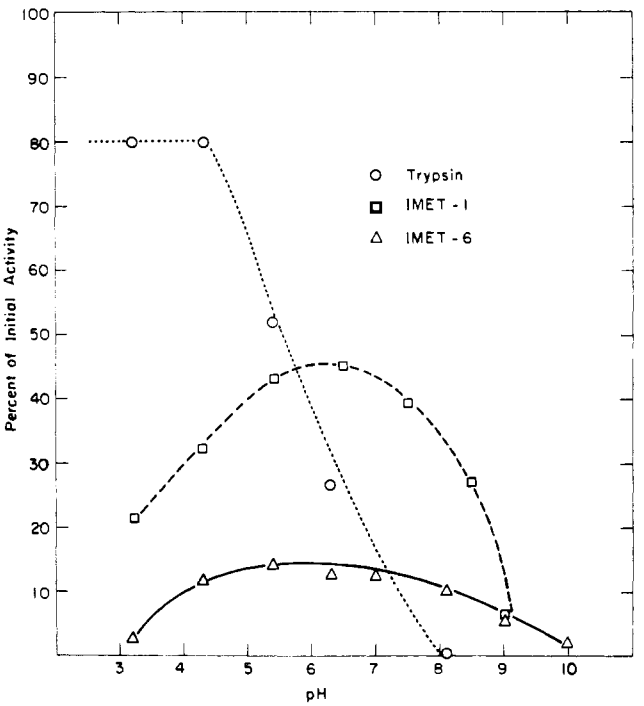

FIG. 3.—Heat inactivation of trypsin, IMET-1, and IMET-6. The reaction mixtures (0.5 ml) in the appropriate buffer (see legend to Fig. 2) containing trypsin (0.5 mg/ml), or an amount of IMET-1 or IMET-6, possessing the same esteratic activity, were kept in boiling water for 10 minutes and then immersed in an ice bath for 30 minutes; 0.1-ml aliquots were withdrawn, and the esteratic activity was determined by the *p*H-stat method using BAEE as substrate (5 ml of a 5.8 × 10^{-3} M solution). NaOH (0.1 N) was used as titrant.

of the bound protein relative to native trypsin (see Table I).

Determination of Tryptic Activity on Casein and Hemoglobin.—This was carried out according to the method of Northrop *et al.* (1948), in which the increase in trichloroacetic acid-soluble peptides is followed spectrophotometrically at 280 mμ. Mixing of the reaction mixtures containing water-insoluble enzyme was effected by shaking in a constant-temperature bath at 35°.

Tryptic Digestion of Lysozyme.—Lysozyme in aqueous solution (0.1%) was denatured by heating to 100° for 8 hours according to McLaren and Estermann (1956). To 2 ml of the denatured protein solution were added 3 ml of 0.1 M phosphate buffer, *p*H 7.5, or 0.05 M Tris buffer, *p*H 9.0, and 1 ml of a solution of trypsin or a suspension of IMET containing the desired amount of enzyme. The reaction mixtures were incubated with shaking at 35°, and the extent of hydrolysis after 20 minutes was estimated by spectrophotometric determination at 280 mμ of the trichloroacetic acid-soluble peptides (Northrop *et al.*, 1948).

Tryptic Digestion of Poly-L-lysine.—The rates of hydrolysis of this polypeptide were determined by the *p*H-stat method at *p*H 7.7, according to Waley and Watson (1953). The reaction mixture (5 ml) contained poly-L-lysine (50 mg), and 0.1 mg trypsin or an amount of IMET possessing the same esteratic activity.

RESULTS

*Stability as a Function of p*H.—The enzymatic activity of the two water-insoluble IMET preparations, IMET-6 and IMET-1, after incubation at 25° for 24 hours at different *p*H values is recorded in Figure 2. For comparison the activities of trypsin and water-insoluble polytyrosyl trypsin, IPTT, incubated under similar conditions, are also included. The data presented show that both IMET-1 and IMET-6 are more stable than trypsin between *p*H 5 and 10. Similar behavior is shown also by IPTT. IMET-1, the preparation rich in carrier, is somewhat more stable than IMET-6, the preparation rich in protein. Thus at *p*H 10, IMET-1, IMET-6, and IPTT retain about 80, 40, and 50%, respectively, of their initial esteratic activities. At the most alkaline *p*H tested, *p*H 11, IMET-1 was found to be the most stable trypsin derivative.

Trypsin is known to undergo reversible denaturation at different *p*H values on short exposure to elevated temperatures (Kunitz and Northrop, 1934; Terminiello *et al.*, 1958). It seemed desirable, therefore, to test the recovery of enzymatic activity of the water-insoluble trypsin derivatives described here after heating to 100° and subsequent cooling. The percentage of activity recovered for IMET-6 and IMET-1, heated to 100° for 10 minutes, at the *p*H values specified, and then kept for 30 minutes at 0° is recorded in Figure 3. The IMET preparations investigated differed markedly from trypsin in their behavior. Trypsin shows highest recovery of enzymatic activity in the *p*H range 3–4. Above *p*H 4 there is a gradual decrease in the residual activity, the enzyme being completely inactivated at *p*H 8.0. The IMET samples, on the other hand, exhibited maximum recovery at *p*H 5–7. Lower residual activities were found at more acid and alkaline *p*H values. The enzymatic activities recovered throughout the *p*H range studied were higher for IMET-1 than for IMET-6. The former trypsin derivative contains a considerably higher fraction of polyelectrolyte carrier than the latter. It should be noted that stability behavior similar to that of the

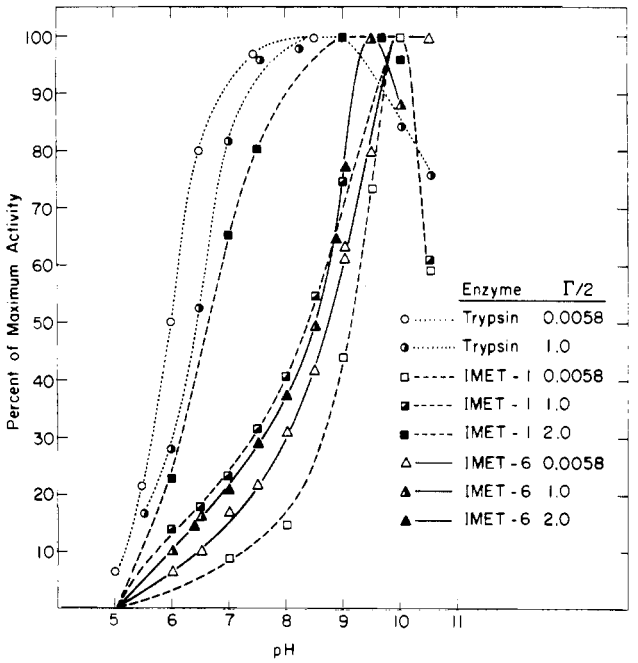

FIG. 4.—*p*H-Activity curves of trypsin, IMET-1, and IMET-6 using BAEE as substrate, at different ionic strengths. Reaction mixtures contained 5.8×10^{-3} M BAEE (5 ml) and trypsin (0.1 mg), or an amount of IMET possessing similar esteratic activity (evaluated at the corresponding *p*H of maximum activity). Ionic strengths were adjusted with NaCl. Substrate hydrolysis, at 25°, was determined potentiometrically using 0.1 N NaOH as titrant. The initial rates recorded at the *p*H value of maximum activity were 3×10^{-6} mole/min for trypsin, 0.68×10^{-6} mole/min for IMET-1, and 1.14×10^{-6} mole/min for IMET-6.

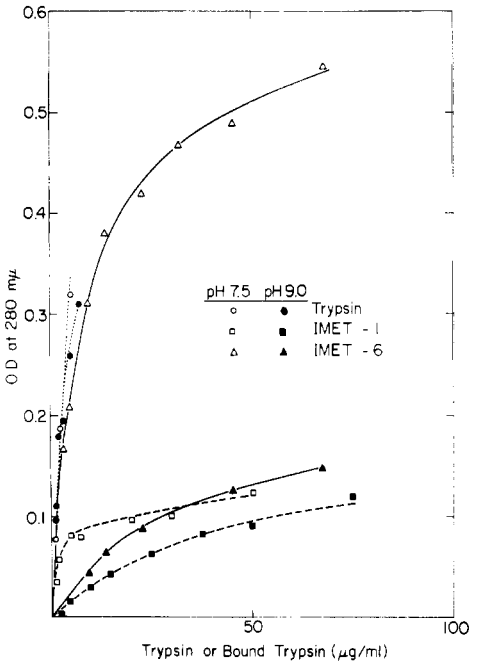

FIG. 5.—Digestion of casein by varying amounts of trypsin IMET-1, and IMET-6 at *p*H 7.5 and *p*H 9.0. The test solutions (2 ml) contained 5 mg/ml, heat-denatured casein in 0.05 M phosphate buffer, *p*H 7.5, or 0.05 M Tris buffer, *p*H 9.0. The reaction mixtures were incubated at 37° with the appropriate amount of trypsin, or IMET possessing the same esteratic activity, for 20 minutes; 3 ml of a 5% solution of trichloroacetic acid was then added, the precipitate was filtered off, and the optical density of the trichloroacetic acid–soluble fraction was determined at 280 mμ.

IMET's has been reported by Terminiello *et al.* (1958) for succinyl trypsin. Excess negative charge seems to play an important role in determining the heat stability at different *p*H values of the two types of enzyme derivatives.

Change in Activity on Prolonged Storage.—An IMET preparation (IMET-2) stored under 0.1 M phosphate buffer, *p*H 7.0, for 3 months at 4° was found to retain practically all of its esteratic activity at the end of this period. IMET-8 retained full activity even on storage for 8 months under the same conditions. IMET-2 lost approximately 20% of its original esteratic activity, when stored for 3 months in the cold under distilled water; a higher loss in activity (about 50%) was found on storage under 10^{-3} M HCl.

Aqueous suspensions of IMET-1 and IMET-6 yield, on lyophilization or drying at room temperature over concentrated sulfuric acid, powders retaining approximately 70–90% activity. Homogenization of the dried powders in water or buffer was required to reconvert the preparations to fine suspensions. The dried preparations retained 60–80% of their esteratic activity after storage for 3 months at room temperature.

Activity in 8 M Urea.—When an amount of IMET-1 possessing an esteratic activity equivalent to that of 40 μg trypsin, was added to 5.8×10^{-3} M BAEE in 8 M urea (5 ml), at 25°, the rate of substrate hydrolysis, as measured *p*H-statically, dropped within 5 minutes to a value corresponding to about 50% of the enzymatic activity measured in the absence of urea. The rate of hydrolysis then remained practically unaltered for at least 30 minutes. It may be noted that when IMET, presuspended in 8 M urea, was added to BAEE in 8 M urea, a lower enzymatic activity corresponding to approximately 15–20% of the activity in the absence of urea was recorded. The enzymatic activity in both cases decreased slowly with time, a few per cent of the initial activity being retained after 24 hours. Upon pretreatment of native trypsin with 8 M urea at room temperature complete inactivation occurred. Addition of intact enzyme to BAEE in 8 M urea, however, led to an esteratic activity corresponding to approximately 5% of that in the absence of urea, which still could be detected in the reaction mixture after 15 minutes at room temperature.

The above data indicate that trypsin bound to a negative polyelectrolyte is considerably more resistant to denaturation by urea than native trypsin. Furthermore, the urea denaturation process appears to be somewhat retarded by the low-molecular-weight substrate BAEE.

*p*H-*Activity Curves.*—The *p*H dependence of the initial rates of hydrolysis of BAEE by trypsin, IMET-1, and IMET-6, at different ionic strengths at 25°, is given in Figure 4. The enzymatic activities for each set of experiments, carried out at constant ionic strength, are expressed as percentage of the maximum activity attained at the appropriate optimal *p*H (*p*H 8.0 for trypsin and *p*H 9–10.5 for the different IMET preparations). The *p*H-activity profile of IMET-1 at low ionic strength ($\Gamma/2 = 5.8 \times 10^{-3}$), when compared with that of trypsin under similar conditions, was found to be displaced by about three *p*H units toward more alkaline *p*H values. At high ionic strength ($\Gamma/2 = 2.0$), the *p*H-activity curve of IMET-1 shifts toward more acid *p*H values, approaching the *p*H-activity curve of trypsin. The displacement of the *p*H-activity curve of IMET-6, at $\Gamma/2 = 5.8 \times 10^{-3}$, toward more acid *p*H values, in comparison to trypsin, is somewhat

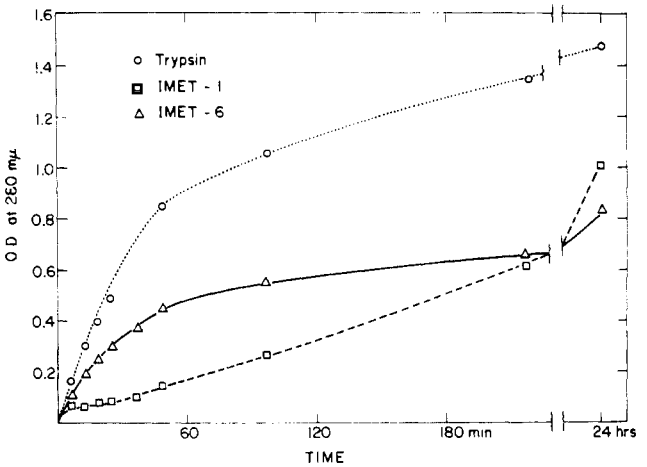

FIG. 6.—Time course of casein digestion by trypsin, IMET-1, and IMET-6 at *p*H 7.5. Reaction mixtures (2 ml) contained 5 mg/ml heat-denatured casein in 0.05 M phosphate buffer, *p*H 7.5, and trypsin (3 μg/ml), or a suspension of IMET-6 or IMET-1 possessing an esteratic activity equivalent to that of 3 μg/ml and 5 μg/ml trypsin, respectively. At the times specified, 3 ml of a 5% solution of trichloroacetic acid was added, the precipitates were filtered off, and the optical density of the trichloroacetic acid–soluble fraction determined at 280 mμ.

smaller than that observed with IMET-1, and is almost independent of the ionic strength of the medium.

Tryptic Digestion of High-Molecular-Weight Substrates.—CASEIN.—The extent of hydrolysis of casein on incubation for 20 minutes at *p*H 7.5 and 35° with different amounts of IMET-1, IMET-6, and trypsin, possessing similar esteratic activities, is given in Figure 5. The data presented show that the rates of hydrolysis of casein by IMET-6, the protein-rich preparation, are similar to those of native trypsin. The rates of hydrolysis of casein by IMET-1, the protein-poor preparation, on the other hand, are markedly lower than those of trypsin or IMET-6. On increasing the *p*H to 9.0 (Fig. 5), the caseinolytic activity of IMET-6 drops markedly, approaching that of IMET-1. The time course of the hydrolysis of casein at *p*H 7.5 by IMET-1, IMET-6, and trypsin, possessing the same esteratic activity, is given in Figure 6. It may be seen that the initial rate of hydrolysis of casein by IMET-1 is considerably lower than by the native enzyme. However, after long times of hydrolysis, IMET-1 hydrolyzed casein to the same extent as did both IMET-6 and native trypsin.

In an attempt to interpret the foregoing results the following variables should be considered: (1) electrostatic repulsion, at *p*H 7.5 and 9.0, between the negatively charged IMET's[2] and the negatively charged casein (isoelectric point 4.5); (2) the size of the substrate molecules and their ability to penetrate the enzyme gel; (3) stability of the enzyme. The evaluation of the relative contribution of each of these factors in determining the overall characteristics of the IMET-casein system is impossible at this stage. It seems, however, that at *p*H 9.0, when the polyelectrolyte carrier is practically completely ionized, electrostatic repulsion plays a predominant role in determining the relatively low rates of digestion of casein by both IMET preparations (see Fig. 5). At *p*H 7.5, at which *p*H the polyelectrolyte carrier is only partially ionized and the electrostatic repulsion between casein and the water-insoluble trypsin derivatives is weaker than at *p*H 9.0, steric factors determined by the macromolecular character of the substrate may play a role. It may perhaps be that since the bound trypsin molecules of IMET-6, the protein-rich preparation, are more accessible to the casein substrate than the enzyme molecules in IMET-1, which contains a high fraction of polyelectrolyte carrier, proteolytic digestion by the former insoluble enzyme is considerably higher than by the latter.

HEMOGLOBIN.—IMET-1 and IMET-6 digested hemoglobin similarly to casein. Hemoglobin is readily hydrolyzed by both IMET-1 and IMET-6 at *p*H 7.5 as well as at *p*H 9.0. The extent of hydrolysis by varying amounts of IMET-6, after 10 minutes at *p*H 7.5, was similar to that found for native trypsin. Considerably lower extents of hydrolysis were recorded under similar conditions for IMET-1. Both IMET preparations displayed rather low proteolytic activity toward hemoglobin at *p*H 9.0.

LYSOZYME.—The behavior of IMET-1 and IMET-6 toward lysozyme differed somewhat from their behavior toward hemoglobin and casein. The extent of hydrolysis of heat-denatured lysozyme (McLaren *et al.*, 1956; 1 mg/ml) by varying amounts of IMET-6, after 20 minutes at *p*H 7.5, was lower than that found with native trypsin. IMET-1 hydrolyzed to an even lesser extent under the same conditions. Raising the *p*H to 9 did not affect the extent of hydrolysis by the two IMET preparations. This difference in the behavior of lysozyme in comparison to casein (Fig. 5) and hemoglobin is probably owing to the fact that lysozyme, which has an isoelectric point of 11.0 (Anderson and Alberty, 1948), is still positively charged at *p*H 9.0.

Preliminary experiments were performed in which the peptides liberated on exhaustive digestion (at *p*H 7.5, 37°, for 18 hours) of heat-denatured lysozyme (1 mg/ml) with trypsin (16 μg/ml), IMET-1, or IMET-6 (possessing an esteratic activity of 30 μg trypsin/ml) were mapped by two-dimensional chrom-electrophoresis (Katz *et al.*, 1959). The three peptide maps closely resembled each other; however, a few spots present in the map of the trypsin digest were found to be missing in the IMET digests.

It is of interest that native lysozyme could be digested by some of the IMET preparations investigated in the presence of 8 M urea, whereas no digestion whatsoever occurred with native trypsin under similar conditions. The hydrolysis (in reaction mixtures containing 100 mg lysozyme in 10 ml 8 M urea, and an amount of IMET possessing the esteratic activity of 2 mg trypsin) at *p*H 7.5 and 37° was followed *p*H-statically. With IMET-1 (enzyme-to-carrier ratio, 1:20), a very fast rate of hydrolysis was observed, about 40 μmoles NaOH being used in the first 6 minutes of the reaction. With IMET-5 (enzyme-to-copolymer ratio, 1:5), a much slower rate of hydrolysis was observed; 20 μmoles of alkali were consumed within the first 30 minutes of the reaction. With IMET-6, the enzyme-rich preparation (enzyme-to-carrier ratio, 3:1), no alkali uptake occurred. These findings indicate that the various IMET preparations are considerably more active in concentrated urea solutions than trypsin. It thus seems feasible to utilize such modified enzymes for the digestion of intact proteins in 8 M urea.

(d) POLY-L-LYSINE.—At *p*H 7.7, IMET-5, IMET-8, and IMET-6 (see Table I) showed similar proteolytic activities toward poly-L-lysine. The initial rates of peptide hydrolysis, as measured *p*H-statically (Waley and Watson, 1953), corresponded to 70–90% of that of native trypsin with a similar esteratic activity.

[2] The potentiometric titration of an ethylene–maleic acid copolymer, at $\Gamma/2 = 0.1$, revealed two buffering zones with apparent dissociation constants $pK_1 = 4.35$ and $pK_2 = 7.5$ (A. Shatkai, unpublished results).

It is worth mentioning that the rates of hydrolysis of poly-L-lysine at *p*H 9.0 by trypsin and by the IMET preparations tested were approximately three to five times lower than those measured at *p*H 7.7.

Activation of Chymotrypsinogen.—Trypsin (50 μg), IMET-1, IMET-6, or IPTT (possessing an esteratic activity equivalent to that of 150 μg trypsin) was added to test tubes each containing 50 mg chymotrypsinogen in 3 ml 0.01 M phosphate buffer, *p*H 7.5, and the reaction mixtures were left for 16 hours at 4° with stirring. The chymotryptic activities obtained were determined after 50-fold dilution, on 0.1-ml aliquots by the *p*H-stat method, using ATEE as substrate (5 ml of an 0.018 M solution, 0.25 M in NaCl; Laskowski, 1955). From the initial rates of ATEE hydrolysis: 10.0 μmoles/minute, 7.9 μmoles/minute, 8.3 μmoles/minute, and 8.2 μmoles/minute, obtained for trypsin, IMET-1, IMET-6, and IPTT activation, respectively, the following per cent chymotrypsinogen conversions were calculated: 100% for trypsin activation, 79% for IMET-1 activation, 83% for IMET-6 activation, and 82% for IPTT activation.

Inhibition by Soybean- and Pancreatic-Trypsin Inhibitors.—SOYBEAN-TRYPSIN INHIBITOR.—In a previous communication (Bar-Eli and Katchalski, 1963) it was reported that the esteratic activity of water-insoluble polytyrosyl trypsin, IPTT, decreased by about 50% on incubation for 30 minutes with a 50-fold excess of soybean-trypsin inhibitor. It seemed desirable, therefore, to investigate the effect of the negatively charged, high-molecular-weight inhibitor on the various IMET preparations studied. The decrease in esteratic activity of IMET-1, IMET-6, and IPTT after incubation for 30 minutes at *p*H 7.5 at 25° with varying amounts of soybean-trypsin inhibitor is presented in Figure 7. IMET-1 retained 90% of it esteratic activity even at an inhibitor–to–bound protein ratio of 50:1 (w/w). IMET-6 retained about 60% of its esteratic activity at the same inhibitor-to-protein ratio. Under similar conditions both IMET preparations seem to display somewhat higher activity in the presence of soybean-trypsin inhibitor than the uncharged IPTT.

The proteolytic activities of IMET-1 and IMET-6, after incubation under the foregoing conditions with varying amounts of soybean-trypsin inhibitor, were tested on casein, using the procedure of Northrop *et al.* (1948, see Experimental). At a 1:1 ratio by weight of inhibitor to bound protein, IMET-6 was completely inactive toward casein. IMET-1 retained about 30% of its proteolytic activity under these conditions. At higher inhibitor–to–bound protein ratios both IMET preparations were found to be completely inactive.

PANCREATIC-TRYPSIN INHIBITOR.—When IMET-1 or IMET-6 were preincubated (at 25°, *p*H 7.8) with BAEE, and pancreatic-trypsin inhibitor was added to the reaction mixtures at a 1:1 ratio by weight of inhibitor to bound protein, no decrease in activity occurred within 20 minutes. However, on reversing the order of addition of reagents, i.e., on preincubation of IMET-1 or IMET-6 with pancreatic-trypsin inhibitor at a 1:1 ratio followed by the addition of BAEE, the esteratic activity of IMET-1 was found to decrease to approximately 8% of its initial value, whereas the activity of IMET-6 dropped to 27% of its initial value. When preincubation of the IMET preparation with the pancreatic inhibitor was carried out at *p*H 9.5, IMET-1 showed a similar residual esteratic activity (~8%), while the activity of IMET-6 under these conditions was further reduced to about 14% of its initial value.

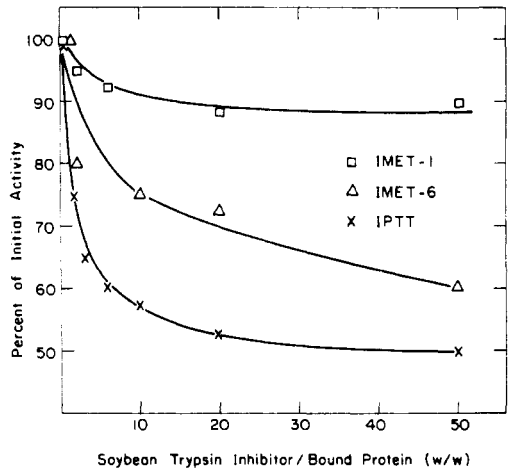

FIG. 7.—Inhibition of IMET-1, IMET-6, and IPTT by soybean-trypsin inhibitor. The incubation mixtures (0.2 ml) in 0.01 M Tris buffer, *p*H 7.8, contained IMET-1, IMET-6, or IPTT, possessing an esteratic activity of 1 mg/ml trypsin and soybean-trypsin inhibitor 1 mg/ml to 50 mg/ml. Each of the mixtures was incubated for 30 minutes at 25° with stirring, 0.1-ml aliquots were withdrawn, and the esteratic activity was assayed by the *p*H-stat method using BAEE as substrate.

DISCUSSION

The preparation of a new type of water-insoluble trypsin derivatives, in which the enzyme is covalently bound to a negatively charged polyelectrolyte, has been made possible by the use of a maleic anhydride–ethylene copolymer as a polyfunctional cross-linking agent. Since the coupling of enzyme with carrier was carried out under mild conditions (i.e., at a low temperature and neutral *p*H) and covalent binding was effected via enzymatically nonessential functional groups (the ε-amino groups of lysine), preparations with high enzymatic activity were obtained (see Table I). Furthermore, the preparative method developed permitted the preparation of water-insoluble copoly-(maleic acid–ethylene)-trypsin derivatives (IMET) of widely different carrier-to-protein ratios, e.g., IMET-1 and IMET-6, possessing a carrier-to-protein ratio of 20:1 and 1:3, respectively. The enzyme-rich IMET preparations may be visualized as a network consisting of enzyme molecules bound by relatively short polyelectrolyte chains; the IMET preparations of low enzyme content, on the other hand, may be looked upon as a loose polyelectrolyte network in which trypsin molecules are embedded.

The stability of all the IMET preparations tested, particularly in the neutral and alkaline range, was markedly greater than that of soluble trypsin (Fig. 2). This fact might be attributed mainly to the prevention of autodigestion as a result of the fixation of the enzyme molecules on the water-insoluble carrier. The fact that a large part of the trypsin lysyl residues are blocked as a result of the coupling of the enzyme to the carrier should also increase resistance to autodigestion. Finally, it should be mentioned that the enhanced stability in the alkaline-*p*H range is also probably owing to the differences in the hydrogen-ion concentration between the domain of the polyelectrolyte gel and the bulk of the solution (see following article, Goldstein *et al.*, 1964).

All of the IMET preparations investigated retained, in contradistinction to water-insoluble polytyrosyl trypsin, IPTT (Bar-Eli and Katchalski, 1963), a considerable fraction of their enzymatic activity on lyophilization or drying. Stability in this case may

well result from the pronounced hydrophylic character of the copoly-(maleic acid–ethylene) carrier.

The partial retention of tryptic activity of the IMET preparations in 8 M urea is somewhat surprising, since native trypsin shows no activity under these conditions. The study of the digestion by IMET of proteins in concentrated urea solutions might be of interest.

The *p*H-activity curve of IMET-1, the water-insoluble trypsin derivative rich in carrier, when determined at low ionic strength ($\Gamma/2 = 5.8 \times 10^{-3}$) using BAEE as substrate, was found to be displaced toward more alkaline *p*H values, in comparison to native trypsin (Fig. 4). This displacement may be explained by the redistribution of hydrogen ions between the negatively charged polyelectrolyte gel (the IMET particles) and the surrounding aqueous medium. Owing to the electrostatic field of the polyelectrolyte carrier, hydrogen ions accumulate in the gel phase so that the "internal *p*H" is lower than the *p*H measured in bulk. The esteratic activity of the bound enzyme is determined by the "internal *p*H" and thus corresponds to the activity of native trypsin at a lower *p*H than that recorded in the bulk of the solution, as discussed in detail in the following paper (Goldstein *et al.*, 1964). This supposition is supported by the finding that the *p*H-activity curve of IMET-1 at high ionic strength ($\Gamma/2 = 2.0$), at which polyelectrolyte effects are suppressed, approaches that of native trypsin under similar conditions. In the case of IMET-6, the water-insoluble trypsin derivative rich in protein, at $\Gamma/2 = 5.8 \times 10^{-3}$ a *p*H-activity profile similar to that of IMET-1, though slightly less displaced relative to native trypsin at the same ionic strength, has been observed. However, since an increase in ionic strength to $\Gamma/2 = 2.0$ caused only a small shift toward the *p*H-activity profile of native trypsin in the case of IMET-6, one must assume that the electrostatic field prevailing in the microenvironment of the bound trypsin in this is considerably smaller than in the case of IMET-1. In this respect IMET-6 seems to resemble acetyltrypsin (Sri Ram *et al.*, 1954; Goldstein *et al.*, 1964) more closely than IMET-1.

In connection with the above it is pertinent to recall the findings of McLaren and Estermann (1957), who observed a displacement in the *p*H-activity curve of chymotrypsin, similar to that recorded by us for IMET-1, when it acted on heat-denatured lysozyme adsorbed to the negatively charged mineral, kaolinite. No displacement in the *p*H-activity curve of the type discussed was found for IPTT, in which trypsin is bound to a noncharged carrier (Bar-Eli and Katchalski, 1963).

Similarly to other water-insoluble enzyme derivatives (Katchalski, 1962), all the IMET preparations studied showed lower enzyme activity toward high-molecular-weight substrates than that to be expected on the basis of their activity on low-molecular-weight substrates. The decrease in proteolytic activity relative to trypsin toward a given high-molecular-weight substrate, of the various IMET preparations investigated, was found to depend mainly on the enzyme-to-copolymer ratio. The decrease in activity was particularly pronounced in the case of the carrier-rich water-insoluble trypsin derivative IMET-1, acting on casein or hemoglobin (Fig. 5). Here it was found that activity of the water-insoluble trypsin derivative was approximately four to five times lower than that to be expected from its esteratic activity using BAEE as substrate. The IMET preparations rich in enzyme, such as IMET-6, on the other hand, showed initial activity toward casein and hemoglobin (Fig. 5) only slightly lower than that of native trypsin. Steric hindrance, caused by the polyelectrolyte network of the carrier, undoubtedly plays an important role in determining the rate of interaction between the bound enzyme molecules and the corresponding high-molecular-weight substrates. Electrostatic repulsion between the negatively charged carrier and the negatively charged casein and hemoglobin, at *p*H 9, further decreased the initial rate of tryptic digestion as revealed by the data summarized in Figure 5.

Chymotrypsinogen could be activated to chymotrypsin by IMET-1 and IMET-6, as well as by IPTT. The activation of the zymogen by the different water-insoluble trypsin derivatives illustrates the possible use of other water-insoluble proteolytic enzymes in the transformation of the suitable proteins into biologically active entities without contaminating the system under investigation with the proteolytic enzyme used for activation.

Our findings that casein and hemoglobin are digested to different extents by trypsin and by IMET-1 and IMET-6 seem to suggest that a new kind of specificity has been superimposed on the bound trypsin molecules when acting on high-molecular-weight substrates. Thus it might be assumed that some of the lysyl and arginyl peptide bonds of casein or hemoglobin located in the neighborhood of negatively charged amino acid residues may perhaps be less prone to hydrolysis by the negatively charged IMET's than by native trypsin. The preliminary observation that denatured lysozyme yields somewhat different peptide patterns when exhaustively digested by trypsin or by IMET-1 and IMET-6 supports the foregoing suggestion.

The easy inhibition of the IMET's by the pancreatic-trypsin inhibitor may be explained by the positive charge (isoelectric point, 10; Green and Work, 1953) and the relatively low molecular weight (~9000; Laskowski and Laskowski, 1954) of the latter.

In the case of soybean-trypsin inhibitor the picture is somewhat more complicated. It would be expected that, steric factors apart, the negative charge of both IMET and soybean-trypsin inhibitor would cause electrostatic repulsion between them resulting in poor inhibition. Soybean-trypsin inhibitor did indeed poorly inhibit the esteratic activity of IMET. However, an explanation based on electrostatic repulsion is not consistent with the finding that soybean-trypsin inhibitor strongly inhibits the caseinolytic activity of IMET, and therefore the important factor here is probably the steric one.

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A Water-insoluble Polyanionic Derivative of Trypsin. II. Effect of the Polyelectrolyte Carrier on the Kinetic Behavior of the Bound Trypsin*

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The mode of action of the water-insoluble derivatives of trypsin (IMET), obtained by the covalent binding of trypsin to a copolymer of maleic acid and ethylene (1:1), has been investigated at 25°. The *p*H-activity profile of IMET at low ionic strength ($\Gamma/2 = 5.8 \times 10^{-3}$), using benzoyl-L-arginine ethyl ester as substrate, was found to be displaced by approximately 2.5 *p*H units toward more alkaline *p*H values, when compared with trypsin under similar conditions. At higher ionic strength, the *p*H-activity curve of IMET shifted toward more acid *p*H values, approaching the *p*H-activity curve of IMET–trypsin at $\Gamma/2 = 1.0$. The Michaelis constant ($K_m = 0.2 \pm 0.05 \times 10^{-3}$ M) measured for the benzoyl-L-arginine amide system at low ionic strength ($\Gamma/2 = 0.04$) and optimal *p*H (*p*H = 9.5) was found to be approximately thirty times lower than that of the trypsin–benzoyl-L-arginine amide system, at its optimal *p*H (*p*H 7.5) at $\Gamma/2 = 0.04$. The Michaelis constant at high ionic strength ($\Gamma/2 = 0.5$), measured for the IMET–benzoyl-L-arginine amide system at the *p*H of optimal activity (*p*H 9.5), approached that for the trypsin–benzoyl-L-arginine amide system ($K_m = 6.8 \pm 1.0 \times 10^{-3}$ M) when measured at its optimal *p*H (*p*H 7.5) and the same ionic strength. The effect of the polyanionic carrier on the *p*H-activity profiles and Michaelis constants of the bound enzymes investigated can be explained as resulting from the effect of the electrostatic potential of the polyelectrolyte carrier on the local concentration of hydrogen ions and positively charged substrate molecules in the microenvironment of the bound enzyme molecules. Theoretical analysis of the kinetic data allowed a quantitative correlation of the displacement in the *p*H-activity curves and the shifts in the Michaelis constants with the electrostatic potential prevailing in the domain of the polyelectrolyte carrier.

The preparation and properties of a new type of water-insoluble trypsin derivatives (IMET),[1] in which the enzyme is covalently bound via its ϵ-amino groups to the carboxyls of a copolymer of maleic acid and ethylene, was described in the preceding paper (Levin *et al.*, 1964). Each active trypsin molecule in this preparation is embedded in a highly negatively charged three-dimensional network formed by the many ionized carboxyl groups of the synthetic carrier. The enzyme is thus exposed to a strong electrostatic field which might markedly affect its mode of action. It seemed of interest, therefore, to investigate the effect of the polyelectrolyte environment prevailing in the IMET preparations on the kinetics of enzymatic hydrolysis of the classical low-molecular-weight trypsin substrates benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-arginine amide (BAA).

The *p*H-activity curves of IMET at low ionic strength, when compared with those of trypsin, were found to be displaced by 2–3 *p*H units toward the alkaline region. The Michaelis constant of IMET, when determined under similar conditions, was markedly lower than that of trypsin. The differences recorded between the modified and intact enzyme could be explained by the effect of the negative electrostatic field on the local concentration of both the hydrogen ions and the positively charged substrate molecules in the domain of the insoluble IMET particles. A

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[1] The abbreviations used are: IMET, water-insoluble copoly-(maleic acid–ethylene)-trypsin; BAEE, benzoyl--arginine ethyl ester; BAA, benzoyl-L-arginine amide.