SIMULTANEOUS HYDROLYSES OF ESTERS AND PROTEINS AT SATURATION LEVELS

By M. CASTAÑEDA-AGULLÓ and LUZ M. DEL CASTILLO

(From the Department of General Physiology, National School of Biological Sciences, National Polytechnic Institute, Mexico, D. F.)

(Received for publication, June 3, 1957)

ABSTRACT

A direct titration method for the determination of proteolytic activity is discussed. This involves the potentiometric measurement of the volume of 0.08 N NaOH required to maintain a constant pH (8.0) during the time of the hydrolysis. It is a sensitive method which presents several advantages; *viz.*, it measures simultaneously protease and esterase activity, it follows the hydrolysis very closely and from the first stages; the titration is continuous and on the same sample. This method determines a constant fraction of the groups titratable by formol titration. The ratio formol:direct titration is represented by a factor "*f*" which is presumed to be distinct for each protein-enzyme system.

Kinetic studies, using this method, revealed that the rates of hydrolysis of mixtures casein-gelatin on one hand, casein-BAEE or gelatin-BAEE on the other, are always larger than those of the corresponding isolated substrates. In many cases the resulting rates are equal or nearly equal to the sum of the individual rates, even though the mentioned rates have been determined within the saturation zones for every substrate.

The former observations are inconsistent with the theory of the formation of an intermediary enzyme-substrate compound, unless it is assumed that the enzyme has a specific active group for each substrate.

In a previous paper (1), evidence was presented to show that Northrop's phenomenon, an anomaly in enzymatic kinetics first described by Northrop (5, 6), holds good for enzymatic systems other than trypsin–casein–gelatin. It was demonstrated that the addition of gelatin does not affect the rate of hydrolysis of casein or hemoglobin by trypsin, papain, or pepsin. It was then thought of interest to investigate whether such independent hydrolysis also occurs in protein and ester systems and in the presence of substrate-saturated enzymes.

The need to determine esterase and protease activity gave rise to a search for adequate methods. The usual procedures for the measurement of proteolytic activity do not determine initial steps of hydrolysis, and these are not the best conditions for comparison with ester hydrolysis which is a very simple

reaction. In this paper a method is presented to measure either protease or esterase activity. Additionally, this method determines the first stages of protein hydrolysis, and comparisons under favorable conditions are made possible. The results of a series of studies on the simultaneous hydrolyses by trypsin in several systems are also given: (1) casein and gelatin in variable concentrations, (2) benzoyl-L-arginine ethyl ester and casein or gelatin, and (3) benzoyl-L-arginine ethyl and methyl esters.

Materials and Methods

Enzyme.—Four times recrystallized trypsin, starting with Worthington preparation once crystalline trypsin with about 50 per cent $MgSO_4$.

Proteins.—Difco gelatin purified according to Northrop and Kunitz' procedure (7) and Hammarsten casein.

Esters.—Benzoyl-L-arginine methyl ester HCl (BAME) lot number 1237 and benzoyl-L-arginine ethyl ester $HCl \cdot H_2O$ (BAEE) lot number 1946 were Mann preparations.

Determination of Proteolytic and Esterase Activities.—A method of direct potentiometric titration, based on the titrimetric determination of esterases (2), was applied to measure proteolytic activity. The procedure was as follows: the protein was dissolved in 0.1 M phosphate buffer at pH 8 and 10 ml. of the desired concentration placed in a vessel in a constant temperature bath. The electrodes of a Beckmann model "G" pH meter were introduced into the liquid, and pH adjusted to 8 (null point). Then 0.1 ml. of 0.08 N NaOH was added and the solution stirred, the galvanometer needle being then deviated. The enzyme dissolved in 0.1 ml. of 0.005 N HCl was then added, and an electrical time recorder ("Precision time-it" which registers 0.01 minute) started when the galvanometer needle returned to zero. A small increment of NaOH was delivered from a microburette with a capillary tip and the solution stirred thoroughly. The continuously appearing hydrolysis products progressively neutralize the excess of alkali. The time was recorded at the precise moment when the solution pH again fell to the null point value; *i.e.*, at the point of total neutralization. Successive portions of NaOH solution, never more than 0.1 ml., were then added and the procedure continued for 30 minutes with proteins or to the end of the reaction for esters. The course of the hydrolysis was represented by the increase in NaOH consumption against time.

As the protein rate of hydrolysis (in opposition to that of the esters) decreased with time, the volume of NaOH solution added each time was progressively diminished in such a way that the time interval between two consecutive points was 2 minutes at most. The pH variation was not greater than 0.05 unit in any case (2 to 3 galvanometer divisions).

Apparently, the direct titration method would yield better results if more dilute buffer were used. The study of the effect of several concentrations of phosphate showed that this supposition was not quite correct. For protein concentrations up to 3 per cent, the direct titration in the presence of 0.01 or 0.015 M phosphate buffer gave satisfactory results. Larger protein concentrations, and especially mixtures, gave

rise to variable results and sometimes the later steps of the hydrolyses were delayed. Probably this effect could be ascribed to great elevations of pH produced by the addition of the adequate volume of NaOH solution without sufficient buffer to balance it. The resulting alkalinity could inactivate the enzyme to greater or lesser extent.

In special cases (isolated proteins or proteins with esters), a low concentration of buffer may be convenient, but for the comparison of various concentrations of isolated and mixed proteins, 0.1 M phosphate was found more satisfactory since it gave constant results in every case.

When comparative determinations were carried out, the same enzyme solution was used; this was kept at about pH 2.5 (0.005 N HCl) and within a container with ice in order that the activity should remain constant.

Formol titration was performed in the following manner: 15 ml. of the selected concentration of protein in 0.1 M phosphate buffer was placed in a container and equilibrated in a constant temperature bath, and 0.15 ml. of the enzyme solution added. Immediately, a 1 ml. sample was drawn off and mixed with 0.5 ml. of neutral formaldehyde solution. Equal samples were drawn at 5 minute intervals. The titration was done potentiometrically with 0.02 N NaOH solution to pH 8.2 as the end point. In this way, fewer variations are obtained as compared with the titration in the presence of phenolphthalein.

Nitrogen determinations on the enzyme solutions were made by the microKjeldahl method.

RESULTS

Direct Titration of Protein Hydrolysis

The direct potentiometric titration was found to be a sensitive method that gave satisfactory results for following the course of hydrolysis of casein and gelatin by trypsin. The results obtained were reproducible within a few hundredths of a milliliter. The direct titration increased with substrate and enzyme concentration and with temperature.

Fig. 1 shows the hydrolyses of different concentrations of gelatin and casein brought about by the same trypsin solution in order to determine saturation curves. The saturation zones for both proteins are observable from a 3 per cent concentration onward and extend from zero to about 4.5 minutes (0.35 ml. of 0.08 N NaOH) for gelatin, or 6 minutes (0.3 ml. of NaOH) for casein.

Comparison of Direct Titration with Formol Titration

A parallel study has been made of direct and formol titrations to determine the correctness of the first method as a measure of protein hydrolysis. The comparative curves for gelatin digestion are plotted in Fig. 2 and for casein in Fig. 3. The quantity of NaOH required for the direct titration was smaller than that used in the formol titration. However, the courses of the hydrolyses followed by both procedures are very similar, in that each two corresponding

points at a given time maintain a constant ratio. This ratio can be expressed as a factor

$$f = \frac{\text{Milliliters 0.02 N NaOH required in formol titration}}{4 \times \text{ml. 0.08 N NaOH used in direct titration}}$$

in which both numerator and denominator values are calculated for the same volume of digestion mixture; *e.g.*, 10 ml.

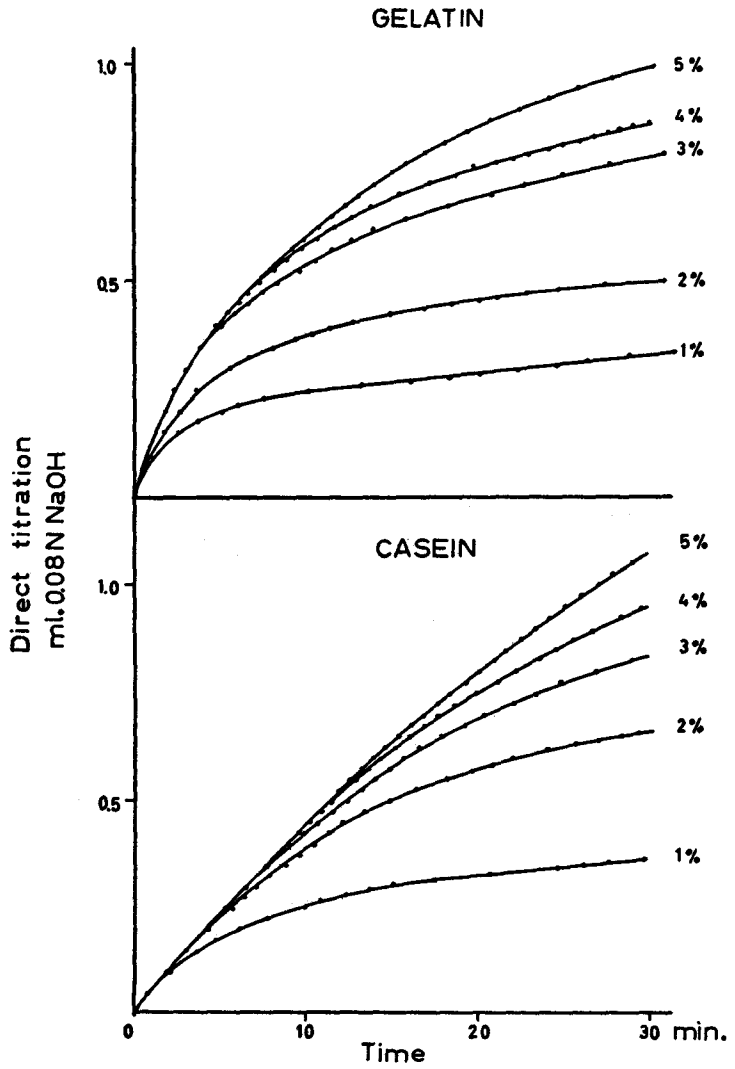

FIG. 1. Tryptic digestion of several concentrations of gelatin and casein using the direct titration method. Temperature, 35°C. Buffer; 0.1 M KH_2PO_4–Na_2HPO_4 pH 8. Volume of digestion mixture, 10 ml.

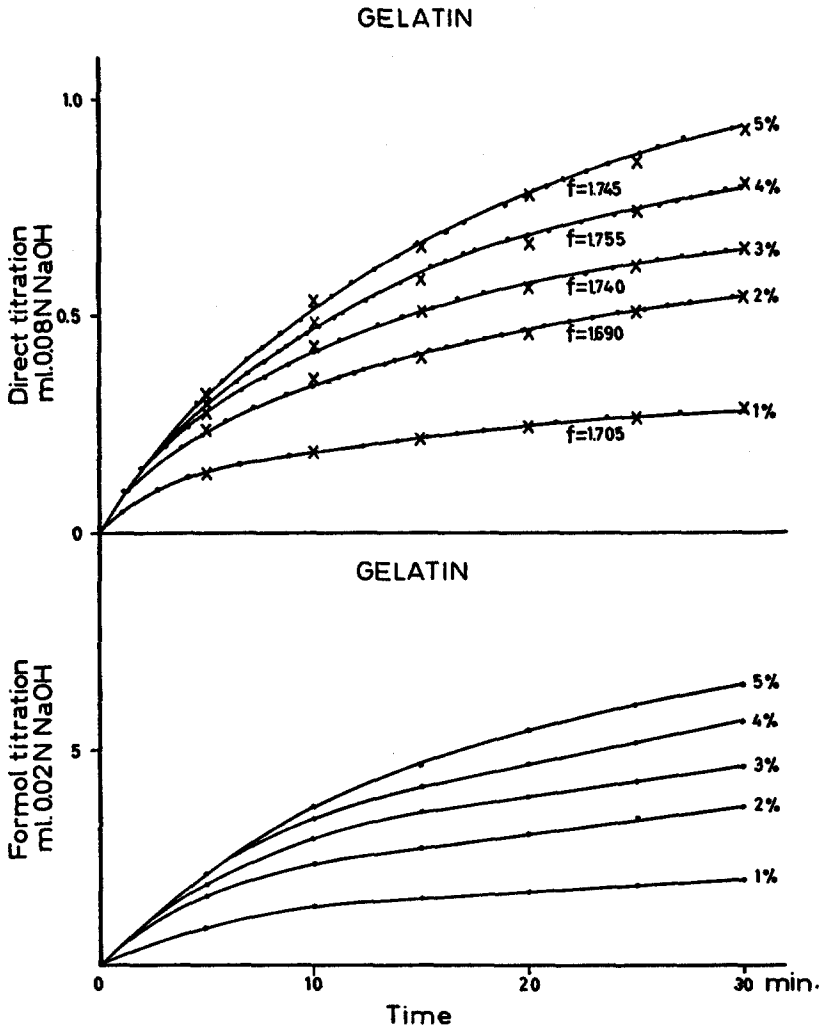

FIG. 2. Comparison of direct and formol titrations with various concentrations of gelatin. The upper figure represents the direct titration curves, and the lower, the formol curves. The *f* value given for each curve is the corresponding average value for the ratio formol:direct titration. The x signs plotted on the direct titration curves are points calculated by dividing the corresponding data of formol titration by *f*. Enzyme, crystalline trypsin. Temperature, 35°C. Buffer, 0.1 M phosphate pH 8. All data refer to 10 ml. of digestion mixture.

The upper portions of Figs. 2 and 3 represent the courses of direct titrations and the lower ones the formal titration curves. The x signs plotted on the upper curves are points calculated by dividing the corresponding data of the formol titration by "*f*." For the calculation of the values of *f*, 20 pairs of determinations

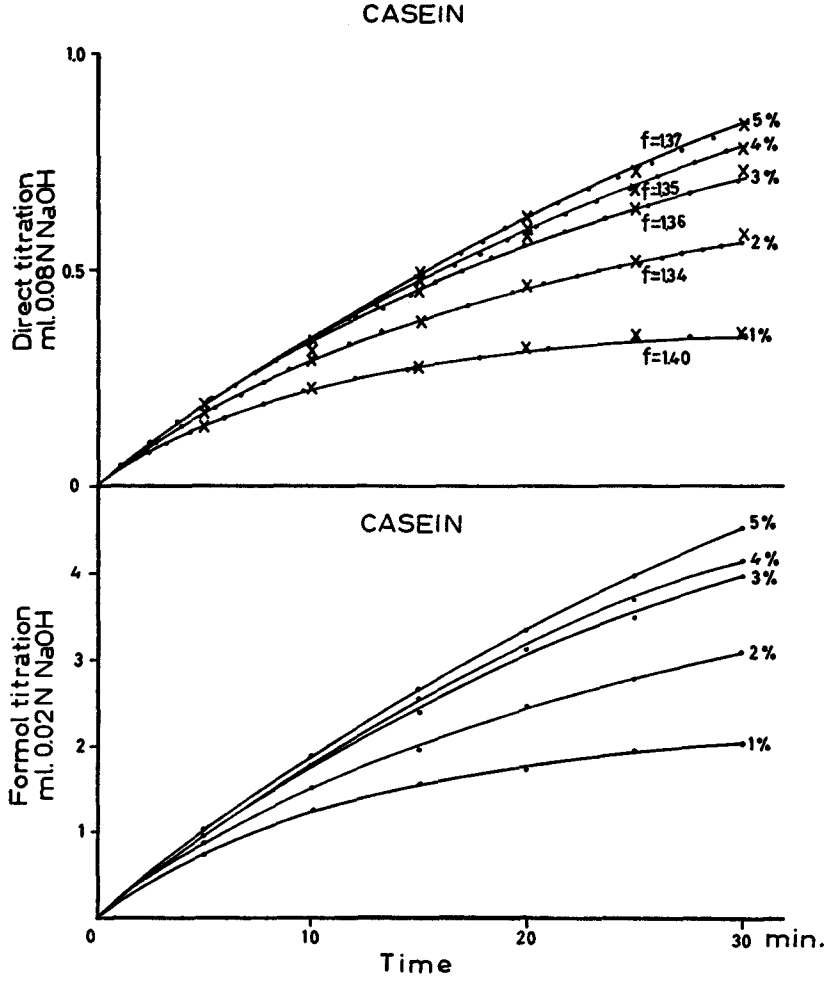

FIG. 3. Comparison of direct and formol titrations for various concentrations of casein. The upper figure shows the direct titration curves, and the lower, the formol titration curves. The *f* values indicated for each curve are the corresponding average values for the ratio formol:direct titration. The x signs plotted on the direct titration curves are points calculated by dividing the corresponding data of formol titration by *f*. Enzyme, crystalline trypsin. Temperature, 35°C. Buffer, 0.1 M phosphate pH 8. All data refer to 10 ml. of digestion mixture.

were performed for each protein, four for every concentration. The same trypsin solution was used for each pair of determinations. The average for every concentration is given for the respective curve.

It may be noted that the factor is independent of the protein concentration. The following average factors were obtained: casein 1.364 with a standard deviation ±0.023, gelatin 1.727 ± 0.028 (mean computed from the individually pooled data for each concentration). The computation of the averages of the whole number of determinations independently of the concentration gave the factors 1.363 ± 0.082 for casein and 1.735 ± 0.048 for gelatin.

Fig. 4 demonstrates the comparative results of direct and formol titrations in mixtures of equal concentrations of casein and gelatin. The final mean factor was 1.575 ± 0.023, a value very similar to the average of casein and gelatin factors, 1.55. For the purpose of determining whether the difference between these values was of statistical significance, Student's "*t*" test was applied. The results showed this difference to have no significance. Hence, it can be concluded that the factor of a mixture of equal concentrations of casein and gelatin is the mean of the individual factors.

The constant ratio between formol and direct titration must be ascribed to distinct groups titrated by the two methods. Though there is no certainty about which groups are titrated directly, the greater consumption of alkali in the presence of formol possibly may be due to those groups which are known to shift their dissociation constants as the effect of formaldehyde.

French and Edsall (3, 4) have presented extensive reviews on the reaction of formaldehyde with amino acids and proteins. In Edsall's review (3), the titration curves of gelatin in aqueous and 1 per cent formaldehyde solutions are presented. Both curves are identical up to pH 5.5, and diverge widely afterwards, the greater difference being observed between pH 8 and 9. This author infers that the point at which the divergence is first apparent represents the end of the zone in which carboxyl groups are being titrated and the beginning of the titration of the imidazole groups. Steinhardt and Zaiser (10) agree in that carboxyls are titrated up to pH 5.5, and imidazolium groups from about 5.5 to 8. Above pH 8 ammonium, phenoxy, sulfhydryl, and guanidinium groups are titrated. In addition to these data, the authors mentioned state that in the formol titration of a protein the apparent dissociation equilibrium of the ϵ-ammonium groups of lysine is displaced 3 units toward acid pH, and that the differential titration with and without formol gives the number of ϵ-ammonium groups. The ionization of imidazolium groups can also be affected, unless the differential titration is carried out at pH 8.5, where there is no interference of imidazolium or α-ammonium groups. Formol does not affect either carboxyl or phenoxyl groups.

The values of the dissociation constants found for titratable groups in peptides and proteins differ from those for free amino acids. The curves of direct

titration in this paper represent the titration of groups that are being freed in the course of hydrolysis. Notwithstanding the different experimental conditions in titration curves of proteins as such and direct titration of enzymatic protein hydrolyses, it may be inferred that the greater titration values of

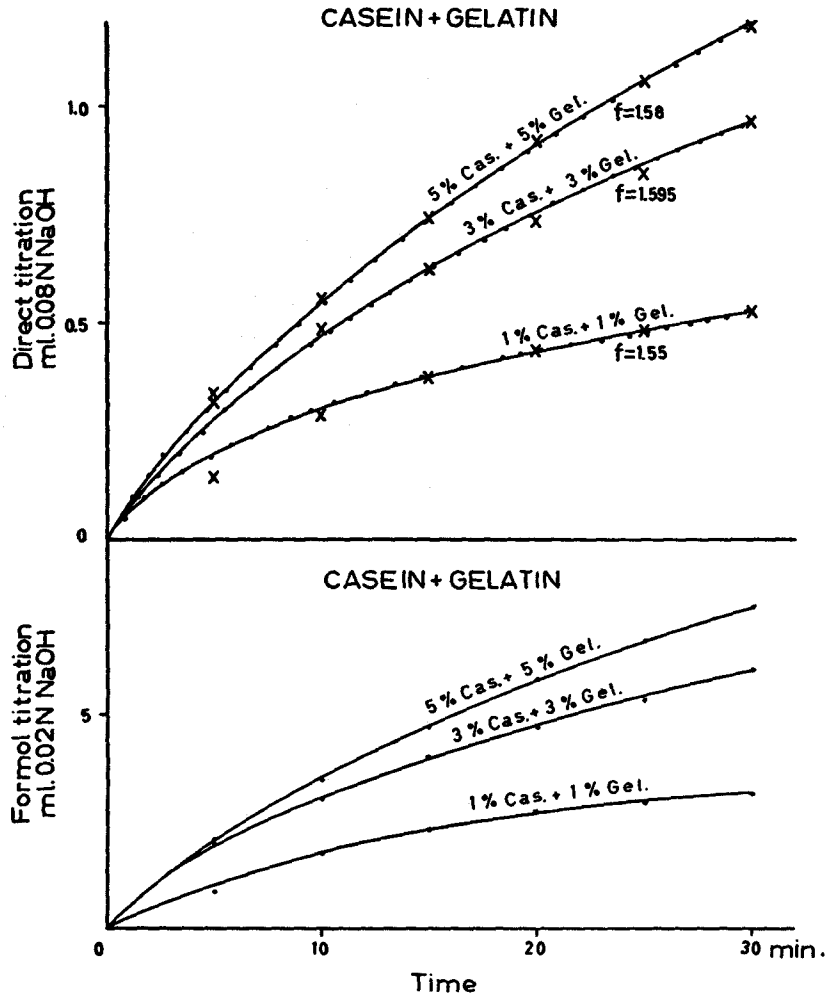

FIG. 4. Comparison of direct and formol titrations for various mixtures of equal concentrations of casein and gelatin. The *f* values indicated for each curve in the upper figure are the corresponding average values for the ratio formol:direct titration. The x signs plotted on the direct titration curves are points calculated by dividing the corresponding data of formol titration by *f*. Temperature, 35°C. Enzyme, crystalline trypsin. Buffer, 0.1 M phosphate pH 8. All data refer to 10 ml. of digestion mixture.

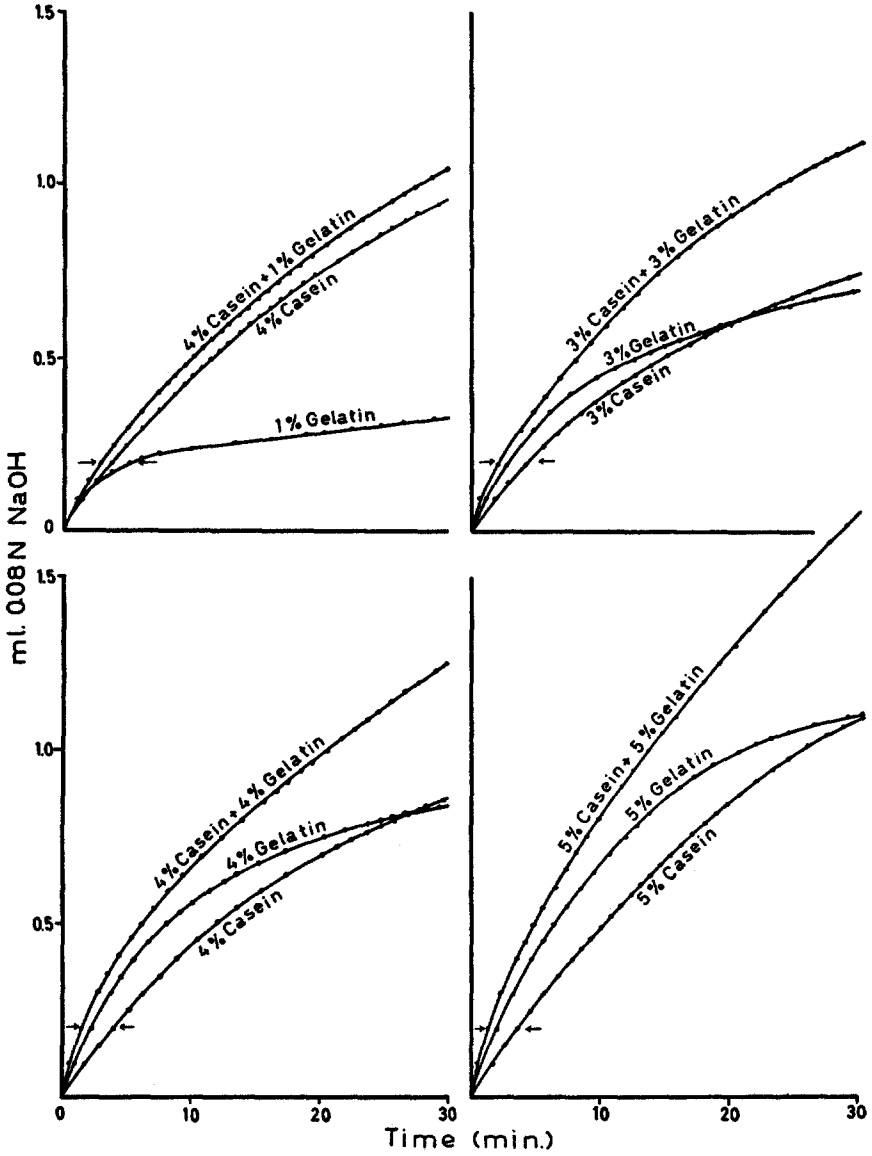

FIG. 5. Tryptic digestion of various mixtures of casein and gelatin, represented by the increase in direct titration of the mixtures and of each protein separately. Arrows indicate the stages at which the rates of hydrolysis given in Table I were calculated. Temperature, 35°C. Buffer, 0.1 M phosphate pH 8.

formol titration are possibly due to ε-ammonium groups and in part to imidazolium groups, since both of these are affected by formol at pH 8.2.

Experiments with Protein mixtures

A series of experiments with casein and gelatin in different proportions was carried out. Throughout all the experiments, at least one of the proteins was at a saturation level; *i.e.*, 3 to 5 per cent. The addition of 1 per cent gelatin to 4 per cent casein increased markedly the titration curve from the first minutes of hydrolysis. The same happened when 3, 4, or 5 per cent gelatin was added to

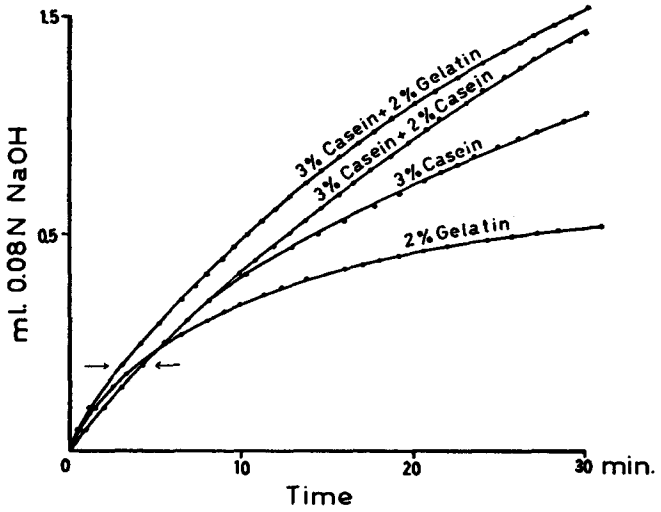

FIG. 6. Effect of the addition of 2 per cent casein and 2 per cent gelatin on the rate of hydrolysis of 3 per cent casein. Direct titration method. The arrows indicate the stages at which the rates of hydrolysis given in Table I were calculated. Temperature, 35°C. Enzyme, crystalline trypsin. Buffer, 0.1 M phosphate pH 8.

the same concentration of casein (Fig. 5). Fig. 6 shows that while the addition of 2 per cent casein to 3 per cent casein caused no change in the rate up to 9 minutes (saturation zone), the addition of the same proportion of gelatin did give rise to an increased rate of reaction. In Table I the estimated rates for each experiment are given. The rates were calculated as the reciprocal of the time necessary for the consumption of 0.2 ml. of NaOH solution. This volume of alkali was chosen as the end point for the calculation of the rates because it is less than the value corresponding to the point at which the saturation stages finish. The arrows plotted on the respective curves indicate the points at which the rates were estimated. A noticeable increment in the rates of hydrolysis in the mixtures 1 per cent gelatin–4 per cent casein and 2 per cent gelatin–3 per

cent casein is observed when compared with the rate of pure casein. This effect is still more marked in the mixtures 3 + 3, 4 + 4, and 5 + 5 per cent of each protein when the resulting rates are equal, or nearly equal, to the sum of the individual rates.

TABLE I

Hydrolysis Rates of Protein and Protein with Ester Mixtures Calculated as the Reciprocal of the Time of Consumption for 0.2 Ml. 0.08 N NaOH

Substrate	Time	Rate 1/T	Sums
	min.		
4 per cent casein	3.9	0.256	0.438
1 per cent gelatin	5.5	0.182	
4 per cent casein + 1 per cent gelatin	2.9	0.345	
4 per cent casein + 1 per cent casein	3.9	0.256	
3 per cent casein	4.4	0.227	0.471
2 per cent gelatin	4.1	0.244	
3 per cent casein + 2 per cent gelatin	3.1	0.323	
3 per cent casein + 2 per cent casein	4.4	0.227	
3 per cent casein	4.4	0.227	0.584
3 per cent gelatin	2.8	0.357	
3 per cent casein + 3 per cent gelatin	2.2	0.455	
4 per cent casein	4.0	0.250	0.666
4 per cent gelatin	2.4	0.416	
4 per cent casein + 4 per cent gelatin	1.5	0.666	
5 per cent casein	3.7	0.270	0.746
5 per cent gelatin	2.1	0.476	
5 per cent casein + 5 per cent gelatin	1.4	0.715	
3 per cent gelatin	4.1	0.243	0.743
0.012 M BAEE	2.0	0.500	
3 per cent gelatin + 0.012 M BAEE	1.5	0.666	
3 per cent casein	6.7	0.150	0.677
0.012 M BAEE	1.9	0.527	
3 per cent casein + 0.012 M BAEE	1.5	0.666	

Experiments with Ester and Proteins

A plot of the reaction rate against weight of trypsin N in the system, similar to that previously reported by Schwert *et al.* (8) for BAME, is given in the upper part of Fig. 7. The results obtained with BAEE bear a great resemblance to those described for BAME. These data confirm further findings of Schwert and Eisenberg (9). The tryptic hydrolyses of both esters are zero order reac-

tions throughout almost their total courses. It has been usually considered that zero order reactions can be ascribed to a complete saturation of the enzyme by the substrate.

Since there is a very close relationship between both esters, not only in struc-

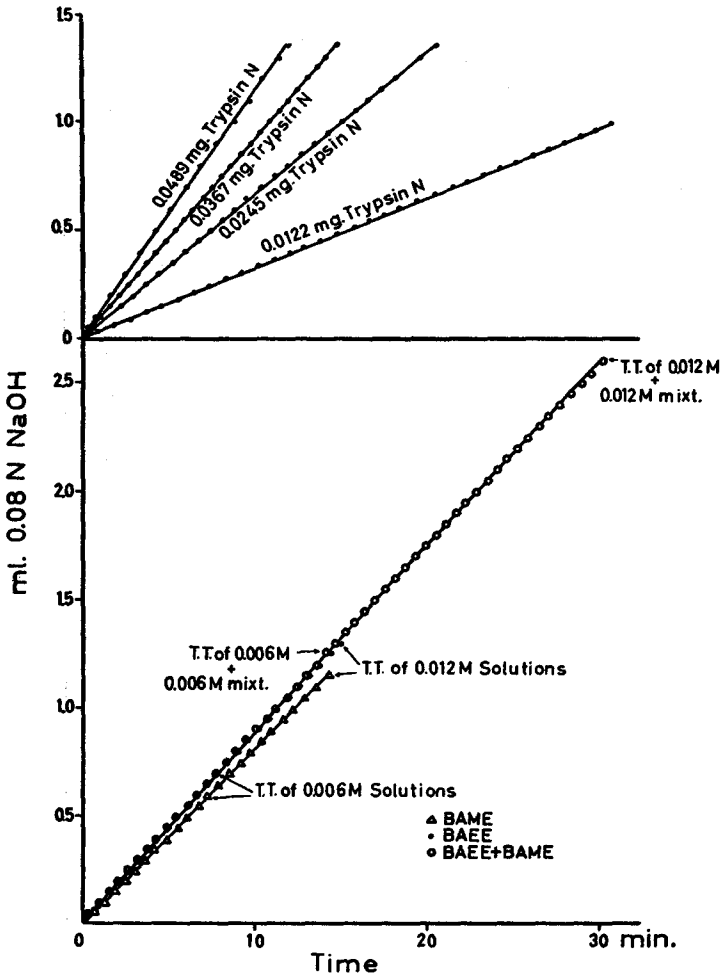

FIG. 7. Upper figure, hydrolysis of 0.012 M BAEE by various concentrations of trypsin expressed for each curve as milligrams of trypsin nitrogen present in the system. Lower figure, hydrolyses of 0.006 and 0.012 M BAEE, 0.006 and 0.012 M BAME, and mixtures of 0.006 and 0.012 M BAME with 0.006 and 0.012 M BAEE respectively, carried out with the same concentration of trypsin. The arrows indicate the end of the reaction for each respective concentration (T. T. = total titration). Temperature, 25°C. Buffer, 0.015 M phosphate pH 8. Digestion mixture, 10 ml.

ture but also in behavior toward trypsin, it was considered interesting to investigate the manner in which the mixture of both substrates would be hydrolyzed. In the lower portion of Fig. 7 the titration curves of two concentrations of each BAEE and BAME, 0.006 and 0.012 M were plotted. The increase of substrate concentration from 0.006 to 0.012 M does not change the speed of

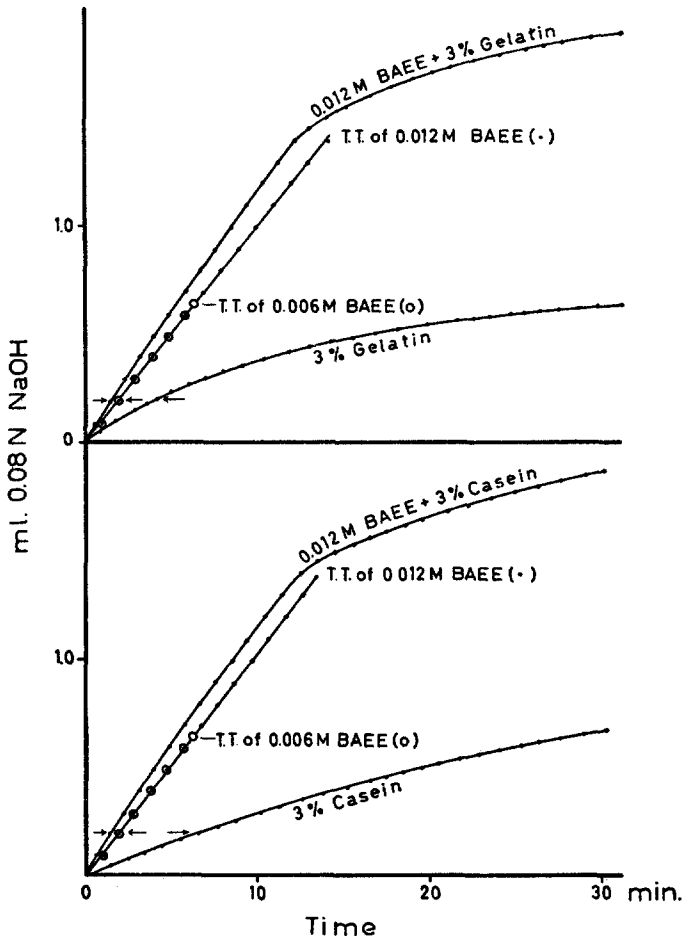

FIG. 8. Hydrolysis of mixtures of BAEE with 3 per cent casein and gelatin, compared with the curves of the separate substrates. Upper figure, digestion curves of 0.006 and 0.012 M BAEE. The end of the reaction is indicated as T. T. (total titration) for each molarity. Digestion curves of 3 per cent gelatin and of the mixture 3 per cent gelatin + 0.012 M BAEE. Temperature, 25°C. Lower figure, the same as the upper with casein instead of gelatin. Temperature, 30°C. Buffer for both casein and gelatin, 0.015 M phosphate pH 8. Enzyme, crystalline trypsin.

the reaction; an additional proof of saturation. Two mixtures, 0.006 M BAEE + 0.006 M BAME, and 0.012 M BAEE + 0.012 M BAME, were studied comparatively with the same concentrations of the separate substrates. No detectable increase in the rate of hydrolysis of the mixtures was observed with reference to that of the individual esters. The effect of the addition of BAME to BAEE was comparable to the addition of larger quantities of the same substrate; *i.e.*, BAME and BAEE behave as if they were a single substrate.

In contradistinction to the absence of effect on the reaction speed of BAEE after the addition of either more BAEE or BAME, the action of casein or gelatin causes an additive or nearly additive effect on the rate of hydrolysis of the ester referred to. Fig. 8 represents the curves of hydrolyses of BAEE–gelatin and BAEE–casein in 0.015 M phosphate buffer and Table I gives the corresponding rates. The hydrolysis of the latter was carried out at 30° on account of viscosity effects of casein at such concentration which render difficult the potentiometric titration at lower temperatures.

DISCUSSION

Direct potentiometric titration is a sensitive and easily reproducible method which presents several advantages: (1) it permits the simultaneous determination of protease and esterase activities, (2) it follows the protein hydrolysis from the first stages, *i.e.* as soon as the protein begins to split and titratable groups appear, (3) as the titration curves render a point per minute or a fraction of a minute, it is possible to observe closely the digestion course and detect any change in the rate that may occur during the saturation period, and (4) the continuous titration can be carried out on the same sample. This cannot be done with the formol titration.

The comparison of direct and formol titration showed that the former may determine correctly protein hydrolysis. It is possible to convert data of direct titration to formol titration values provided that the corresponding conversion factor f has been determined for the substrate-enzyme system being studied.

Though the factors of only two systems, trypsin-casein and trypsin-gelatin, are given in this paper, the assumption is possible that each particular protein-enzyme system has a distinct factor, depending upon the molecular constitution of the protein and the groups attacked by the enzyme.

An interesting observation is the fact that the factor of the mixtures of equal concentrations of casein and gelatin does not differ significantly from a statistical point of view, from the average of the factors for each individual protein. This fact would indicate that equal proportions of each protein are being hydrolyzed simultaneously; otherwise the factor of the mixture would approach either one factor or the other.

The results of the experiments on protein hydrolyses herein described show that for the studied systems, the phenomenon of saturation of enzyme by sub-

strate is observable only when there is a single substrate. Saturation no longer appears when a second protein is added to the system. Divergence of direct titration curves within the saturation zone is produced by the addition of even small concentrations of gelatin (1 to 5 per cent) to casein at concentrations above the saturation level (from 3 per cent onward). The rates of hydrolysis (though calculated for a stage of the reaction far below the end of the saturation phase), demonstrate that both proteins are being hydrolyzed independently, even within the so called saturation phase. This effect is more noticeable in the concentrated mixtures (such as those containing 4 or 5 per cent of each protein) when the rates are practically additive.

The present study of kinetic characteristics of the tryptic hydrolysis of BAEE gave results very similar to those previously reported by Schwert *et al.* for BAME (8) and BAEE (9). These authors concluded that "the hydrolysis of BAME is catalyzed by trypsin itself and by the same active surfaces which catalyze the hydrolysis of benzoyl-L-arginine amide." They state further that "If zero order kinetics are ascribed to a complete saturation of the enzyme by the substrate at all ratios of enzyme-substrate concentration that have been studied, the higher rate and lower order of reaction in the case of the esters must indicate a considerably higher affinity of the enzyme for these substrates." On account of the close resemblance in the behavior of BAME and BAEE, the above statements could be applied to BAEE.

No modification in the speed of the reaction was observed on addition of 0.006 M BAEE to 0.006 M BAEE, that is to say that 0.006 M is sufficient to saturate the enzyme present. On the contrary, the addition of 3 per cent casein or gelatin to 0.012 M BAEE gave rise to an increase in the rate. Moreover, the resulting rate is equal, or nearly equal, to the sums of the rates of each separate substrate, as in the case of protein mixtures.

The addition of 0.006 or 0.012 M BAME to the same molarity of BAEE does not modify the reaction rate of the second ester. The resulting curves are similar to those produced by an increase in the concentration of the same BAEE. Since the chemical structure of both esters is very similar and their behavior in the presence of trypsin almost identical, it was expected that their mixture would behave as a single substrate. The experimental facts confirmed such a supposition.

The facts reported in this paper are inconsistent with the theory which assumes that enzyme and substrate combine to form a more or less persistent compound, unless it is further supposed that there are separate active groups in the enzyme molecule, corresponding to different substrates.

SUMMARY

1. A direct titration method for the measurement of proteolytic activity is discussed. This method titrates a constant fraction of the groups titratable by

formol; this fraction may vary with the protein, and possibly with the enzyme used. The ratio formol:direct titration is expressed as a factor f which is presumed to be distinct for each protein-enzyme system. The procedure mentioned makes it possible to follow the protein hydrolysis from the first stages.

2. The factor f of mixtures of equal concentrations of casein and gelatin is the average of the factors of the separate proteins. This may indicate that trypsin is hydrolyzing equal proportions of each protein simultaneously.

3. The rates of hydrolysis of the mixtures casein-gelatin at saturation levels are always larger than those of the isolated proteins; in some cases and especially with the larger concentrations studied, the rate of the mixtures is equal to the sum of the individual rates.

4. The rates of hydrolysis of the mixtures BAEE–casein and BAEE–gelatin are practically additive with reference to the rates of the separate substrates, in spite of having been determined within the saturation phase for each substrate.

5. The rate of the mixture of two very closely related substrates, such as BAEE and BAME, is the same as in a single substrate.

6. For the systems studied, the so called saturation of the enzyme by the substrate is observable only when there is a single substrate in the system. Saturation is no longer apparent when a second substrate is added, except in the case of a close relationship between both substrates.

7. The above observations are inconsistent with the theory of the formation of an intermediary substrate-enzyme compound, unless it is assumed that there are different active groups in the enzyme.

BIBLIOGRAPHY

1. Castañeda-Agulló, M., *J. Gen. Physiol.*, 1956, **39,** 361.
2. Davis, N. C., and Smith, E. L., *in* Methods of Biochemical Analysis, (David Glick, editor), New York, Interscience Publishers, Inc., 1955, **2,** 230.
3. Edsall, J. T., *in* (E. J. Cohn and J. T. Edsall, editors), Proteins, Aminoacids and Peptides as Ions and Dipolar Ions, New York, Reinhold Publishing Corp., 1943, 493.
4. French, D., and Edsall, J. T., *Advances Protein Chem.*, 1945, **2,** 277.
5. Northrop, J. H., *J. Gen. Physiol.*, 1922, **4,** 487.
6. Northrop, J. H., *J. Gen. Physiol.*, 1932, **16,** 339.
7. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1928, **11,** 477.
8. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, 1948, **172,** 221.
9. Schwert, G. W., and Eisenberg, M. A., *J. Biol. Chem.*, 1949, **179,** 665.
10. Steinhardt, J., and Zaiser, E. M., *Advances Protein Chem.*, 1955, **10,** 155, 168.