[CONTRIBUTION FROM THE DIVISION OF BASIC BIOCHEMISTRY OF THE PALO ALTO MEDICAL RESEARCH FOUNDATION, PALO ALTO, CALIFORNIA]

Salt Reversible Inhibition of Chymotrypsin by Serum Albumin and Other Proteins[1]

BY B. H. J. HOFSTEE

RECEIVED FEBRUARY 8, 1960

Serum albumin and other proteins in concentrations of the order of that of the enzyme strongly inhibit the hydrolysis of acetyltyrosine ethyl ester and of methyl hippurate by chymotrypsin. This inhibition is prevented by salts, especially by bivalent anions and cations. Evidence is presented that the primary structure of the proteins is involved.

The *p*H-stat technique[2] that obviates the presence of buffer has made it possible to study the influence of low salt concentrations on enzymic reactions that produce changes in the hydrogen ion concentration. Few such studies have been carried out with chymotrypsin, although it has been found that certain salts tend to prevent the association of chymotrypsin molecules that occurs at enzyme concentrations of the order of 0.1% or higher.[3] It has been found also that various salts up to concentrations of ≈ 1 *M* have a kinetic influence on the monomeric form of the enzyme.[4] The present investigation, however, is concerned with enzyme concentrations not higher than 0.01% and salt concentrations not higher than 0.1 *M* and deals mainly with the influence of salt on the reaction of chymotrypsin with other proteins.

Experimental

Activity Determinations.—Initial rates of acid formation at constant *p*H were measured at 30° with the use of a Radiometer *p*H-stat Type TTTla, standardized with buffer based on U. S. National Bureau of Standards certified buffers and recorded with an Ottesen recorder.[2] To minimize leakage of KCl a calomel electrode with upwards bent capillary tip[2] was used. In the absence of CO_2 blank values were negligible at all *p*H values applied, *i.e.*, alkali (0.1 *N* NaOH) was taken up at a measurable rate only when both enzyme and substrate (0.01 *M*) were present. The final concentration of salt formed during the titration could be kept below the concentration ($\approx 10^{-3}$ *M*) at which the salt effect under study becomes noticeable (see below).

Substrates.—N-Acetyl-L-tyrosine ethyl ester (Mann Research Laboratories) was chromatographically pure, with a m.p. of 101–102° and specific rotation of +28° in methanol. The ester, referred to as ATEE, is soluble in water up to a concentration of ≈ 0.02 *M* at 30° and was applied in aqueous solution without the aid of alcohol.

N-Benzoylglycine methyl ester (H. M. Chemical Co.), grade CP is more soluble in water than ATEE. It is referred to as MH (methyl hippurate).

Enzyme; Reaction Mixture.—Salt free crystalline bovine α-chymotrypsin (Armour) was kept in the refrigerator as stock solutions in 10^{-3} *N* HCl. With the aid of Carlsberg pipettes not more than 0.1 ml. of the appropriate stock solution was added to 10 ml. reaction mixture.

When only a few μg. enzyme is used, *e.g.* in the case of ATEE, the system is extremely sensitive to traces of contaminating material. For instance, in a reaction mixture with 0.1 *M* of an "analytical" grade KCl (0.0005% heavy metal expressed as Pb), the reaction rate was found to decrease considerably during the time of measurement. Versene had a protecting effect and the inactivation was more pronounced in alkaline than in acid solutions. Thus the inactivation could at least in part be ascribed to the action of heavy metals[5] that are present in such solutions in a concentration ($\approx 10^{-7}$ *M*) of the same order as that of the enzyme. However, some inactivation occurred even with 10^{-4} *M* Versene in the reaction mixture. For this reason serum albumin that has a high and almost universal binding power[6] was used as a protecting agent. With only 0.01% albumin the rate in 0.1 *M* KCl was constant (see Fig. 1) and strictly proportional to the enzyme concentration. Thus, the decrease in rate observed in the absence of salt (Fig. 1), but in the presence of serum albumin, indicates a genuine change in the system. A reaction of the enzyme with serum albumin as the substrate (see below) did not cause a measurable *p*H change, at least not at such a low albumin concentration. Neither did it affect the protecting power of the protein.

With MH as the substrate, whereby of the order of 1 mg. enzyme must be used to obtain a convenient reaction rate, the addition of a protecting agent is not necessary. Nevertheless, precautions against contamination must, also here, be taken since it was found that even at this higher enzyme concentration 10^{-5} *M* $CuSO_4$ caused $\approx 50\%$ inhibition.

Results and Discussion

Inhibition by Proteins in the Absence of Salt.—For the data in Fig. 1 the activity of chymotrypsin with 0.01 *M* ATEE as the substrate was measured in the presence of 0.01% serum albumin that was added as a protecting agent (see Experimental). As can be seen, in the absence of salt the rate decreases slowly until after 20–30 minutes it becomes constant. At *p*H 7 the extent of inhibition is about 80% as compared to the rate in the presence of 0.1 *M* KCl. Even with $K_M >> (S)$ in the absence of salt,[4] it follows from the equation[7] $(\Delta v)/v = I/K_i (1 + S/K_M)$ that an inhibition (Δv) of this magnitude, caused by competition with serum albumin (I) as a substrate, would correspond to an apparent inhibitor constant (K_i) of the order of 10^{-7} *M*.

The Michaelis constants of chymotrypsin are usually in the range of 10^{-2}–10^{-4} *M*.[8-10] Although these constants are not necessarily dissociation constants, it seemed nevertheless reasonable to assume[1] that reaction of the enzyme with serum albu-

(1) Presented, in part, under the title of "Kinetic Effects of Salt on Chymotrypsin," at the 136th National Meeting of the American Chemical Society in Atlantic City, Sept. 13–18, 1959.

(2) For a comprehensive description of this technique along with the original references see: C. F. Jacobson, J. Léonis, K. U. Linderstrøm-Lang and M. Ottesen in "Methods of Biochemical Analysis," David Glick, Ed., Vol. 1V, Interscience Publishers, Inc., New York, N. Y., 1957, p. 171.

(3) G. W. Schwert, *J. Biol. Chem.*, **179**, 655 (1949); G. W. Schwert and S. Kaufman, *ibid.*, **190**, 807 (1951); E. L. Smith, D. M. Brown and M. Laskowski, *ibid.*, **191**, 639 (1951); E. L. Smith and D. M. Brown, *ibid.*, **195**, 525 (1952); V. Massey, W. F. Harrington and B. S. Hartley, *Discussions Faraday Soc.*, **20**, 24 (1955); K. A. Booman and C. Niemann, *Biochem. Biophys., Acta*, **26**, 439 (1957); I. Tinoco, Jr., *Arch. Biochem. Bophys.*, **68**, 367 (1957); F. L. Aldrich, Jr., and A. K. Balls, *J. Biol. Chem.*, **233**, 1355 (1958).

(4) R. B. Martin and C. Niemann, THIS JOURNAL, **79**, 4814 (1957); *ibid.*, **80**, 1481 (1958).

(5) Similar observations on the influence of metal ions on chymotrypsin activity have been made by N. M. Green, J. A. Gladner, L. W. Cunningham, Jr., and H. Neurath, *ibid.*, **74**, 2122 (1952).

(6) F. Karush, *ibid.*, **72**, 2705 (1950).

(7) B. H. J. Hofstee, *Enzymologia*, Vol. XVII, 278 (1956).

(8) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(9) Few data are available on the Michaelis constants of proteolytic enzymes with protein-substrates, although for trypsin a value of $\approx 10^{-4}$ *M* has been found.[10]

(10) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1949).

min as a substrate could be neglected at the applied albumin concentration ($\approx 10^{-6}$ *M*) and that its only effect was a protection of the enzyme against traces of inactivating contaminants. The observation that increasing the albumin concentration had little or no further effect was taken as a confirmation of this point of view. Preincubation of the enzyme (stored at *p*H 3 and 0°) at the *p*H and temperature of the reaction mixture did not abolish the gradual decrease in the reaction rate upon subsequent mixing with the substrate and 0.01% serum albumin. Thus, since the change in environment of the enzyme could not account for the phenomenon, it was speculated[1] that inactivation was induced by the substrate (ATEE). However, further experimentation showed that indeed a reaction of the enzyme with the albumin is involved.

Using MH as the substrate, which requires an enzyme concentration of the order of 250 times higher than in the case of ATEE, the presence of a protecting agent is not necessary. Thus it is possible to measure rates in the absence of serum albumin and to determine its effect on the reaction.

TABLE I

INFLUENCE OF VARIOUS TREATMENTS OF SERUM ALBUMIN ON ITS INHIBITION (IN 0.01% CONCENTRATION) OF 1 MG. CHYMOTRYPSIN WITH NO SALT ADDED[a]

	Inhibition, %
Untreated serum albumin	52–56
Boiled at *p*H 7.5[b]	59–67
Dialyzed 3 days	62
Supernatant of ppt. at *p*H 5–6	5
Trypsin treated[c]	30–35
2–3 hr. at 100° in 0.1 *N* NaOH[d]	55
2–3 hr. at 100° in 0.1 *N* HCl	51
12–15 hr. at 100° in 0.1 *N* HCl	44
Dialyzed 20 hr.	34
Dialyzed 2 days	33
8 hr. at 100° in 5 *N* HCl[e]	13
20–24 hr. at 100° in 5 *N* HCl[e]	3

[a] Substrate: 10 ml. 0.012 *M* MH; *p*H 7.5. [b] At this *p*H the protein remains in solution. [c] Trypsin crystals were added to a boiled 1% serum albumin solution in 10^{-2} *M* tris buffer of *p*H 7.5 and the mixture was left overnight at room temp. [d] An H_2S-like odor was observed upon neutralization of this solution. [e] After treatment the hydrochloric acid was evaporated and the residue taken up in water.

It was found that the addition of 1 mg. of serum albumin to 1 mg. of α-chymotrypsin in 10 ml. reaction mixture of *p*H 7.5 decreased the rate of hydrolysis of MH by 60–70%, while in the presence of 0.1 *M* KCl the albumin had no effect. Similar results were obtained with δ-chymotrypsin. Although, in contrast to the case of ATEE, the inhibition needed no measurable time to develop (see below), the following observations indicate nevertheless that also in this latter case the inhibition is caused by the serum albumin: (1) A maximum inhibition of about 80% is found in both cases. The fact that this maximum is reached at a lower albumin concentration in the case of ATEE could be related to the lower enzyme concentration. (2) In both cases the inhibition is prevented by KCl in concentrations of 0.03 *M* or higher. (3) Pretreatment of the serum albumin with protein denaturating agents affects the inhibition in both cases (see below).

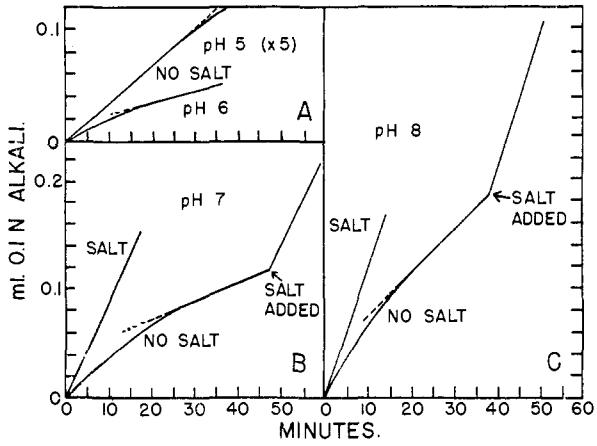

Fig. 1.—Recorder tracings of the alkali uptake in the presence of 0.01% serum albumin, with and without added salt, (0.1 *M* KCl) with 0.01 *M* ATEE as the substrate; 4 μg. enzyme was used at *p*H 6, 7 and 8 and 20 μg. enzyme at *p*H 5.

The data of Table I and Table II are presented as evidence that the inhibition is caused by the protein and not by a contaminant of the particular preparation used. Heat, acid and alkali treatment, dialysis and the effect of trypsin (Table I) indicate that the inhibition is a property of the intact, although denatured, protein that is more inhibitory than its split products.

TABLE II

INHIBITION OF 1 MG. CHYMOTRYPSIN BY 1 MG. OF VARIOUS NATIVE PROTEINS WITH NO SALT ADDED[a,b]

	Inhibition, %
Whole human serum (0.014 ml., ≈1 mg. protein)	45
Crystalline human serum albumin, NBC[c]	35
Crystalline bovine serum albumin, NBC	46
Crystalline bovine serum albumin, Armour	52
Bovine albumin, "Fraction V," NBC	46
Recrystallized ovalbumin, NBC	48
β-Lactoglobulin, NBC	35
Hemoglobin, NBC	10

[a] Substrate: 10 ml. 0.012 *M* MH; *p*H 7.5. [b] No inhibition occurred with any of these protein preparations at KCl concentrations > 0.04 *M* with the exception of whole serum that showed 10% inhibition in 0.04 *M* KCl. [c] Nutritional Biochemicals Corporation.

Table II shows that the inhibition occurs with all serum albumin preparations tested. It is given also by several other proteins which presents further evidence that the inhibition, of the same order of magnitude in all cases, is connected with the protein molecule. The exceptionally low inhibition by hemoglobin could mean that the globin is less inhibitory or that its inhibitory groups are masked by the heme.

Influence of Salt on the Inhibition.—Fig. 1 shows that with 4 μg. enzyme and ATEE as the substrate the inhibition is completely reversed by the addition of 0.1 *M* KCl. However, with 1 mg. enzyme and MH as the substrate it was found that complete reversal could not be obtained if, in the presence of albumin, the enzymic reaction was left to proceed for an extended period of time in the absence of salt. For instance, after a contact of

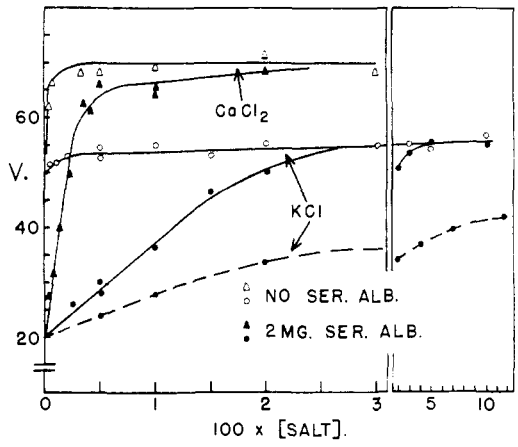

Fig. 2.—Influence of varying concentrations of KCl and $CaCl_2$ on the activity (v, microliter 0.1 N alkali per 5 min.) of 1 mg. α-chymotrypsin and on its inhibition by 2 mg. native serum albumin with 10 ml. 0.012 M MH as the substrate; pH 7.5. The points on the solid lines refer to separate short term runs whereby the enzyme and the albumin were added to the salt solution. The dotted line represents a single long term run whereby the rate was measured after each increment of the salt concentration.

about 15 minutes the inhibition was, within experimental error, completely reversible, but after 70–80 minutes it could be reversed for only about 60%. In view of the high enzyme concentration in the case of MH this would indicate that after the initial salt reversible reaction of the enzyme with the albumin a further and even slower reaction occurs that is not completely reversible by salt (see below). Thus the determination of the influence of the salt concentration on the reversibility must be carried out in short term experiments. Otherwise results are obtained as indicated by the dotted curve in Fig. 2.

It was found also that prolonged treatment of serum albumin with chymotrypsin decreased its inhibitory power. These complications do not apply to the case of ATEE where the enzyme concentration is so much lower.

In any event, with sufficient salt added to the reaction mixture *before* the albumin, the inactivation could always be prevented completely, even in the case of MH, as is seen in Fig. 2 for KCl and $CaCl_2$. This holds true for the other proteins in Table II and occurs with the native as well as with the heated protein. It may be noted that at suboptimal concentrations $CaCl_2$ is much more effective than KCl even when solutions of the same ionic strength are compared. The salts increase also the activity of the control in the absence of albumin.

In Table III the influence of various salts are compared at an ionic strength of 0.01. It can be seen that of the ones tested, the bivalent cations, especially Co++, are more effective than the monovalent cations. Also, the bivalent anion $SO_4^=$ is more effective than Cl^-.

In contrast to the influence on the inhibition, Ca^{++}[5] and Sr^{++} have a more or less specific activating effect on the rate in the absence of inhibitor (Table III). It should be noted that this is not connected with the prevention of possible autolysis

TABLE III

INFLUENCE OF VARIOUS SALTS IN 0.01 IONIC STRENGTH ON THE INHIBITION OF 1 MG. CHYMOTRYPSIN BY 2 MG. SERUM ALBUMIN[a]

Salt[b]	Activity (no. ser. alb.)	Plus 2 mg. ser. alb.	Protection by salt, %[c]
None	1.0	0.4	..
$CoCl_2$	1.07	1.08	100
$MgSO_4$	1.10	1.01	87
$CaCl_2$	1.36	1.22	83
K_2SO_4	1.12	0.95	75
$BaCl_2$	1.17	0.96	70
$SrCl_2$	1.29	1.04	68
$MgCl_2$	1.14	0.91	67
NaCl	1.06	0.71	45
KCl	1.07	0.72	45

[a] Substrate: 10 ml. 0.012 M MH; pH 7.5. [b] $NiSO_4$ has a slight inhibitory effect on the activity but like $CoCl_2$ completely prevents inactivation by serum albumin. [c] Multiple determinations indicated that the maximal error in these figures is not larger than 5%.

of the enzyme since there was no decrease in rate during the time of rate measurement in the absence of salt.

Preliminary Observations on the Reaction between Chymotrypsin and Serum Albumin.—The initial rate of hydrolysis of the substrate in the presence of albumin and in the absence of salt (Fig. 1), immediately after adding the enzyme, cannot be determined with great accuracy. However, it would appear that this rate, at least at pH 8, is not very much lower than that in the presence of salt. Considering the fact that KCl has a slight activating effect regardless of the presence of albumin (Fig. 2), it is possible that the native albumin has little or no inhibitory action. With heated or urea treated serum albumin, or after pretreatment of the albumin with the enzyme,[11] the time needed for the inhibition to develop fully was considerably less, indicating that this time element is connected with the denaturation of the albumin, possibly by the enzyme acting as a "denaturase".[12] The apparent absence of the time factor in the case of MH as the substrate would be due to the higher rate of this process at the higher (250 fold) enzyme concentration. On the basis of this time element surface denaturation can be excluded since the albumin was present at the same concentration in both cases.

It is a well known fact that after denaturation, proteins are more readily attacked by proteolytic enzymes.[13] Thus, the above observations suggest that the inhibitory action is associated with the reaction of the enzyme with the protein as a substrate. However, it was found that salt that prevents the inhibition of the hydrolysis of ATEE and MH by the native as well as that by the heated serum albumin prevents the base uptake due to

(11) In order not to introduce kinetic complications[3] the results reported in this paper refer to systems containing no alcohol that is usually applied to obtain concentrated stock solutions of the substrate. These concentrated solutions can be added to the reaction mixture without considerable dilution of the system. When this procedure was used in the case of ATEE, it was found that incubation of the enzyme with the serum albumin, prior to adding the substrate, abolished the time lapse otherwise needed for the inhibition to develop.

(12) For a review on this subject see: N. M. Green and H. Neurath in "The Proteins," H. Neurath and K. Bailey, Eds., Vol. II, part B, Academic Press, Inc., New York, N. Y., 1954, p. 1057.

(13) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **17**, 399 (1934).

splitting of peptide bonds only when the native but not when the heated protein is used as the substrate. In these experiments the alkali uptake by a mixture of 1 mg. chymotrypsin and 10 ml. of a 1% solution of serum albumin was measured at *p*H 7.5.

Thus it would seem that in the presence of salt the denatured albumin can still act as a substrate but not as an inhibitor. The inference would be that the inhibition by serum albumin involves salt sensitive groups that are not essential in its reaction as a substrate. This duality is also suggested by the observation that the Michaelis constants of a variety of substrates (see above) are of the order of 10^3–10^5 times as high as the apparent inhibitor constants (I_{50}-values) of serum albumin and the other proteins of Table II.

Comparison with Other Inhibiting Proteins.—An inhibitory action by protein fractions from serum and other sources on various proteolytic enzymes, including chymotrypsin, has been observed on numerous occasions.[14] However, to the author's knowledge a potent inhibition of the type shown above has never been ascribed to the serum albumin molecule or to other proteins, such as those in Table II. The reason for this could be that salts, applied as buffers or otherwise, were present in concentrations too high to observe this inhibition.

Since it occurs at protein concentrations of the order of 10^{-7} *M*, the inhibition is of the same order of magnitude as that by a typical trypsin and chymotrypsin inhibitor, such as the one that has been obtained from soybean.[15] However, the latter inhibition is of a different type because the effect of soybean inhibitor decreases by heating and is not reversed by salt. An experiment at *p*H 7.5 with 1 mg. of chymotrypsin, 0.1 mg. of soybean inhibitor and 10 ml. of 0.012 *M* MH as the substrate showed an inhibition of 43%, while the heated inhibitor gave less than 10% inhibition. The inhibition was 100% with 1 mg. soybean inhibitor, even in the presence of 0.04 *M* KCl. Thus it would seem that, in contrast to our case, the inhibition by the soybean protein is connected with its secondary or tertiary structure and that ionic forces may not play a predominant role.

(14) For a review with references on this subject see: M. Laskowski and M. Laskowski, Jr., "Adv. in Protein Chemistry," M. L. Anson and K. Bailey, Eds., Vol. IX, Academic Press, Inc., New York, N. Y., 1954, p. 203.

NOTE ADDED IN PROOF.—In the meantime it has been found that a salt reversible inhibition, similar to that by serum albumin, is also shown by other charged polymers such as carboxymethylcellulose (soluble sodium salt) and nucleic acids. This would give support to the supposition that the inhibition by serum albumin is unrelated to the fact that it is a substrate for the enzyme.

Acknowledgments.—The preliminary work for this investigation was carried out in the Department of Chemistry of the Carlsberg Laboratory in Copenhagen, Denmark. The author gratefully acknowledges the hospitality of the late Professor K. U. Linderstrøm-Lang and the coöperation of the Carlsberg Staff, especially Dr. M. Ottesen and Civilingeniør A. Johansen. This investigation was supported by Grant Nr. C 2289 (C6) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(15) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948, p. 162.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY, EVANSTON, ILLINOIS]

"Denaturation" of a Synthetic Polymer and its Relationship to Protein Denaturation

BY IRVING M. KLOTZ AND VIRGINIA H. STRYKER

RECEIVED JANUARY 2, 1960

A conjugate has been prepared having a $(CH_3)_2N$- group attached to a polymer (polyvinylpyrrolidone) which cannot form intramolecular hydrogen bonds. The acidity constants of this polymer conjugate in water and in urea have been compared with those of a protein conjugate. Parallel shifts in pK_a are observed with polymer and protein. Since urea denatures proteins, it must also "denature" the polymer lacking hydrogen bonds. It is suggested, therefore, that the primary step in denaturation is a perturbation of solvent–(macromolecular) solute interactions, rather than a disruption of intramolecular hydrogen bonds.

Introduction

It has been suggested recently[1] that many aspects of the behavior of proteins in solution (*e.g.*, masked groups, reversible denaturation, shifts in pK_a's) might be interpreted advantageously in terms of the crystalline nature of the hydration water of the macromolecule. Analogy was drawn between the stabilizing effect of non-polar molecules (for examples, see Table I) on the lattice structure of water leading to crystalline hydrates,[2–4] and the proposed stabilizing influence of side chains of non-polar amino-acid residues (for examples, see Table I) on the lattice of hydration water of a protein molecule. From this viewpoint, the denaturing effect of urea is considered to involve first a disruption of the hydration lattice, followed, when denaturation is irreversible, by a disruption of the framework of the macromolecule itself.

In this connection an intriguing experiment would be to examine the effect of urea in some macromolecular system which does not have both hydrogen donor and hydrogen acceptor groups to stabilize its structure. In such a system any influence of urea could not be attributed to disruption of intramolecular hydrogen bonds but would have to be ascribed to some other cause.

(1) I. M. Klotz, *Science*, **128**, 815 (1958).

(2) W. F. Claussen and M. F. Polglase, THIS JOURNAL, **74**, 4817 (1952).

(3) M. v. Stackelberg and H. R. Müller, *Z. Elektrochem.*, **58**, 25 (1954).

(4) M. v. Stackelberg and B. Menthen, *ibid.*, **62**, 130 (1958).