0.9426) but still containing a small amount of halogen. This material was lost in attempting to remove the last traces of halogen.

The substance appeared to form a binary with water boiling at 88–90° and an attempt was made to utilize this property but the results were not as satisfactory as with the magnesium chloride method.

The use of potassium carbonate as a drying agent for the supposed cyclopropanol was abandoned early in the work because it converted the product to higher boiling, water-insoluble substances that lost water during further purification by distillation. In a control experiment, in which 0.97 mole of 1-bromo-3-chloro-2-propanol was dissolved in 700 cc. of ether, the mixture saturated with water and dried with potassium carbonate for two weeks, 46% of the halohydrin was converted to epichlorohydrin, b. p. 114–117°.

Anhydrous copper sulfate did not give satisfactory results either and attempts to freeze the water out of ether solutions of the cylopropanol fraction with dry-ice gave no ice crystals. Calcium sulfate was not tried because in one experiment 4-heptanol was changed to an olefin on being distilled after drying over Drierite. A similar experience with 3-methyl-2-butanol was encountered in this Laboratory.[12]

One cyclopropanol fraction after drying over sodium sulfate, later found to be quite neutral, gave, on several distillations through a 3-bulb Snyder column, a 7% yield of propionaldehyde, b. p. 46–52°, based on the bromochloropropanol used. The propionaldehyde was identified by conversion to the 2,4-dinitrophenylhydrazone and application of the mixed melting point method.

Attempts to Convert Cyclopropanol to Other Substances.—The substance absorbed bromine in carbon tetrachloride without evolving hydrogen bromide. Distillation produced considerable coke-like material but no well-defined product.

Concentrated hydrochloric acid, hydrochloric acid–zinc chloride reagent and phosphorus pentachloride all decomposed the compound and no definite product could be isolated. An attempt to acetylate a 6 cc. portion of impure material with 9 cc. of acetyl chloride resulted in a product with an ester-like odor but which distilled over a wide range, 90–118°, and left a residue. When formic acid (1 cc.) was heated with an equal amount of the alcohol, a strong odor of aldehyde was produced.

Attempt to Isolate 1,4-Cyclohexanediol.—An aqueous layer, that remained after hydrolysis and ether extraction of a bromochloropropanol experiment, was treated with potassium carbonate until precipitation was complete. The filtrate was concentrated by distillation, evaporated to dryness and the residue extracted thoroughly with a 50/50 mixture of anhydrous ether and absolute alcohol. Evaporation of the organic solvents gave no significant amounts of organic material.

(12) L. S. Powell, Doctor of Philosophy dissertation, 1936, Rutgers University, p. 51.

Summary

The theory that ethylene oxides react spontaneously with both the alkylmagnesium and magnesium bromide bonds of the Grignard reagent was found completely satisfactory when applied to the reaction of epichlorohydrin with ethylmagnesium bromide.

Evidence found during the investigation pointed to the presence of cyclopropanol as a product of the reaction of epichlorohydrin and of 1-bromo-3-chloro-2-propanol with ethylmagnesium bromide.

NEW BRUNSWICK, N. J. RECEIVED JULY 21, 1941

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF DUKE UNIVERSITY]

Inhibition of the Proteolytic Action of Trypsin by Soaps

BY ROBERT L. PECK[1]

It has long been known[2] that soaps are capable of inhibiting the proteolytic activity of trypsin. Earlier workers,[3] using trypsin which had not been crystallized, showed that the amount of inhibition brought about in a given system was proportional to the amount of soap present, and that the inhibiting effect appeared to be greater the more unsaturated the soap. The phenomenon has been reinvestigated using crystalline trypsin and pure soaps. A simple quantitative system is described, and some results obtained are discussed.

(1) Present address: Merck & Co., Inc., Rahway, N. J.

(2) J. Neumann, *Berl. Klin. Wchnschr.*, **65**, 2066 (1908).

(3) J. W. Jobling and W. Petersen, *J. Exp. Med.*, **19**, 239 (1914).

Experimental

Materials.—In all of the experiments here described, crystalline trypsin was used.[4] The substrate stock was a 5% solution of casein in 0.1 *M* phosphate buffer, *p*H 7.6, prepared as described by Northrop and Kunitz.[5] The soap solutions were prepared just before use, by neutralization of a weighed sample of fatty acid with the calculated amount of aqueous potassium hydroxide solution and dilution. Oleic and linoleic acids were distilled in nitrogen

(4) (a) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **16**, 267 (1932); **19**, 991 (1935). (b) The preparation employed was a three times recrystallized sample very kindly furnished by Dr. J. H. Northrop, of the Rockefeller Institute for Medical Research. The small amount of magnesium sulfate present could be removed by dialyzing the solution of trypsin in 0.01 *N* HCl against 0.01 *N* HCl.

(5) J. H. Northrop and M. Kunitz, *ibid.*, **16**, 362 (1932).

atmosphere at 0.1 mm. pressure, and had iodine numbers of 89.0 and 181.0, respectively. Linolenic acid was prepared and purified as described by Erdmann and Bedford,[6] and had an iodine number of 254.0. Linoleic and linolenic acids were also prepared from tetrabromostearic and hexabromostearic acids[7] as described by Kaufmann and Mestern.[8] Stearic acid, m. p. 69.5–70.5, neutral equivalent 284.8, was prepared by catalytic reduction of the purified oleic acid. Dibromostearic acid was obtained by bromination of the purified oleic acid in cold light petroleum ether.[9] (Calcd. for $C_{18}H_{34}O_2Br_2$: Br, 36.15. Found: Br, 37.10.) Tuberculostearic acid, m. p. 10–12°, neutral equivalent 299 (calcd. for $C_{19}H_{38}O_2$: 298), and phthioic acid, m. p. 20–21°, neutral equivalent 395.7 (calcd. for $C_{26}H_{52}O_2$: 396), both from tubercle bacilli,[10] were obtained as described by Spielman[11] and Spielman and Anderson.[12] The unsaturated fatty acids of *Blastomyces dermatitidis* were isolated as described by Peck and Hauser,[13] and had an iodine number of 114 and a neutral equivalent of 282. These have been shown[13] to be a mixture of oleic and linoleic acids. All of the soaps formed clear solutions (0.5%) with the exception of stearate and dibromostearate, which were not very soluble. Iodine numbers were determined by the Rosenmund and Kuhnhenn method.[14] A borate buffer[15] was used for dilutions in the experiments. All *p*H measurements were made with a glass electrode

Procedure.[16]—Into a 50-cc. Erlenmeyer flask was pipetted the amount of buffer solution calculated to give a volume of 30 cc. when the other solutions were added. One cc. of a solution of crystalline trypsin, 0.17 mg., in 0.01 *N* HCl was added and mixed with the buffer. The soap solution was then added. After mixing contents, the flask was left at room temperature (30°) for ten minutes. At the end of this period, 5 cc. of the 5% casein was mixed with the solution and duplicate 1-cc. aliquots were taken at once for determination of the total undigested protein. The flask was left at room temperature (which was nearly constant at 30°) throughout the experiment. Duplicate 1-cc. aliquots, taken at intervals, permitted measurement of the rate and extent of the digestion. Intervals were measured with a stop watch.

Each aliquot for determination of undigested protein was run into a 4-inch Wassermann tube containing 1 cc. of 10% trichloroacetic acid and the tube was shaken. Two cc. of 5% trichloroacetic acid was then used to wash down into the suspension particles of precipitated protein adhering to the walls of the tube. The tube was allowed to stand in an ice-water-bath for one-half hour and was then centrifuged. The supernatant solution was discarded and the tube carefully washed with 2 cc. of cold 5% trichloroacetic acid, centrifuged and drained. The precipitate was then suspended in about 2 cc. of water and poured into a 100-cc. Kjeldahl flask. The remaining particles of precipitate were rinsed into the flask and the tube was finally washed with a little dilute sodium hydroxide to ensure quantitative transfer of all protein material to the Kjeldahl flask.[17]

TABLE I

AMOUNT OF PROTEIN NITROGEN REMAINING AFTER DIGESTION OF CASEIN (36.9 MG. OF PROTEIN N) BY CRYSTALLINE TRYPSIN (0.17 MG.) IN THE PRESENCE OF VARIOUS QUANTITIES OF SOAP

Volume 30 cc.; *p*H 7.8. All tubes contained 1.23 mg. protein N per cc. at start.

Amount of soap in flask, mol. × 10^{-5}	Mg. of protein N precipitated per cc. by trichloroacetic acid after digestion for four minutes in the presence of potassium soaps			Mg. of protein N precipitated per cc. by trichloroacetic acid after digestion for two hours in the presence of potassium soaps		
	Oleate	Linoleate	Linolenate	Oleate	Linoleate	Linolenate
0.0	1.16	1.10	1.11	0.78	0.71	0.77
0.9	1.21	1.20	1.19	0.84	0.79	0.83
1.8	1.23	1.21	1.22	0.99	0.86	1.06
2.7	1.23	1.22	1.22	1.17	1.04	1.17
3.6	1.22	1.23	1.22	1.22	1.20	1.22
4.5	1.23	1.23	1.23	1.23	1.23	1.23

TABLE II

PERCENTAGE OF DIGESTION OF CASEIN (36.9 MG. OF PROTEIN N) BY CRYSTALLINE TRYPSIN (0.17 MG.) IN THE PRESENCE OF VARIOUS QUANTITIES OF SOAP

Interval of digestion, two hours; volume 30 cc.; *p*H 7.8.

Amount of soap in flask, mol. × 10^{-5}	Percentage of digestion after two hours in the presence of potassium			
	Oleate	Linoleate	Linolenate	Soaps of *B. derm.* unsatd. acids
0.0	100	100	100	100
0.9	87	85	87	90
1.8	53	71	37	50
2.7	13	37	13	42
3.6	2	6	2	17
4.5	0	0	0	0

Results.—The results of typical experiments on the potassium soaps of oleic, linoleic and linolenic acids are summarized in Tables I and II. Similar results were obtained with linoleic and linolenic acids prepared by both methods. In Table II the data on the ratio of mg. of protein digested in the flasks that contained soap, to mg. of protein digested in the control flask that contained no soap are expressed as percentage of digestion. The amount of digestion in the control

(6) E. Erdmann and F. Bedford, *Ber. Chem. Ges.*, **42**, 1324 (1909); *Ann.*, **464**, 214 (1928).

(7) Samples of pure tetrabromostearic and hexabromostearic acids were very kindly given by Dr. D. H. Wheeler, of the United States Department of Agriculture.

(8) H. P. Kaufmann and H. E. Mestern, *Ber.*, **69B**, 2684 (1936).

(9) R. H. Snyder and W. R. Bloor, *J. Biol. Chem.*, **99**, 562 (1933).

(10) Pure samples of both acids were kindly provided by Dr. R. J. Anderson of Yale University.

(11) M. A. Spielman, *J. Biol. Chem.*, **106**, 87 (1934).

(12) M. A. Spielman and R. J. Anderson, *ibid.*, **112**, 759 (1936).

(13) R. L. Peck and C. R. Hauser, THIS JOURNAL, **60**, 2599 (1938).

(14) K. W. Rosenmund and W. Kuhnhenn, *Z. Untersuch. Nahr. Genussm.*, **46**, 154 (1923).

(15) See W. M. Clark, "Determination of Hydrogen Ions," 3rd ed., Williams & Wilkins Co., Baltimore, Md., 1928, p. 213.

(16) Part of the procedure was adapted from the quantitative technique used in transferring immune precipitates to Kjeldahl flasks. For example, see M. Heidelberger, *et al.*, *J. Exp. Med.*, **52**, 477 (1930).

(17) The Kjeldahl digestion was carried out with sulfuric acid, potassium sulfate and copper sulfate. The ammonia, collected in 2% boric acid, was titrated with 0.01 *N* hydrochloric acid.

flask is considered 100% as a basis for calculation. When the percentage of digestion is subtracted from 100, a figure is obtained which may be arbitrarily termed the *percentage of inhibition.*

In order to compare inhibiting effectiveness of the various soaps, we chose as a basis the minimum amount of soap which was capable of causing complete inhibition of the proteolytic activity of a standard amount of trypsin (0.17 mg. of pure trypsin in 30 cc. of solution, *p*H 7.8) for two hours. Table III lists this figure for the soaps studied.

TABLE III

MINIMUM AMOUNTS OF SOAPS CAPABLE OF CAUSING COMPLETE TWO-HOUR INHIBITION OF 0.17 MG. OF CRYSTALLINE TRYPSIN

Potassium soaps	Molecular weight of fatty acid in soap	Amount of soap causing two-hour inhibition, mol. $\times 10^{-5}$
Linolenate	278	4.5
Linoleate	280	4.5
Oleate	282	4.4
B. derm. unsatd. acids	282	4.4
Stearate	284	Incomplete[a]
Tuberculostearate	298	4.2
Phthioate	396	3.2
Dibromostearate	442	Incomplete[a]

[a] Owing to poor solubility of soaps.

From the above data it can be seen that (a) solubility of a soap determines to a certain extent the inhibiting power of the soap. (Note that the relatively insoluble soaps stearate and dibromostearate and the insoluble calcium soaps—see reversibility experiment—show poor inhibiting power.) The soluble soaps show good inhibiting power.[18] (b) Double bonds, the main difference between stearate and the series oleate, linoleate and linolenate, appear to have a solubilizing effect and do not appear specifically to influence inhibiting power otherwise. In Table III it is evident that the inhibition is about the same for 18-carbon soaps with one, two or three double bonds. Also the tuberculostearate (soap of 10-methylstearic acid) which is a soluble but saturated soap also has good inhibiting power, about equal to that of the unsaturated soaps just mentioned. (c) The molecular weight of soluble soaps appears to have a specific influence on inhibiting power of soaps. The three unsaturated soaps mentioned and the saturated tuberculostearate are, roughly speaking, of about the same molecular weight and show about the same percentage of inhibition Phthioate, a much higher molecular weight soap, shows a correspondingly higher inhibiting power. (d) The structure of the fatty acids in soaps must have some specific effect on the inhibiting ability of the soluble soaps. This must be taken into consideration in interpreting the preceding inference. Phthioic acid is a complex branched acid whose structure is not yet known. It is unfortunate that no soluble normal chain soap of similar molecular weight was available for comparison.

It would be of interest to compare a number of soaps of several homologous series, including both normal and branched chain types. Such data would appear to be necessary in order to establish the truth of the above inferences and to decide on the mechanism of the inhibition reaction.

Reversibility.—When calcium chloride was added to mixtures of soap, enzyme and substrate showing complete two-hour inhibition, an insoluble calcium soap was precipitated and some digestion occurred. The sooner the calcium chloride was added after mixing the enzyme and the soap, the more nearly complete was the recovery of digestive activity. This indicates reversibility of soap inhibition. It also appears from this experiment that at the *p*H used, namely, 7.8, a slow alkaline denaturation takes place, as is to be expected.[19]

In experiments with amounts of soap just sufficient to cause complete two-hour inhibition, some digestion was observed after twenty-four hours. This was also true in experiments using twice that amount of soap. Thus it appears that the reaction is reversible or that the rate of digestion under these conditions is too slow to be measured over the arbitrary two-hour period.

Order of addition of components in the standard system was quite significant. When mixed in the order already described, 4.5×10^{-5} mole caused complete two-hour inhibition. With soap and substrate mixed before addition of enzyme, there was only 20% inhibition. With substrate and enzyme mixed before addition of soap, there was only 10% inhibition. It cannot at present be definitely decided whether the inhibition is a competitive or a non-competitive effect. It would appear that the former effect is not unlikely; this would fit in with a general soap-protein reaction.

(18) A single experiment carried out since with sodium ricinoleate did not show a very high inhibiting power. This may have been due to the fact that the only sample of this soap obtainable was not very pure.

(19) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **17**, 591 (1933–4).

Acknowledgment.—The author wishes to express his thanks to Dr. C. R. Hauser, of Duke University, for suggesting this problem and for his useful criticism during the work.

Summary

The inhibition of the proteolytic action of trypsin by soaps has been studied using crystalline trypsin and pure soaps. The effect appears to be reversible. A simple system for the quantitative study of the phenomenon has been described. The inhibition of tryptic action by soaps may be an example of a general soap-protein reaction.

PLAINFIELD, N. J. RECEIVED NOVEMBER 12, 1941

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA]

The Photolysis of Methyl Acetate

BY WALTER L. ROTH AND G. K. ROLLEFSON

In a recent paper Royal and Rollefson[1] reported observations on the photolysis of methyl formate. They showed, by means of experiments with metallic mirrors, that free radicals are formed in the decomposition of that compound and also in the photolysis of methyl acetate. In this paper we wish to present the results of analyses of the decomposition products of the latter compound and to interpret these results in terms of a reaction mechanism. In our experiments the energy necessary to bring about the reaction was introduced into the methyl acetate molecule in two ways. These were (1) by the absorption of light by the acetate molecule, and (2) by the absorption of the 2537 Å. line by mercury vapor in the reaction vessel followed by the reaction of this activated atom with the acetate molecules. Measurements of the absorption of light by the system showed that in the absence of mercury vapor there was no appreciable absorption at 2537 Å. Hence the two modes of activation can be studied separately.

Experimental

Apparatus and Materials.—The apparatus was essentially the same as that of Royal and Rollefson. Two types of light sources were used: (1) a quartz capillary mercury arc of the kind described by Atwood and Rollefson,[2] and (2) a low pressure gas filled resonance arc[3] which was operated at 25 milliamperes and 15,000 volts. The second arc was used for the mercury sensitized reaction in which the effective wave length was the 2537 line. In the capillary arc the resonance radiation was reversed and the other radiation near 2537 Å. was not absorbed so the effective energy was that supplied by the lines in the mercury spectrum between 2537 and the transmission limit of quartz. Since no filters were used all of these lines were involved but the principal one is that at 1942 Å.

The methyl acetate was from the same lot as had been used for the mirror experiments. The nitric oxide was obtained from Dr. Atwood, who had prepared it by the method of Johnston and Giauque.[4]

Procedure.—The reaction vessel and connecting tubing were evacuated to a pressure of 10^{-5} mm. or less, methyl acetate introduced to the desired pressure, and the temperature noted. The system was illuminated until a suitable amount of reaction had occurred as shown by the pressure change. The products were separated into three fractions as follows.

−185° Fraction.—The cell was immersed in liquid air and those gases not condensed were pumped into the gas buret. The residual products were warmed to room temperature and the pressure noted. In all cases, the moles of gas measured in the buret agreed satisfactorily with the value calculated from pressure differences. The fraction then was forced into a small gas holder and subsequently analyzed.

−117° Fraction.—This fraction represents those gases not condensable at the melting point of ethyl alcohol. The residual reaction mixture was distilled into a small (1-cc. capacity) tube equipped with a stopcock. A Dewar flask containing a solid-liquid ethyl alcohol mixture was placed around the tube, and after temperature equilibrium was established the stopcock was opened and the non-condensed gases expanded into the 500-cc. volume of the Toepler pump. Then the stopcock was closed, the gases in the pump forced into the gas buret, and the process repeated. (The quantity of gases measured in the buret was usually found to be somewhat less than that calculated from the pressure data. The buret values were far more consistent and were used for subsequent calculations.)

Residual Fraction.—This fraction represents those products which are condensable at room temperature and atmospheric pressure. After their pressure as gases was measured in the reaction vessel, they were distilled into a 50 cc. flask which contained 10 cc. of water. The flask was attached to the vacuum line through two ground joints and a stopcock, arranged so the flask could be detached and opened for analysis.

Analytical Methods.—Gas analyses were made in a microburet of the type described by Blacet and Leighton, and

(1) Royal and Rollefson, THIS JOURNAL, **63**, 1521 (1941).
(2) Atwood and Rollefson, *J. Chem. Phys.*, **9**, 506 (1941).
(3) Noyes and Leighton, "The Photochemistry of Gases," Reinhold Publishing Corp., New York, N. Y., 1941, p. 38.
(4) Johnston and Giauque, THIS JOURNAL, **51**, 3194 (1929).