sary for the "ordering" of water molecules. The specific mechanism involved, as yet unknown, merits serious investigation.

Summary.—The proton signal of the water in agar gels, studied in high resolution NMR, differs from that of pure water in that the line width is significantly broadened and the amplitude is decreased; T_1 is not influenced but T_2 is decreased. This effect in agar is not shown by a variety of other gels and viscous solutions studied. The signal of the methyl protons from tetramethylammonium chloride is essentially equivalent when studied in agar gels or in pure water in marked contrast to the proton signal from water. These findings demonstrate that a possible heterogeneity of the internal field, resulting from compartmentalization of water, diamagnetic anisotropy, or the presence of paramagnetic impurities, are not responsible for the NMR changes observed in the water of agar gels. The NMR data can best be explained at present on the basis that water in an agar gel is in a modified state with properties of structural rigidity and mobility intermediate between "free" water and ice.

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[10] There is a chemical shift as temperature is varied in the 3 per cent agar, the water signal shifting downfield as temperature decreases (methyl peak as standard); similar results are obtained, however, with water-tetramethylammonium chloride. The similar chemical shift observed with both 3 per cent agar and water relative to the methyl signal does not necessarily mean that the degree of hydrogen bonding of water in the two cases is necessarily the same.

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*STUDIES ON BEEF SPLEEN CATHEPSIN A**

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Previous studies on the action of proteolytic enzymes in animal tissues (spleen, kidney, liver) on synthetic substrates for well-defined proteinases (pepsin, trypsin, chymotrypsin) have led to the identification of three cathepsins, designated A, B, and C, respectively.[1] Cathepsin A (termed cathepsin I in earlier papers[2–4]) was characterized by its optimal action on carbobenzoxy-L-glutamyl-L-tyrosine

(a substrate for pepsin[5]) near pH 5.6, and the absence of a requirement for activation by cysteine or of inhibition by iodoacetic acid.[2] This substrate also appears to be cleaved by a carboxypeptidase present in spleen and kidney, and which requires activation by SH compounds.[3] Cathepsin B (termed cathepsin II in earlier papers[3, 4]) hydrolyzes benzoyl-L-argininamide (a substrate for trypsin[6]) at pH values near 5.3 and requires activation by SH compounds.[7] Cathepsin C deamidates glycyl-L-tyrosinamide (a substrate for chymotrypsin[8]) in the pH range 4–8, and is activated by SH compounds.[9] Highly purified preparations of cathepsins B and C have been obtained from beef spleen,[7, 10] and have been shown to cleave proteins; studies of the specificity of these two enzymes toward synthetic substrates, however, have shown significant differences from those of trypsin and chymotrypsin respectively.[7, 11] Although cathepsin A has been considered to be similar to pepsin in its specificity, highly purified preparations of this enzyme have not been available for careful specificity studies, and work has therefore been undertaken in this laboratory directed to the purification and characterization of cathepsin A.

The present preliminary communication is prompted by the publication of a report by Press, Porter, and Cebra[12] on the preparation, from beef spleen, of a proteolytic enzyme, designated by them cathepsin D. Although highly purified preparations of this enzyme cleaved the B chain of insulin at peptide bonds compatible with the pepsin-like specificity originally postulated for cathepsin A,[2] the above authors were unable to observe any hydrolysis, at pH 5.0, of any of a number of synthetic substrates for pepsin. They reported, moreover, that assays for cathepsin A activity, with carbobenzoxy-L-glutamyl-L-tyrosine as the substrate, showed only minimal amounts to be present in initial spleen extracts, and that no cathepsin A could be detected after the first stage of purification. Because of their failure to detect this enzyme with certainty in the crude spleen extracts prepared by them, and the absence of hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by purified cathepsin D, Press *et al.* concluded that "it is clear that cathepsin D is unrelated to cathepsin A and the status of the latter enzyme is uncertain."

In view of this report, we wish to describe some recent experiments on the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine and related substrates by beef spleen preparations. These experiments have confirmed the many earlier observations that beef spleen contains readily measurable amounts of an enzyme that hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine near pH 5.7, and which does not require activation by SH compounds and is not inhibited by iodoacetic acid. Furthermore, evidence has been obtained for the endopeptidase action of such cathepsin A preparations through the finding that carbobenzoxy-L-glutamyl-L-tyrosylglycine is cleaved at the glutamyl-tyrosyl bond, a pepsin-like specificity consonant with that postulated for cathepsin A, and that demonstrated by Press *et. al.*[12] for the action of cathepsin D on the B chain of insulin. The status of cathepsin A is therefore less uncertain than suggested by Press *et al.*, and re-investigation of the action of cathepsin D on synthetic substrates seems desirable in order to establish more clearly the relationship of cathepsin D to cathepsin A.

Materials and Methods.—The following synthetic peptides were used in these studies, and had been prepared in this laboratory: carbobenzoxy-L-glutamyl-L-tyrosine,[13] carbobenzoxy-L-glutamyl-L-tyrosylglycine,[5] carbobenzoxyglycyl-L-tyrosine,[14] and L-tyrosylglycine.[15] The enzyme preparation was obtained from

beef spleen extracts by $(NH_4)_2SO_4$ precipitation in the manner described previously.[2] Stock enzyme solutions were prepared by dissolving 1 gm. of the precipitate in 15 ml of water followed by dialysis at 4°C against distilled water for about 24 hr. The dialyzed enzyme solution was filtered; the protein content of the filtrate was about 15 mg of protein per ml as measured by the biuret method,[16] with crystalline bovine serum albumin as a standard.

The enzymic activity of such solutions was tested at 38°C in a reaction mixture which contained 0.034 *M* (34 μmoles per ml) carbobenzoxy-L-glutamyl-L-tyrosine (or related compound) and 0.086 *M* citrate buffer of appropriate pH; the enzyme concentration in the test solution was about 3 mg of protein per ml. Samples (0.3 ml) were withdrawn at time intervals, diluted to 2 ml with water, and 0.1 ml portions used for photometric determination of liberated amino groups; 0.5 ml of ninhydrin reagent[17] were added, and the mixture was heated in a boiling water bath for 20 min. The cooled mixtures were diluted with 5 ml of *n*-propanol-water (1:1) and the color was measured in a Coleman Junior spectrophotometer at 570 mμ, using tyrosine as a standard. Experiments in which enzyme or substrate had been omitted were run in parallel, and the blank values were subtracted. Control experiments showed that the presence of carbobenzoxy-L-glutamic acid in the test sample did not affect the results obtained by this method. Separate experiments showed that the same results were obtained when the samples of the incubation mixture were diluted to 2 ml with 1 per cent picric acid and centrifuged.[18]

The extent of hydrolysis is expressed in μmoles of substrate (per ml of incubation mixture) cleaved at one peptide bond. When carbobenzoxy-L-glutamyl-L-tyrosylglycine was used as the substrate, L-tyrosylglycine was used as the standard for calculation of the extent of hydrolysis.

Results.—At pH 5.6, the crude enzyme solution readily effected the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine; under the conditions given above, the following values were obtained: 30 min, 10 μmoles; 60 min, 14.5 μmoles; 120 min, 20 μmoles. The liberation of tyrosine was confirmed by paper chromatography, using Whatman No. 1 paper and butanol-acetic acid-water (25:6:25) as the solvent. The pH optimum for the action of the enzyme preparation on carbobenzoxy-L-glutamyl-L-tyrosine is about 5.7; the values for the extent of hydrolysis in 2 hr were: pH 4.8, 5 μmoles; pH 5.3, 17 μmoles, pH 5.7, 21 μmoles; pH 6.1, 17 μmoles; pH 6.3, 9.5 μmoles.

As shown in Table 1, the enzymic cleavage of carbobenzoxy-L-glutamyl-L-

TABLE 1

EFFECT OF IODOACETAMIDE AND 2-MERCAPTOETHANOL ON ENZYMIC HYDROLYSIS OF CARBOBENZOXY-L-GLUTAMYL-L-TYROSINE BY BEEF SPLEEN PREPARATION

Concentration of iodoacetamide (*M*)	Concentration of 2-mercaptoethanol (*M*)	Hydrolysis μmoles./ml.
...	...	12.5
0.001	...	12.5
0.0015	...	12.5
...	0.02	19
...	0.04	20
0.001	0.02	12.5
0.0015	0.04	12.5

pH 5.2; time, 2 hr.

tyrosine is not inhibited by iodoacetamide at the concentration levels tested. Addition of 2-mercaptoethanol caused an increase in the rate of hydrolysis, presumably by activating the SH-dependent carboxypeptidase. Upon addition of iodoacetamide to the mercaptoethanol-activated system, the observed rate was the same as that found in the absence of added SH compound.

The fact that the enzyme preparation contains an endopeptidase capable of cleaving a glutamyl-tyrosyl bond is shown by the finding that carbobenzoxy-L-glutamyl-L-tyrosylglycine is hydrolyzed (Table 2) and the demonstration that

TABLE 2

ACTION OF BEEF SPLEEN PREPARATION ON SYNTHETIC SUBSTRATES

Substrate	Time (hr)	Hydrolysis (μ moles/ml)
Carbobenzoxy-L-glutamyl-L-tyrosine	2	20.5
Carbobenzoxy-L-glutamyl-L-tyrosyl-glycine	2	4
	20	12
Carbobenzoxyglycyl-L-tyrosine	2	2
	24	6

pH 5.6.

tyrosylglycine is a product of the reaction. Chromatographic analysis of the incubation mixture (20 hr) in the manner mentioned above gave a single ninhydrin-reactive spot whose R_F (0.41) corresponded to that of tyrosylglycine. Although this peptide has the same R_F value as tyrosine in the solvent system employed, there was no evidence in the chromatograms, even after prolonged incubation with the enzyme preparation, of the appearance of glycine (R_F 0.21). Furthermore, elution of the material of R_F 0.41, followed by acid hydrolysis (6 N HCl, 105°C, 24 hr) and paper chromatography of the hydrolysate gave two spots of R_F 0.43 and 0.21, corresponding to tyrosine and glycine respectively.

Although further work is needed to establish unequivocally that the same enzyme is responsible for the cleavage of carbobenzoxy-L-glutamyl-L-tyrosylglycine and carbobenzoxy-L-glutamyl-L-tyrosine at the glutamyl-tyrosyl bond, it appears likely that both synthetic substrates are hydrolyzed by the enzyme designated cathepsin A, and having a pepsin-like specificity. In this connection, it is of interest that the beef spleen preparation hydrolyzes carbobenzoxyglycyl-L-tyrosine much more slowly than carbobenzoxy-L-glutamyl-L-tyrosine, a behavior similar to that observed for pepsin.[5] Previous data have shown that carbobenzoxyglycyl-L-tyrosine is more readily cleaved by the SH-activatable carboxypeptidase of animal tissues.[2, 4]

Summary.—Beef spleen extracts contain enzymic activity toward carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-tyrosylglycine, the glutamyl-tyrosyl bond being cleaved in both cases. This action is similar to that of pepsin on these substrates, and is assigned to the spleen enzyme designated cathepsin A, which acts optimally near pH 5.7 and does not require activation by sulfhydryl compounds.

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*EVIDENCE FOR THE EXISTENCE OF A SINGLE-STRANDED STAGE OF T2 BACTERIOPHAGE DURING REPLICATION**

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There are theoretical and experimental indications that it is possible to distinguish between single and double-stranded DNA by means of ultraviolet action spectra.[1, 2] The wavelength of minimum efficiency for affecting single strands is 2,400 Å, as in the phage ØX174, and that for affecting double strands, as in T2 or calf thymus DNA, is close to 2,350 Å. The present work is an application of this idea to the investigation of development of intracellular virus. If, in the replication of a bacterial virus, there exist times when the viral DNA is double-stranded and other times when it is in a single-stranded configuration, this change in the state of the DNA should be detected by a shift in the position of the minimum of the action spectrum. There have been several previous determinations of action spectra for T2,[3, 4] and there have also been many previous attempts to follow the intracellular development of bacterial viruses by irradiating the complex of bacterium and virus at various stages in the latent period with ultraviolet light and determining the survival of the complex as a function of dose.[5–9] The experiments reported here differ from the others mentioned in that inactivations were carried out below 2,600 Å with seven different wavelengths. Action spectra have been obtained for complexes of T2r$^+$ and *E. coli* B at various times after DNA injection. A shift was observed in the position of the minimum of the action spectrum.

Materials and Methods.—Suspensions of T2r$^+$ or T2r$^+$ complexes were irradiated in buffered saline solution.[10] Complexes were made in the following way. *Escherichia coli* B was grown in aerated glycerol medium[11] to a concentration of