D, supernatant at 10,000 × gravity (probably soluble and microsomal)

D contained about 50% of the small activity found in the whole homogenate, while A, B, and C shared the other 50% approximately equally. The summation of the net activities of the four components was not greater than the net activity of the whole brei, which indicated that there was no inhibition of one component by another. Fraction D was used in some fortification attempts using nicotinamide, fluoride, magnesium, cytochrome C, and adenosine triphosphate, but it behaved exactly like the whole homogenate.

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Literature Cited

(1) Aldridge, W. N., *Biochem. J.*, **53,** 117 (1953).
(2) Artom, C., and Lefland, H. B., *Federation Proc.*, **13,** 176 (1954).
(3) Assoc. Offic. Agr. Chemists, "Official Methods of Analysis," 7th ed., Method 22.49 (a), 1950.
(4) Augustinsson, K. B., *Biochem. et Biophys. Acta*, **13,** 303 (1954).
(5) Beck, S. D., Stahmann, M. A., and Casida, J. E., Entomol. Soc. America, 1st Annual Meeting, Los Angeles, December 1953.
(6) Borsook, H., *J. Biol. Chem.*, **110,** 481 (1935).
(7) Brown, A. W. A., *J. Exptl. Biol.*, **14,** 87 (1937).
(8) Casida, J. E., Allen, T. C., and Stahmann, M. A., in manuscript.
(9) Casida, J. E., Chapman, R. K., Stahmann, M. A., and Allen, T. C., *J. Econ. Entomol.*, **47,** 64 (1954).
(10) Casida, J. E., and Stahmann, M. A., J. Agr. Food Chem., **1,** 883 (1953).
(11) Chiancone, F. M., *Boll. soc. ital. biol. sper.*, **15,** 579 (1940).
(12) Dyer, W. J., *J. Fisheries Research Board Can.*, **6,** 361 (1945).
(13) Fleisher, J. H., and Jandorf, B. J., *Federation Proc.*, **11,** 212 (1952).
(14) Hartley, G. S., Section 13, Pesticides, XIIth International Congress of Pure and Applied Chemistry, New York, 1951.
(15) Hobbiger, F., *Brit. J. Pharmacol.*, **6,** 21 (1951).
(16) Huerga, J. de la, and Popper, H., *J. Clin. Invest.*, **30,** 463 (1951).
(17) Jandorf, B. J., and McNamara, P. D., *Federation Proc.*, **8,** 210 (1949).
(18) Kilby, B. A., *Chemistry and Industry*, No. **33,** 856 (1953).
(19) Kunitz, M., *J. Gen. Physiol.*, **35,** 423 (1952).
(20) March, R. B., Metcalf, R. L., and Fukuto, T. R., J. Agr. Food Chem., **2,** 732 (1954).
(21) March, R. B., Metcalf, R. L. Fukuto, T. R., and Sprott, M. B., Entomol. Soc. America, 1st Annual Meeting, Los Angeles, December 1953.
(22) Mazur, A., *J. Biol. Chem.*, **164,** 271 (1946).
(23) Metcalf, R. L., and March, R. B., *J. Econ. Entomol.*, **46,** 288 (1954).
(24) Mounter, L. A., *J. Biol. Chem.*, **209,** 813 (1954).
(25) Mounter, L. A., Floyd, C. S., and Chanutin, A., *Ibid.*, **204,** 221 (1953).
(26) O'Brien, R. D., thesis, University of Western Ontario, 1954.
(27) O'Brien, R. D., and Spencer, E. Y., J. Agr. Food Chem., **1,** 946 (1953).
(28) Rockstein, M., and Herron, P. W., *Anal. Chem.*, **23,** 1500 (1951).
(29) Ronold, O. A., and Jakobson, F., *J. Soc. Chem. Ind.*, **66,** 160 (1947).
(30) Spencer, E. Y., and O'Brien, R. D., J. Agr. Food Chem., **1,** 716 (1953).

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RADIATION STERILIZATION

Influence of Gamma Radiation on Proteolytic Enzyme Activity of Beef Muscle

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Approximately 70% of the proteinase activity in beef muscle tissue could be extracted with citrate buffer at pH 9.6. Irradiation with cobalt-60 at dosages of 1.6 × 10^6 rep. reduced the apparent proteinase activity of beef muscle about 50%. This loss occurred largely in the fraction of the enzyme that was extractable at pH 9.6. At lower irradiation dosages (5 × 10^5 rep.) there was little reduction in proteinase activity as measured by liberation of tyrosine from casein substrate. Irradiation reduced the amount of tyrosine extractable from beef, which suggests that the amino acid is changed by irradiation.

Spoilage microorganisms in many foods can be killed by ionizing radiations at dosage levels of 10^5 to 2 × 10^6 rep. (*5, 7, 10, 12*). However, radiation sterilization cannot be applied generally to foods unless enzyme systems that may catalyze undesirable changes during extended storage are also inactivated.

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Studies on purified proteolytic enzyme systems have indicated that these biological catalysts are much more resistant to ionizing radiations than are microorganisms (*3, 10*). However, there have been no investigations on the effect of ionizing radiations on the proteolytic enzyme activity of beef muscle tissue. In fact, very little is known about the proteolytic enzymes in muscle tissue, even though the tenderizing influence of aging meat is usually attributed to the action of these enzymes (*8, 9*). Balls (*2*) reported that the proteinase of beef muscle could be liberated by autolysis of the tissue. He found that the optimal pH for its activity was 4.1 and suggested that it was probably a cathepsin.

The investigations reported here were undertaken to determine the influence of gamma radiation of the over-all proteolytic enzyme activity in beef muscle

Table I. Tyrosine Liberated by Meat Autolyzates and Autolyzate Residues

(Casein substrate, pH 4.6, 37° C. 24 hours, samples shaken)

Autolysis Time, Hours	Tyrosine Liberated, Mg./G. Meat: By autolyzate	By autolyzate residue
44	0.06	0.11
68	0.07	0.11
187	0.09	0.07

tissue. This information is essential for the evaluation of ionizing radiations as a means of commercial sterilization of raw meat.

Materials and Methods

For all studies reported here, beef muscle tissue (usually top round, U. S. Good or Choice grade, obtained from a local retail market) was freed from all separable fat and connective tissue, triple ground through a grinder with plates having holes 1/8 inch in diameter, and well mixed. The ground meat was used for the enzyme source for both irradiated and nonirradiated samples.

Earlier work in this laboratory (*1*) had shown that the liberation of nonprotein nitrogen (nitrogen of compounds soluble in 4% trichloroacetic acid) was not rapid enough or sufficiently great to serve as a sensitive and accurate index of over-all proteolytic activity. Consequently, an attempt was made to use the liberation of tyrosine from meat or from casein as an index of proteolytic activity. This amino acid was selected because rapid sensitive chemical methods are available for its determination and because it is liberated more rapidly than some others during proteolysis of beef muscle tissue (*6*).

As preliminary studies (*1*) had indicated that the optimal pH for proteolysis in beef muscle tissue was near pH 4.5 and the optimal temperature for a reaction time of 24 hours was near 40° C., test conditions of pH 4.6, a temperature of 37° C., and a reaction period of 24 hours were selected for measurement of enzymatic activity. For all enzyme tests, ground meat, extracts from ground meat, or residues from extracted meat were used at a level equivalent to 7.5 or 15 grams of raw meat in 50 ml. of acetate buffer, pH 4.6. When casein was used as an added substrate, it was added at the level of 1.0 gram per test. For maximum activity, particularly with added casein, all test suspensions were stirred or shaken continuously during the incubation period.

Table II. Extraction of Proteolytic Enzymes from Meat with Alkaline Extracted Agents

(Results expressed as tyrosine liberated by incubation at 37° C. for 24 hours at pH 4.6 with shaking)

Extracting pH	Tyrosine Liberated, Mg./G. Meat: In residue	In extract + casein
8.6	0.027	0.075
9.0	0.028	0.123
9.8	0.016	0.142
11.2	0.019	0.147

One milliliter of a preservative consisting of 1 part of fluorotoluene, 1 part of ethylene chloride, and 2 parts of butyl chloride (*11*) was used to prevent microbial growth in all test suspensions during incubation. After incubation the solutions were filtered, the residue was washed thoroughly at pH 4.6, and tyrosine was determined (*4*) in a suitable aliquot of the filtrate.

The ground meat was irradiated in a cobalt-60 irradiation source, refrigerated so that the temperature of the product was maintained at 5.5° ± 2.0° C. The total activity of the source was 561 curies, with a radiation intensity inside the source of approximately 97,000 roentgens per hour.

Separation of Proteinase from Beef Muscle Tissue

Dependable characterization of an enzyme requires that the enzyme be separated, at least in part, from other constituents of the tissue. Consequently, attempts were made to separate the proteolytic enzymes without inactivation from beef muscle tissue. Preliminary tests indicated that autolysis of the meat could liberate the proteolytic enzymes present (Table I). This agrees with results reported by Balls (*2*). However, after autolysis for 187 hours, more than 40% of the proteolytic activity, as indicated by liberation of tyrosine from casein, still remained in the insoluble meat residue.

Next, attempts were made to extract the proteolytic enzymes. Acid and salt extractions were not successful, but it was found that most of the activity could be obtained in alkaline extracts. For these extractions, 37.5 grams of meat was cut in a Waring Blendor with 100 ml. of citrate buffer of the desired pH for 3 minutes. The suspension was centrifuged, the supernatant poured off, and the residue extracted again with 50 ml. of buffer. A third extraction was made and the combined supernatants were adjusted to pH 4.6 with acetic acid, made to 250 ml. with deionized water, and allowed to stand overnight at room temperature. The inactive pigmented material which separated was filtered off and suitable aliquots of the filtrate were used for enzyme activity testing by incubation at 37° C. with constant shaking.

Table II shows that increasing proteolytic activity was obtained in the extract as the pH of the extracting medium was increased from pH 8.6 to pH 9.8. Nearly 90% of the total proteinase activity was extracted from the meat at pH 9.8 and was only slightly increased by extraction at pH 11.2.

Additional studies showed, however, that extraction of the proteinase at

Table III. Influence of Shaking and Stirring during Incubation on Apparent Extraction of Proteolytic Enzymes from Beef with Citrate Buffer at pH 9.6

(Results expressed as tyrosine liberated from casein at 37° C. for 24 hours at pH 4.6)

Incubation Mixing	Sample No.	Tyrosine Liberated, Mg./G. Meat: By residue	By extract	Enzyme Extracted, %
None	1	...	0.038	...
	2	...	0.040	...
	3	...	0.016	...
Shaking	4	0.016	0.107	87
	5	0.018	0.121	87
Stirring	6	0.057	0.131	69
	7	0.060	0.117	66

Table IV. Proteolytic Enzyme Activity of Citrate Buffer (pH 9.6) Extracts and Residues of Beef before and after Irradiation of 1.6 × 10^6 Rep.

(Incubation at 37° C. for 24 hours at pH 4.6 with stirring)

Sample No.	Nonirradiated Extract: Tyrosine present, mg./g. meat	Nonirradiated Extract: Tyrosine liberated from casein, mg./g. meat	Irradiated Extract: Tyrosine present, mg./g. meat	Irradiated Extract: Tyrosine liberated from casein, mg./g. meat	Tyrosine Liberated, Mg./G. Meat: Non-irradiated residue + casein	Tyrosine Liberated, Mg./G. Meat: Irradiated residue + casein
2	0.190	0.085	0.133	0.019	0.087	0.080
3	0.195	0.046	0.122	0.012	0.059	0.037

alkaline pH was not so effective as the data just given would suggest. When the enzyme substrate mixture was stirred vigorously during the incubation period, considerably more activity was exhibited, particularly by the residue, than in tests where no agitation or shaking was used during incubation (Table III).

Attempts to purify the proteinase further by fractional ammonium sulfate precipitation have not been successful, primarily because the low level of activity in extracts made it extremely difficult to determine accurately the activity of the various fractions.

Influence of Irradiation on Proteinase Activity

Preliminary experiments on irradiated beef muscle tissue indicated that irradiation reduced the activity of the proteinase and the amount of extractable tyrosine present (Table IV). There are three possible explanations for the observed results:

The enzyme(s) could have been inactivated during irradiation.

The tyrosine molecule could have been destroyed or altered, so that it could not be determined by the method used.

The altered tyrosine molecule could have acted as an inhibitor for enzyme.

If the third explanation were valid, then a mixture of extracts from irradiated and nonirradiated samples should show less proteinase activity than an extract from the nonirradiated sample. This was not found to be the case (Table V). In fact, this series of samples did not exhibit the same loss of apparent enzyme activity with irradiation that was observed with earlier samples. This may be explained either by difference in samples or by the lower radiation dosages used (0.5 × 10^6 rep. as compared to 1.6 × 10^6 rep). Some support is afforded for the first possibility by the fact that proteinase activity of all the extracts from this series of samples was much lower than that of extracts from other samples treated in exactly the same manner. The activity remaining in the residue after alkaline extraction was similar to that previously observed.

Consideration of all the data indicates that there was little, if any, inactivation of the proteinase in beef by irradiation at a dosage of 0.5 × 10^6 rep. At a higher dosage level (1.6 × 16^6 rep.) there was approximately 50% loss in apparent activity in some samples. Even this level of activity would probably be sufficient to catalyze proteolytic changes in raw meat, if attempts were made to sterilize meat by irradiation and store the meat for long periods of time without refrigeration.

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Literature Cited

(1) Andrews, F. A., and Doty, D. M., American Meat Institute Foundation, unpublished data.

(2) Balls, A. K., *Ice and Cold Storage*, **41** (4), 85 (1938).

(3) Brasch, A., Huber, W., Friedmann, U., and Traub, F. B., *Proc. Rudolf Virchow Med. Soc.*, **8,** 3 (1949).

(4) Doty, D. M., *Ind. Eng. Chem., Anal. Ed.* **13,** 169 (1941).

(5) Dunn, C. G., Campbell, W. L., Fram, H., and Hutchins, A., *J. Appl. Phys.*, **19,** 605 (1948).

(6) Ginger, Irene, Wachter, J. P., Doty, D. M., Schweigert, B. S., Beard, F. J., Pierce, J. C., and Hankins, O. G., *Food Research*, **19,** 410 (1954).

(7) Goldblith, S. A., Proctor, B. E., Davison, S., Lang, D. A., Kan. B., Bates, C. J., and Karel, M., *Radiology*, **60,** 732 (1953).

(8) Hankins, O. G., and Hiner, R. L., *Food Inds.*, **12,** 49 (1940).

(9) Hiner, R. L., Madsen, L. L., and Hankins, O. G., *Food Research*, **10,** 312 (1945).

(10) Huber, W., *Electronics*, **21** (3), 74 (1948).

(11) Hutner, S. H., and Bjerknes, C. A., *Proc. Soc. Exptl. Biol. Med.*, **67,** 393 (1948).

(12) Morgan, B. H., Natl. Canners Assoc., *Research Rept. 7–53* (1953).

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Table V. Proteolytic Enzyme Activity of Citrate Buffer (pH 9.6) Extracts and Residues of Meat before and after Irradiation of 0.5 × 10^6 Rep.

(Results expressed as mg. of tyrosine liberated per gram of meat during incubation at 37° C. for 24 hours at pH 4.6 with added casein substrate and stirring)

Sample No.	Extract: Non-irradiated	Extract: Irradiated	Extract: Non-irradiated + irradiated	Residue: Non-irradiated	Residue: Irradiated
4	0.032	0.026	0.035	0.075[a]	0.064[a]
5	0.035	0.035	0.043	0.062	0.056
6	0.034	0.030	0.024	0.063	0.058
7	0.037	0.021	0.040	0.076	0.075
8	0.028	0.033	0.027	0.076	0.076

[a] Casein not added as additional substrate.