A Fluorometric Method for Microgram Quantities of Ethylenediamine Residues in Milk

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In a fluorometric method for the determination of microgram quantities of ethylenediamine, milk samples are enzymatically digested, then extracted with ethanol. Ethylenediamine is removed from the aqueous alcoholic solution with a weakly acidic ion exchange resin. The diamine, eluted with trichloroacetic acid, reacts with adrenochrome under alkaline conditions to form a fluorescent product which is measured spectrofluorometrically at activation and emission wavelengths of 460 and 510 mμ, respectively. The response is linear from 0 to 10 μg. of ethylenediamine. Recoveries ranged from 88 to 140% and average recovery was 109%. The average apparent ethylenediamine value in control milk was 7.7 p.p.b. The limit of detectability was approximately 5 to 6 p.p.b.

Ethylenediamine has been proposed as an ingredient in infusion formulations for the treatment of mastitis in cows. Registration of the formulation for commercial use necessitated the development of a very sensitive method—below 10 p.p.b.—for residual ethylenediamine in milk.

The direct quantitative measurement of microgram quantities of ethylenediamine, because of the simplicity of the molecule, is a singularly difficult task. The formation of a derivative (*3–5*) or condensation product which can be measured by gas chromatography, visible or ultraviolet spectrophotometry, or fluorometry to obtain the required sensitivity was investigated. Combes (*2*) made ethylenediamine react with acetylacetone and copper acetate and obtained a violet precipitate which could be partitioned into chloroform. The sensitivity of this method is about 100 μg. per ml. Colorimetric procedures, based on the reaction of 1-fluoro-2,4-dinitrobenzene with primary amines, have been reported by McIntire *et al.* (*8*) and other investigators (*1*, *7*). This nonspecific assay was used in the authors' laboratory in conjunction with a weak cationic ion exchanger and resulted in a specific method for ethylenediamine in milk. Recoveries at 0.1 to 0.5 p.p.m. were between 90 and 110%. The method, however, could not be made to give the desired sensitivity (at least 0.010 p.p.m.).

Fluorometric procedures for the determination of adrenaline-like substances in biological materials using ethylenediamine as a reagent have been reported by Weil-Malherbe and Bone (*10*, *11*), Harley-Mason and Laird (*6*), and Yagi *et al.* (*12*). The authors report a sensitivity of 1 μg. per liter for adrenaline.

This report deals with the development of a reproducible, specific method for the quantitative determination of ethylenediamine in milk. In principle, the fluorometric detection method is the same as that described by Weil-Malherbe and Bone (*10*, *11*). However, conditions were reversed, in that adrenochrome was used as the reagent to detect small concentrations of ethylenediamine.

The method as modified and after cleanup of milk extracts can detect (*9*) 5 to 6 p.p.b. (0.5 μg. per 100 ml.) of ethylenediamine in milk.

Analytical Procedure

Reagents. Anhydrous 3A alcohol (95% ethanol–5% methanol) or 95% ethanol.

Cytase 40 enzyme complex, Enzymes Inc., Cherry Hill, N. J.

Amberlite XE-89 C.P. ion exchange resin, Mallinckrodt Chemical Works, New York, N. Y.

Trichloroacetic acid, Baker analyzed reagent, 15% aqueous solution.

n-Butyl acetate, b.p., 124–26° C., Matheson Coleman & Bell.

Triethanolamine, J. T. Baker Co.

Adrenochrome Base, (P)-M2085, Mann Research Laboratories, Inc., 136 Liberty St., New York, N. Y. Make an aqueous solution by dissolving 4.5 mg. of reagent in 25 ml. of water. This gives a concentration of approximately 180 μg. of adrenochrome per ml. of solution.

Apparatus. Spectrophotofluorometer, Aminco-Bowman, slit arrangement 5.

Absorption cells, quartz, 1 cm.

Chromatography columns, 20 × 300 mm., 250-ml. reservoir, with polytetrafluoroethylene stopcocks.

Separatory funnels, 125-ml., with polytetrafluoroethylene stopcocks.

Calibration Curve. Accurately weigh 1 ml. of ethylenediamine by difference from a Smith pipet. Transfer the sample directly from the pipet into a 1-liter volumetric flask containing about 600 to 800 ml. of distilled water. Dilute to the mark with distilled water. Mix well. (Stock Solution *A*.) Pipet 1 ml. of Stock Solution *A* into a second 1-liter flask and dilute to volume with distilled water. Mix well. (Solution *B*.) (The standard ethylenediamine has a concentration of

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about 0.8 to 0.9 μg. per ml.) Pipet 0- to 10-ml. aliquots (in 1-ml. increments) into separate 100-ml. beakers. Add by pipet 15 ml. of 15% trichloroacetic acid and enough distilled water to make a volume of approximately 30 ml. Place the beakers in an ice bath and cool to 0° to 4° C.

Saturate several hundred milliliters of *n*-butyl acetate with distilled water, separate the phases, and cool the *n*-butyl acetate to 0° to 4° C. in the ice bath. Transfer the reagent blank and standard solutions to 125-ml. separatory funnels. Add 25 ml. of cold *n*-butyl acetate and shake vigorously for 1 minute. Permit the phases to separate and collect the lower (water) phase in a clean, 100-ml. beaker. Cool solutions to 0° to 4° C. Discard the *n*-butyl acetate layer. Transfer the aqueous solution to the original separatory funnels and extract with another 25-ml. portion of *n*-butyl acetate. Thoroughly rinse the separatory funnels with distilled water before re-use. Repeat the extraction of the aqueous phases with a third 25-ml. portion of *n*-butyl acetate. Collect the aqueous phases in clean, 100-ml. beakers and let stand until the solutions clear. Check the pH of the aqueous phase with a pH meter. The pH should be approximately 2.3 to 2.5 to indicate adequate removal of trichloroacetic acid. If the pH is less than 2.2, partition trichloroacetic acid into *n*-butyl acetate once again.

Transfer the clear solutions to 50-ml. graduated cylinders. Using an eyedropper, add 60 drops (3 ml.) of triethanolamine (which adjusts the pH to approximately 9.7) to each solution. Add 1 ml. of adrenochrome solution, dilute to 50 ml. with distilled water, and shake vigorously. Place the graduated cylinders in a water bath to a depth sufficient to cover the sample and heat for 3 hours at 55° C. Cool the solutions in the cylinder to 25° C. and read the fluorescence intensity with the Aminco-Bowman spectrophotofluorometer in a 1-cm. cell at an activation wavelength of 460 mμ and a fluorescence wavelength of 510 mμ. Subtract the "zero" reading, and, using linear graph paper, plot the curve of micrograms of ethylenediamine per 50-ml. volume *vs.* fluorescence intensity at the meter multiplier reading of 0.01.

Preparation and Extraction of Samples. Pour 100 ml. of milk (recovery sample, field trial, or control) into a 250-ml. beaker and place in a water bath at approximately 33–37° C. After the milk has warmed to the temperature indicated above, add 15 to 18 mg. of Cytase 40 enzyme and stir the milk solution occasionally to break up the gel formed during coagulation. Normally, coagulation of the milk should begin after about 20 to 30 minutes' reaction. If coagulation does not occur, add a few more milligrams of Cytase 40 enzyme. Leave the milk sample in the water bath approximately 1.5 hours, stirring occasionally during the hydrolysis reaction. Transfer the sample to a 250-ml. centrifuge bottle and centrifuge at approximately 2000 r.p.m. for 20 to 25 minutes to remove the coagulated solids. Transfer the aqueous supernatant to a 1-liter polyethylene bottle. Add 200 ml. of 3A alcohol to the aqueous supernatant and stir mechanically using a magnetic stirrer or shake vigorously on a reciprocating shaker for 30 minutes. Centrifuge the solution in the polyethylene bottle for 25 to 30 minutes at 2000 r.p.m. to remove the solids precipitated.

Prepare 20 × 300-mm. chromatography columns by placing a glass wool plug in the bottom and adding Amberlite XE-89 resin slurried in 3A alcohol to a resin bed height of 5 cm. Place a glass wool plug at the top of the resin bed. Do not allow the resin to become dry. Pass the alcoholic extract through the resin at a rate of approximately 1 to 2 drops per second. When the level of the solution reaches the top of the resin column, wash the column with two 25-ml. aliquots of water. Discard the alcoholic percolate and water washes. When the last water wash reaches the surface of the column, elute the ethylenediamine with 15 ml. of 15% trichloroacetic acid followed by 15 ml. of distilled water. Collect the acid eluate and the water wash in a 100-ml. beaker. Let the column drain to remove all the aqueous solution. Cool the solution in the beaker to 0° to 4° C. and continue as outlined under Calibration Curve, beginning with "Saturate several hundred milliliters of *n*-butyl acetate . . ." Subtract the reagent blank reading from the sample reading and determine the micrograms per 50 ml. of ethylenediamine in the sample from the standard curve. Note: If the fluorescence intensity of the sample solution is too intense and does not fall on the standard curve (which will cause self-quenching), dilute the sample with distilled water so that it will. Make the necessary adjustment for dilution in the calculations.

Calculations.

$$\text{P.p.b. diamine} = \frac{\mu\text{g. diamine from std. curve} \times 1000}{\text{ml. of milk}}$$

Recovery Determinations. Recovery samples were prepared by the addition of aqueous standard solutions to normal cow's milk: 106, 88, 71, 52, 44, 35, and 18 p.p.b. The recovery of ethylenediamine dihydrochloride also was investigated (Table I). Control values were obtained from the milk of several cows.

Control values averaged 7.7 p.p.b. apparent ethylenediamine. Recoveries ranged from 88 to 140% and average recovery was 109%. The limit of detectability (*9*) was approximately 5 to 6 p.p.b.

Analysis of Treated Samples. Six cows were treated with infusions of an experimental mastitis formulation 12 hours apart. The "zero hour" milk sample was taken just prior to the third and last infusion, then milk samples were taken at 10- to 12-hour intervals for 144 hours and analyzed by the previously described analytical procedure (Table II). The concentration of the diamine in all six cows reached 10 p.p.b. at 60 hours and all samples were below 5 p.p.b. at 82 hours, which is the corrected limit of detectability (*9*).

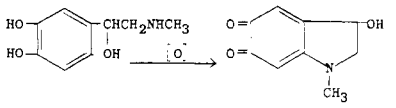
Discussion of Analytical Procedure

Preliminary investigative work indicated that a sensitive fluorometric method for ethylenediamine (EDA) was possible based upon the reaction of EDA with adrenaline (I) or its oxidation product adrenochrome (II) in an alkaline medium as outlined by Weil-Malherbe and Bone (*11*). These authors measured the fluorescence of 7,8-dihydro-6-methyl-6*H*-pyrrolo[2,3-*g*]quinoxalin-8-

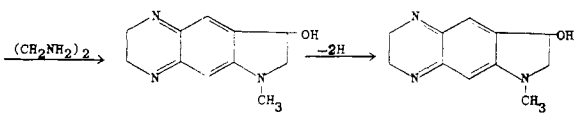
Table I. Recovery of Ethylenediamine from Milk (100 Ml.)

Diamine Added, P.P.B.	Fluorescence Meter Intensity × 0.01 Obsd.	Corr. for reagent	Recovery μg.	Apparent, p.p.b.	Corrected, p.p.b.	%
Reagent blank	6.1	...	...	...	...	...
	6.6	...	...	...	...	...
	4.4	...	...	...	...	...
	5.1	...	...	...	...	...
	5.1	...	...	...	...	...
	5.6	...	...	...	...	...
	6.2	...	...	...	...	...
	Av. 5.6					
0	8.3	2.7	0.52	5.2	...	...
	8.8	3.2	0.62	6.2	...	...
	13.0	7.4	1.45	14.5	...	...
	10.8	5.2	1.03	10.3	...	...
	9.3	3.7	0.70	7.0	...	...
	10.9	5.3	1.06	10.6	...	...
	9.9	4.3	0.86	8.6	...	...
	9.1	3.5	0.69	6.9	...	...
	9.1	3.5	0.69	6.9	...	...
	9.0	3.4	0.68	6.8	...	...
				Av. 7.7		
106	59.1	53.5	10.6	106	98	92
	70.0	64.4	12.8	128	121	114
88	48.0	42.4	8.4	84	77	88
	60.0	54.4	10.8	108	100	114
70	39.9	34.3	6.8	68	61	87
	59.0	53.4	10.6	106	98	140
52	41.3	35.7	7.1	71	63	121
44	29.4	23.8	4.7	47	39	87
	36.2	30.6	6.1	61	54	123
35	25.2	19.6	3.9	39	32	91
	29.9	24.3	4.8	48	40	114
35	30.2	24.6	4.9	49	41	117
	31.8	26.2	5.2	52	44	126
18	17.8	12.2	2.4	24	17	94
	19.8	14.2	2.8	28	20	111
	20.9	15.3	3.0	30	23	128
17	19.4	13.8	2.7	27	19	112
						Av. 109

ol (III) following reaction of adrenaline in an aqueous ethylenediamine medium at pH 10.0. The reaction is postulated to be as follows:



I II



III

Weil-Malherbe and Bone (*11*) state that the reaction is between ethylenediamine dihydrochloride and adrenochrome, and that the free base is added to produce the required degree of alkalinity (pH of the mixture = 10.4) and may be replaced by ammonia.

The object of this study was to attempt to reverse the conditions, in that adrenochrome was to be used as the reagent to detect small concentrations of ethylenediamine. Investigation on the reaction of ethylenediamine base or ethylenediamine dihydrochloride with various alkaline reagents such as ammonia, triethanolamine, and sodium hydroxide indicated that, of the three, only triethanolamine yielded the desired fluorescent derivative. Initially, the derivative was formed when ethylenediamine reacted with adrenochrome or adrenaline in an aqueous medium adjusted to pH 10.0

Table II. Ethylenediamine Residues in Milk, P.P.B.

Cow No.	Hours Postmedication													
	−24	0	10	24	36	48	58	72	82	96	106	120	132	144
2	7	528	922	417	64	17	7	6	<5	<5	<5	<5	<5	<5
4	10	282	726	53	13	<5	<5	<5	<5	<5	<5	<5	<5	<5
5	9	616	904	108	13	<5	<5	<5	<5	<5	<5	<5	<5	<5
6	6	732	1030	437	64	62	12	7	<5	<5	<5	<5	<5	<5
8	7	457	528	69	19	18	<5	<5	<5	<5	<5	<5	<5	<5
12	7	463	804	99	25	7	<5	<5	<5	<5	<5	<5	<5	<5

The corrected limit of detectability in this study was 5 p.p.b.

and incubated at 50° C. for 2 hours. The derivative, however, could not be partitioned into isobutyl alcohol as reported by Weil-Malherbe (*11*) nor into other water-immiscible solvents such as ethyl acetate, chlorinated hydrocarbons, ether, methyl isobutyl ketone, and butanol from either aqueous or saturated salt solutions.

The fluorescence characteristics of the adrenochrome-ethylenediamine derivative were investigated with the Aminco-Bowman spectrophotofluorometer. The activation wavelength was established at 460 mμ. Other wavelengths gave greater response (Figure 1), but they were either due to, or affected by, the solvent and the reagents. The 460-mμ wavelength appeared to be attributable only to the fluorescent derivative. With activation at 460 mμ, the maximum fluorescent emission wavelength was at 515 mμ (Figure 2).

Aqueous ethylenediamine standards, adjusted to pH 10 with 4 drops of triethanolamine, were made to react with a known excess of aqueous adrenaline or adrenochrome solution at different temperatures for varying periods of time. The fluorescence intensities of the various solutions showed maximum response when the condensation was carried out at 55° C. for 3 hours. Similar fluorescence intensity was observed in samples left overnight (17 to 18 hours) at room temperature. Samples reacted for 1 hour at 55° C. still retained the initial intense fluorescence due to unreacted adrenochrome in water, while those reacted for 2 hours did not attain the maximum fluorescence of the reaction product. Samples reacted for 4 hours showed decreased intensity comparable to samples left at room temperature for more than one day. At 75° C. and above, the reaction yielded more than one reaction product as shown by the activation and emission spectra. The intensity of fluorescence of the derivative was stable over a considerable length of time, although it had a tendency to decrease after one day.

Aqueous ethylenediamine standards containing from 0 to 60 μg. of ethylenediamine, after being adjusted to pH 10, were made to react with concentrations of adrenochrome ranging from 11.0 to 375.0 μg. The optimum

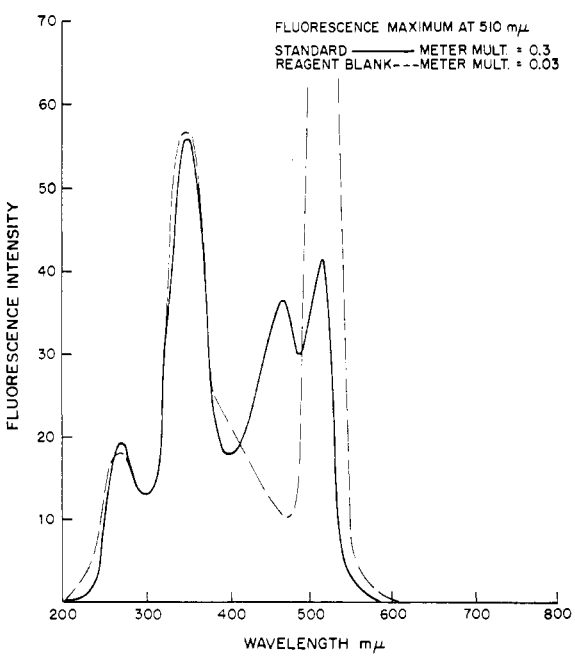


Figure 1. Activation scan of ethylenediamine-adrenochrome derivative

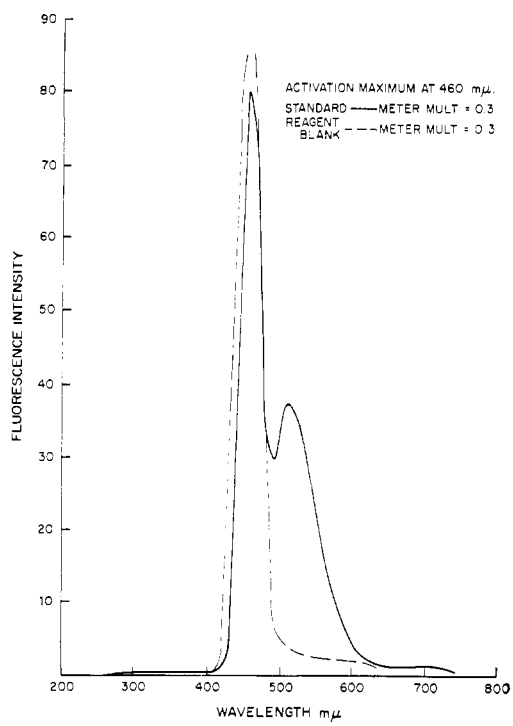


Figure 2. Emission spectrum of ethylenediamine-adrenochrome derivative

response was obtained at a mole-for-mole (ethylenediamine-adrenochrome) relationship which for 60 μg. of ethylenediamine required 150 to 180 μg. of adrenochrome. This concentration of adrenochrome did not affect the reagent blank significantly and was applicable to even trace concentrations of ethylenediamine. However, at adrenochrome concentrations greater than 180 μg., the fluorescence intensity of the reagent blank increased significantly, and the intensity of the derivative decreased. It was decided that 1 ml. of a 180 μg. per ml. aqueous adrenochrome solution would suffice for most ethylenediamine residues in milk extracts.

The standard curve for the fluorescence intensity of the ethylenediamine-adrenochrome derivative is shown in Figure 3. Fifteen milliliters of 15% aqueous trichloroacetic acid was added to 15 ml. of aqueous standard solutions containing from 0.881 to 8.81 μg. of ethylenediamine. The trichloroacetic acid was removed with *n*-butyl acetate, and the fluorescence was developed. A linear standard curve was obtained from 0 to 10 μg. of ethylenediamine per 50-ml. volume. The slope of the curve was relatively flat in this range but increased significantly with higher concentrations. Fluorescent values at higher concentrations were also more erratic because of quenching effects. Although the curves for standards not involving trichloroacetic acid were similar to those reported, trichloroacetic acid partitioning tended to stabilize the fluorescence intensity of the ethylenediamine-adrenochrome derivative in the range reported in Figure 3.

Trichloroacetic acid, if not neutralized or removed from solution, contributed to a dark, intense green fluorescence in the adrenochrome solution. The excess acid was removed from aqueous solutions by partitioning it into three equal 25-ml. aliquots of *n*-butyl acetate at 0° to 4° C. (*13*). Quantitative partitioning also occurred at room temperature, but the low temperature partitioning resulted in lower apparent values in control milk extracts than did room temperature extraction. After removal of the trichloroacetic acid, the aqueous solutions had a pH of about 2.3 to 2.5, but were easily adjusted to pH 9.5 to 10 with 60 drops (3 ml.) of triethanolamine and showed no effects of residual trichloroacetic acid in the development of fluorescence.

The visible spectrum of aqueous adrenochrome standard solution with maximum absorbance at 470 to 480 mμ indicated the reagent was stable for at least 20 days if kept refrigerated; however, with frequent intermittent warming and cooling, the stability of this solution was notably reduced. At room temperature this solution was relatively unstable and showed significant differences in the spectrum after 2 days' storage. The best procedure was to prepare only 25 ml. of adrenochrome solution at one time and to keep it refrigerated when not in use.

Amberlite XE-89 was satisfactory in removing and retaining ethylenediamine from up to 400-ml. volumes of water and alcohol as evidenced by negative values upon development of fluorescence in these eluates. Ethylenediamine was quantitatively eluted from the column with 15 ml. of 15% aqueous trichloroacetic acid.

Preliminary recoveries of ethylenediamine from milk were not quantitative and were erratic. This was attributed to irregularities in the cleanup procedure, but after each procedural step was evaluated, the extraction step seemed to be the source of the problem. Studies were conducted in which ethylenediamine was added to whole milk or the milk extract at each step of the cleanup and fluorescence-developing procedure. Freeze-dried samples of milk containing ethylenediamine also were extracted with alcohol and other solvents. Ethylenediamine was not quantitatively extracted from milk.

Separate 100-ml. aqueous preparations of 5% lactose, 4% butterfat, 3.3% calcium caseinate, and 10% powdered, instant nonfat milk were fortified with ethylenediamine, precipitated, and extracted with 200 ml. of 3A alcohol, and cleaned by the Amberlite XE-89 column–trichloroacetic acid elution procedure prior to fluorescence development. The control values for all solutions were satisfactory. Low recoveries from milk were due to casein binding of ethylenediamine.

Enzymatic systems were investigated as a means of liberating the ethylenediamine. Several systems were

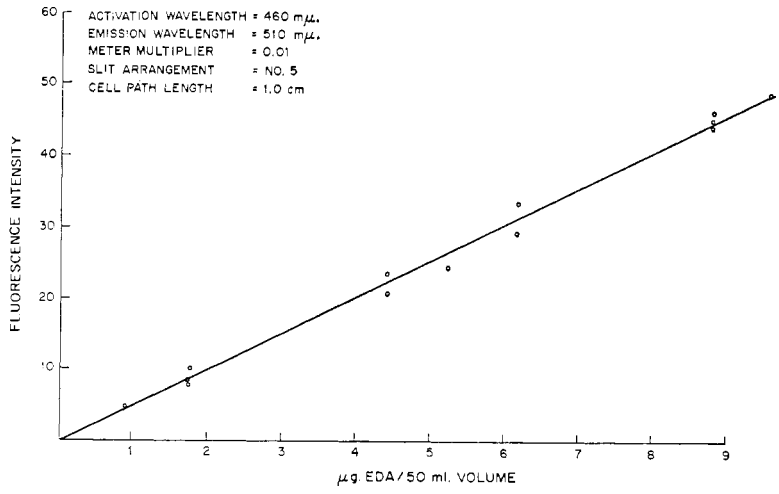


Figure 3. Standard curve for ethylenediamine-adrenochrome derivative

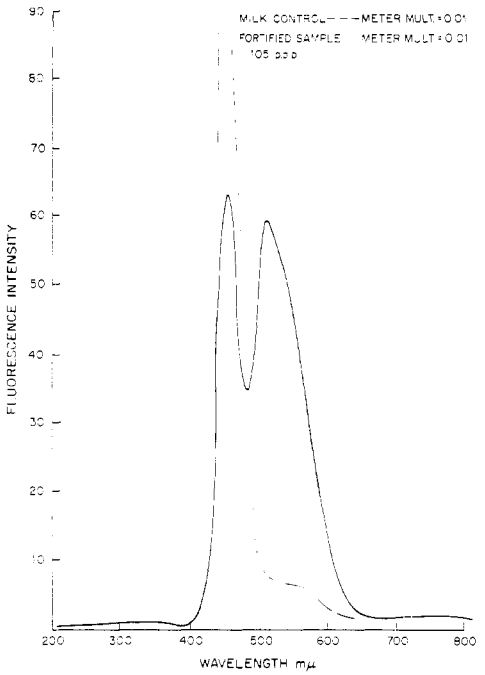


Figure 4. Fluorescence curves for ethylenediamine-extracted milk

satisfactory, but because Cytase 40 enzyme complex causes enzymatic hydrolysis of several constituents of milk in addition to the protein fraction, it was used in this work. One-hundred-milliliter samples of milk were fortified with 8.81 μg. of ethylenediamine, and 15 mg. of Cytase 40 enzyme complex was added with stirring to the solutions warmed to 37° C. The samples were kept at 37° C. in a water bath during the enzymatic hydrolysis and stirred occasionally to break up any coagulate that had formed. Coagulation began after 20 to 30 minutes. The samples were left in the water bath for 75 to 90 minutes. They were then centrifuged to remove the coagulated solids. Both the coagulate and the aqueous supernatant were shaken separately with alcohol (100 and 200 ml., respectively) for 30 minutes and then were recentrifuged. The supernatant of all samples was passed through Amberlite XE-89 columns, the columns were eluted with trichloroacetic acid, the acid was removed, and fluorescence was developed as previously discussed. Results showed that the ethylenediamine was in the aqueous phase following enzymatic hydrolysis. The solid phase was devoid of ethylenediamine and the fluorescence intensities of both the sample and control were relatively low. Recovery of ethylenediamine from the aqueous phase ranged from 89 to 119%. Later recoveries (Table I) of ethylenediamine at concentrations of 1.74 to 10.57 μg. per 100 ml. of milk showed that the enzyme complex hydrolyzed the milk proteins sufficiently to release ethylenediamine quantitatively. Typical fluorescence emission spectra for ethylenediamine extracted from milk and milk controls are shown in Figure 4.

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