ery periods. 2) One hour after injection, operated rats showed a greater depletion of adrenal vitamin than intact animals. If the lower values in operated-stressed rats are due to failure to rebuild ascorbic acid, then analyses of adrenals of operated animals at later periods of time should also be lower. Such is not the case. Six hours after treatment, thyroidectomized rats show the same degree of recovery of adrenal ascorbic acid as intact animals. The basis for the difference in response of the thyroidectomized rat to a given stress has not been determined.

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Use of Copper-Amino Acid Complexing for Determining Amino Acid Esterase Activity.* (22565)

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Spies and Chambers(1) described an adaptation of a method for the quantitative determination of amino acids which is based on the formation of a colored copper-amino acid complex. The method is limited to the determination of N-unsubstituted amino acids which form soluble complexes. Since copper does not form a complex with amino acid esters, the method lends itself to the determination of an amino acid liberated as a result of ester hydrolysis. The present paper describes an application of the copper method to the determination of lysine resulting from the enzymatic hydrolysis of lysine ethyl ester. In our laboratory, this procedure was found to be simple and easily adaptable to routine enzymatic analysis.

Materials. Copper phosphate suspension is prepared by the addition of 0.21M cupric chloride to 1600 ml of 0.18M trisodium phosphate with stirring. After centrifugation for 5 minutes at 2000-3000 rpm, the precipitate is washed by resuspension in 2000 ml of 0.1M borate buffer, pH 7.2. The precipitate is collected, again washed and then suspended in 2000 ml of the buffer. Two hundred and forty grams of reagent grade sodium chloride are added and the suspension is adjusted to pH 7.2 with 5N sodium hydroxide. The preparation is similar to that used by Schroeder *et al.*(2). L-Lysine ethyl ester dihydrochloride (LEE) is prepared as described by Werbin and Palm(3) and L-lysine monohydrochloride was purchased from Nutritional Biochemicals. Both were stored in a desiccator over phosphorous pentoxide. Twice recrystallized trypsin (Worthington) was used as the LEE esterase.

Method. Two ml of copper phosphate suspension are added to 2 ml of the test solution contained in a 15 ml conical centrifuge tube. The tube is shaken intermittently by hand for 5 minutes and excess copper phosphate removed by centrifugation for 2 minutes at 2000 rpm. The blue colored supernatant fluid is decanted into a Klett microtube and read in the Klett Summerson Photoelectric Colorimeter using a #66 (Red) filter.

Lysine standard curve. Since many of our hydrolysis experiments were carried out between pH 6.2 and 7.3, we investigated the effect of pH on the color intensity of the copper-lysine complex. Lysine standard curves

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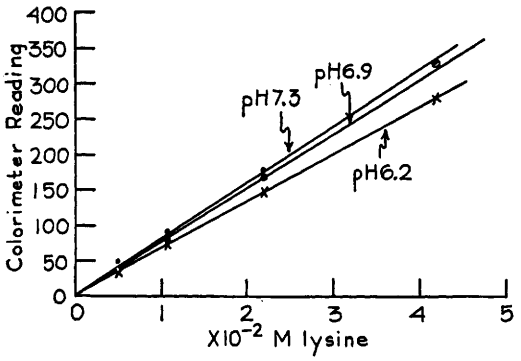

FIG. 1. Effect of pH on the lysine standard curve.

were prepared at pH 6.2, 6.9 and 7.3 in 0.15M phosphate buffers. As shown in Fig. 1, the curves are linear although the color intensity varies with the pH of the copper lysine complex.

An effect of phosphate on the copper-amino acid complex has been reported(2). In the present studies it was found that the use of 0.5M phosphate buffer produced a non-linear relationship between lysine concentration and optical density. However, at a concentration of 0.15M phosphate, no such effect was observed.

For the following experiments standard curves were prepared from lysine dissolved in pH 7.0, 0.1M phosphate buffer at concentrations of 0.042M to 0.00525 lysine. The highest concentration of lysine represented 100 per cent hydrolysis of the LEE used in the hydrolysis experiments reported below.

Results. Reproducibility of the method. Lysine standard curves at pH 6.5 (Table I) were determined 6 times during a period of 2 weeks using a standard 0.042M lysine solution. The same copper phosphate was used throughout the experiments since no instability of the suspension was observed. It is calculated that the 95% confidence limits for the concentration of lysine in an unknown sample using a single lysine standard curve and a single reading on the unknown sample are ± 3.5%, irrespective of the lysine concentration. This figure may be taken as the probable maximum limit of error, taking into account possible errors in reading the unknown sample and in determining the lysine standard curve. If the 6 standard curves are averaged and plotted as one, the error in determining the standard curve will be reduced to a negligible amount, leaving only the error in reading the unknown sample. In this case the 95% confidence limits were calculated to be ± 2.5%.

TABLE I. Lysine Standard Curves Used for Statistical Analysis.

Molarity of lysine solution	Colorimeter readings 1	2	3	4	5	6
.042	312	305	307	306	309	315
.021	152	157	150	149	151	153
.0105	72	79	73	71	73	76
.00525	36	41	35	33	34	37

Rate of spontaneous hydrolysis of LEE. A 0.042M solution of LEE dihydrochloride dissolved in pH 7.8 0.1M phosphate buffer to yield a solution of pH 7.0. During incubation at 37°, 2 ml aliquots were removed at the indicated intervals and analyzed. The lysine content of each aliquot was determined by comparison with a lysine standard curve and the per cent hydrolysis of LEE plotted against the incubation time. The rate of 'spontaneous' hydrolysis of LEE is shown in Fig. 2. The data demonstrate that some hydrolysis of the ester occurs under the experimental conditions in the absence of enzyme. The hydrolysis value at zero time suggests either that the LEE contains a small amount of free lysine or that the addition of buffer or copper phosphate causes the formation of free lysine.

Hydrolysis of LEE by Trypsin. A 5 mg% solution of trypsin was prepared by dissolving the enzyme in 0.0025 N hydrochloride acid. LEE dihydrochloride was dissolved in pH

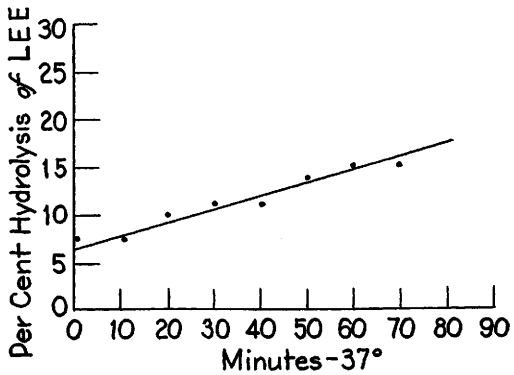

FIG. 2. Spontaneous hydrolysis of LEE at pH 7.

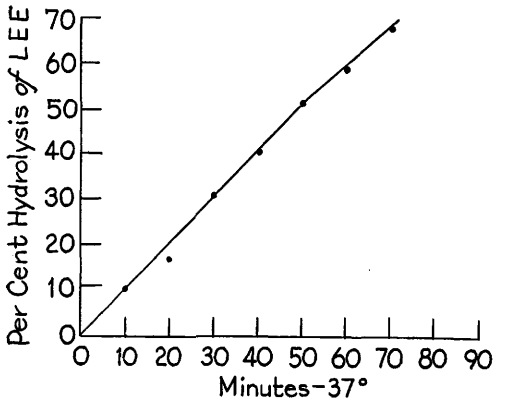

FIG. 3. Hydrolysis of LEE by trypsin at pH 7.

7.8, 0.4M sodium phosphate buffer to yield a 0.168M solution. The reagents were incubated separately at 37°C for 5 minutes with the exception of LEE which was prepared just prior to starting the test. The reaction mixture was prepared by combining 5 ml of enzyme solution, 10 ml of water and 5 ml of LEE solution. The substrate control contained 5 ml of 0.0025N HCl in place of the trypsin and the enzyme blank contained 5 ml of pH 7.0 0.4M phosphate buffer in place of the LEE. The final pH's of solution in all tubes was 7.0. After the addition of the LEE a timer was started and as incubation proceeded 2 ml aliquots were removed and analyzed. The colorimeter readings of the substrate control and enzyme blanks were subtracted from the colorimeter readings of reaction mixture and the corrected value was converted to per cent hydrolysis of LEE. The results are shown in Fig. 3. The data demonstrate that enzymatic breakdown of LEE may be conveniently followed by complexing the liberated lysine with copper.

The present studies employed trypsin as the lysine ethyl esterase but any lysine ethyl esterase may be used. The breakdown of LEE by plasmin (serum proteolytic enzyme) has also been studied by the above technic. Furthermore, by substituting the appropriate amino acid ester as substrate in the procedure described above, it has been possible to study the action of other amino acid esterases(4).

Summary. 1. The determination of lysine by reaction with copper phosphate has been described. 2. Within the range of concentrations studied the reproducibility of the method was found to be ± 3.5%. 3. The rate of hydrolysis of lysine ethyl ester by trypsin was determined by measurement of liberated lysine. 4. Application of the method to studies with lysine ethyl esterases and other amino-acid esterases was briefly discussed.

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Effect of Digitoxin and Quinidine on Intracellular Electrolytes of the Rabbit Heart.* (22566)

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Digitalis and quinidine preparations are much employed in the treatment of cardiac disorders(1). The beneficial effects of these drugs are ofttimes nullified by toxic phenomena which occasionally cause death(2,3). Although the exact mechanism for either toxicity or death is unknown, it has been suggested that electrolyte imbalance in the cardiac muscle results in establishing condi-

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