certain unexplained post-surgical deaths may be due to a rapid reduction in CO_2 tension which has become elevated during anesthesia. It would be interesting to know what changes take place in plasma potassium concentration in the human during prolonged exposure to severe hypercapnia. It seems likely that it is elevated but it would be important to know how rapidly and to what extent, as a function of time and CO_2 concentration, since this would have a bearing on the susceptibility of the heart to fibrillation or arrest when the CO_2 tension is reduced rapidly.

Summary and conclusions. 1. Dogs regularly show an increase in plasma potassium concentration during breathing of 30% and 40% CO_2 mixtures. Immediately following return to air breathing, there is a further sharp rise in this concentration. This secondary rise reaches a peak after 5 minutes of air breathing. 2. Concentration of plasma potassium following 4 hrs of high CO_2 breathing is not as high as that which is necessary to produce fibrillation or arrest when KCl solution is administered as an intravenous drip to normal or acidotic dogs. 3. Rapid return to air breathing following 30 minutes of high CO_2 breathing does not produce cardiac arrhythmias, ventricular fibrillation, or cardiac arrest in dogs. However, when this rapid elevation in blood pH and/or fall in pCO_2 takes place in the presence of sub-lethal but elevated plasma potassium concentrations, cardiac arrest or ventricular fibrillation may occur. Although this does not provide an explanation of the mechanism involved, it appears that the combination of elevated but sub-lethal plasma potassium concentration and a rapid fall in CO_2 tension and/or hydrogen ion concentration, work together to produce the damage to the heart.

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Photometric Method for Estimation of Elastase Activity.* (22022)

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The presence of an enzyme in animal pancreatic extracts which solubilizes elastin was first described by Balo and Banga(1). The activity was measured gravimetrically and by Kjeldahl nitrogen. Using the same technics, Hall(2), and later Lansing *et al.*(3), confirmed the presence of this enzyme in extracts of hog pancreas. Since both methods are somewhat cumbersome, a simpler colorimetric procedure was devised; it permits the direct measurement of the amount of dyed elastin which goes into solution when the dyed substrate reacts with pancreatic extracts.

Material and methods. Substrate. Purified elastin was prepared by the Lansing, Alex and Rosenthal(4) modification of the method of Lowry *et al.*(5). The ligamentum nuchae of horses was freed of the thin investing fascia and then ground into small pieces in a meat

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grinder. This well-ground material was covered with methanol and refluxed over a hot water bath for one hour. The alcohol was decanted, the residue covered with ether and refluxed for another hour. The residue was allowed to dry, added to 0.1 N sodium hydroxide which had been preheated to 93-98°C and the mixture kept at this temperature for 45 minutes. The sodium hydroxide was decanted and the residue washed thoroughly with water. This elastin (20 g) was air-dried, ground to 40-mesh size in a Wiley mill, and suspended in an orcein solution composed of 1 g of orcein in 100 ml of 70% ethanol to which 1 ml of concentrated hydrochloric acid had been added. The stained elastin was then washed repeatedly with large volumes of 70% ethanol and distilled water until both the ethanol and the aqueous washings were color-free. The stained elastin was finally washed with 70% ethanol and the air dried residue ground in a mortar. Samples of this substrate incubated in an enzyme-free system, as described in the Procedure, gave no color change in the supernatant. Quadruplicate nitrogen determinations of this elastin preparation by the Conway micro-diffusion method(6) gave an average of 14.5%.

Buffers: Tris (hydroxymethyl) aminomethane buffer 0.2M, pH 8.8, prepared according to Gomori(7). This buffer will hereafter be referred to as Tris. Phosphate buffer 0.7M, pH 6.0, prepared according to Sörenson (8).

Enzyme Preparation: This was prepared by extracting 10 g Viobin† (defatted autolyzed pork pancreas) with 50 ml N/40 hydrochloric acid for 20 minutes on a magnetic stirrer at room temperature and centrifuging the preparation at 12,000 rpm in an International Model PR-1 high-speed refrigerated centrifuge. The residue was reextracted with another 50 ml N/40 hydrochloric acid for 20 minutes and recentrifuged. The pooled clear supernatant measured approximately 90 ml. This crude extract had a pH of about 5, and was adjusted to pH 8.8 with 1N sodium hydroxide before use. There was no appreciable loss of elastolytic activity by keeping this preparation in the refrigerator for several weeks. Using our assay procedure, 10 g of Viobin extracted with 100 ml N/40 hydrochloric acid yielded a total of 12,000 to 13,000 elastase units when one elastase unit is defined as the amount of enzyme activity required to solubilize 1 mg of elastin substrate in 20 minutes at 37°C under our experimental conditions.

Procedure. Twenty mg portions of the dyed substrate were placed in small test tubes (13x125 mm) and 1 ml of Tris buffer, pH 8.8, 1 ml water, and 1 ml of suitably diluted enzyme preparation adjusted to pH 8.8 added to each. The tubes were then stoppered, placed in the horizontal position in a mechanical shaking device, which was immersed in a constant temperature water bath at 37°C. The shaker moves at a uniform rate of 46 excursions per minute. At the end of 20 minutes the tubes were removed from the bath and 2 ml of 0.7M phosphate buffer, pH 6, added to each tube to stop the enzyme reaction. With this drop in pH the color of the reaction mixture changed from a bluish purple to a reddish purple. This mixture was then centrifuged and the per cent transmission of the supernatant read on a Model DU Beckman Spectrophotometer at 590 mu. The results were then converted into specific activity, *i.e.*, elastase units per milligram enzyme preparation used.

Standard curve: Duplicate portions of the substrate, ranging from 5 mg to 25 mg, were placed in the test tubes. To each tube 1 ml of 0.2M Tris buffer, 1 ml water and 1 ml of enzyme preparation were added and the mixture incubated as described until all the elastin went into solution. This process took from 24 to 48 hours, depending on the strength of the enzyme preparation used. Then 2 ml of the phosphate buffer and 5 ml of water were added, giving a final dilution of 1:10. In all tubes a very small amount of insoluble material remained and this was attributed to non-elastin contamination of the substrate. This insoluble residue was estimated by nitrogen determination and gave an average of 3.8%.

Results. Following centrifugation the du-

† Viobin was generously contributed by the Viobin Corp., Monticello, Ill.

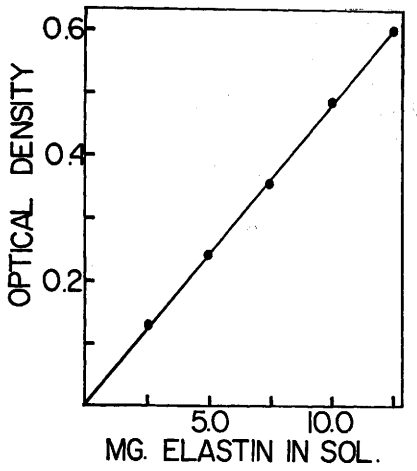

FIG. 1. Relationship between mg elastin dissolved and optical density (590 mu) of the orcein dye liberated.

plicate samples were read at 590 mu on the Beckman Spectrophotometer and the readings plotted as optical density vs mg elastin per 5 ml solution (Fig. 1). The duplicate determinations were in good agreement. Regardless of the analytical procedure, it would seem that standardization of a new elastin preparation would always be indicated, since there may be slight variation in the constituents of any given batch, and there is no reason to assume that any of these preparations represents pure elastin.

Time curve. By using a stop watch, tubes containing the substrate, buffer, water and enzyme mixture, as described, were withdrawn from the water bath at intervals and the reaction stopped by the addition of the phosphate buffer. Results were recorded as time in minutes vs milligrams elastin in solution (Fig. 2).

Dilution curve. Dilutions of a lyophilized enzyme preparation, varying from 0.2 to 5.0 mg elastase powder per ml, were used, with an incubation time of 20 minutes. The results were plotted as milligrams dissolved elastin vs enzyme concentration, and they followed a zero order reaction when less than 7 mg or 35% of the 20 mg substrate used was solubilized (Fig. 2).

pH Optimum. Hall and Gardiner(9) showed that the pH optimum of elastolytic activity lies between 8.7 and 9.2, with a different peak for substrates of different animal origin. On horse ligamentum nuchae, prepared as described, the pH optimum for our enzyme preparation lies between 8.7 and 9.4. The inadequacy of the 0.1N carbonate buffer, pH 10.23, used by Balo and Banga, was verified by us, using the colorimetric method. The pH after incubation dropped to levels varying from 9.8 to 8.9. Buffers of sufficient molarity to hold the pH of the reaction mixture at 10.23 gave minimal elastolytic activity.

Comparison of methods. Different amounts of the elastin substrate were weighed on an analytical balance and exposed to the enzyme buffer mixture until varying amounts of the elastin had gone into solution. The supernatant was removed after centrifugation and the amount of solubilized elastin determined by the colorimetric method described. The nitrogen content was assayed by the Conway micro-diffusion method, using 1 ml of the supernatant. Appropriate controls consisting of identical mixtures of the enzyme buffer solution were also assayed for their nitrogen content. For the gravimetric procedure the residues were oven-dried at 100°C and weighed on an analytical balance, the amount of elastin in solution being the difference between the original weights and that of the residues. The results of all three methods are recorded in Table I.

Discussion. The assay procedure described

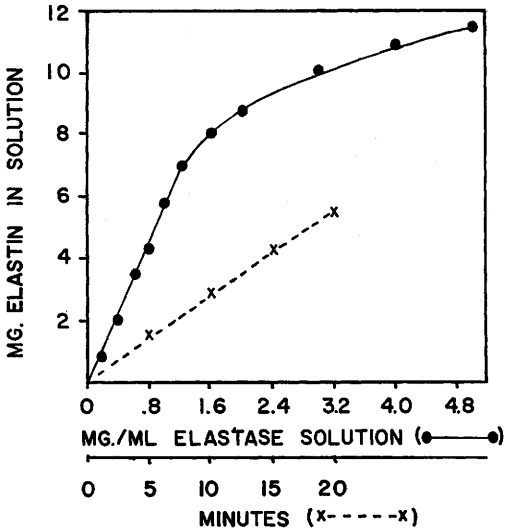

FIG. 2. Effect of time and of elastase concentration on dissolution of elastin.

TABLE I. Milligrams Elastin Dissolved by Action of Elastase as Measured by:

Gravimetric procedure	Colorimetric method	Total nitrogen in solution
21.8	21.8	21.3
21.3	22.7	25.6
19.7	20.2	19.3
17.9	18.2	17.8
18.0	17.5	17.6
10.1	10.9	11.1
8.9	7.9	8.2
12.5	12.5	12.7
11.0	11.6	11.1
4.5	3.7	4.8
3.0	2.9	2.4
5.1	4.3	5.9
18.8		17.8
18.0		19.1
26.8		26.0
33.0		33.1
	19.9	19.5
	3.4	2.4
	11.2	12.7
17.9		17.3
11.8		11.0
19.7		18.5

here has the advantage of rapidity, simplicity and reproducibility, as well as the production of a visible reaction, so that one gauges at a glance whether activity is present and to what degree, without first having to complete the procedure. Hall(10) assayed the enzyme preparation by incubation for 18 hours, without agitation. It would appear that one cannot determine with accuracy the specific activity of the enzyme preparation under conditions where the dissolution of elastin is no longer linear. The risk of bacterial contamination is reduced by using the shorter incubation period in our method. No inactivation of the enzyme was noted by the amount of agitation used by us; instead, it was found that the enzymatic activity was increased up to 100% as compared to incubation without agitation.

When the same enzyme solution is tested against batches of horse ligamentum nuchae substrate prepared at different times, the activity changes. This is most probably a result of variation in particle size and in the amount of dye absorbed by the elastin. This variable may be cited as a disadvantgae of the method, but standardization of the substrate each time a new batch is prepared would appear to be necessary, irrespective of the analytical procedure used. The problem can be minimized by preparing larger quantities of the substrate each time.

Summary. A colorimetric method for the estimation of elastase activity has been presented. When dyed elastin substrate prepared from horse ligamentum nuchae is used, the pH optimum lies between 8.7 and 9.4, and the reaction velocity is linear only when less than 35% of the elastin is dissolved. When compared with the gravimetric and nitrogen procedures, this method has the advantages of rapidity and simplicity.

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