Experimental

The assay procedure used was essentially that given by Tauber[9] since it was found that with fresh skimmed milk sharper end-points were obtained than with reconstituted skimmed milk, as recommended by Kunitz.[10] To 90 ml. of fresh skimmed milk at 25° was added 10 ml. of a 0.5 *M* acetic acid–0.5 *M* sodium acetate buffer to give a solution of *p*H 4.8. A mixture of 1 ml. of inhibitor solution, 1 ml. of enzyme solution, containing 0.052 mg. of protein nitrogen per ml., and 5 ml. of buffered milk solution was gently shaken in a thermostat at 25° until clot formation was observed. The inhibitors were those prepared previously[7,8] and the crystalline α-chymotrypsin was an Armour preparation of bovine origin.

(9) N. Tauber, "Chemistry and Technology of Enzymes," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 136.

(10) M. Kunitz, *J. Gen. Physiol.*, **18**, 459 (1935).

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Peculiar Kinetics of Color Formation in Glycerol–Catechol Condensation

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During a study of the kinetics of color development for a microcolorimetric analysis of glycerol[2a] based on the Hovey and Hodgins reaction[2b] a very peculiar stepwise phenomenon was observed. Although this anomaly has been investigated only superficially, it is felt that a note concerning its behavior is warranted.

Experimental

Procedure.—A series of test-tubes were selected for uniformity of diameter and wall thickness in order to ensure equal rates of heat transfer, and the tubes were immersed in an ice-bath after 1 ml. of a solution containing 100 μg. of glycerol per ml. had been added to each. To each was then added 1 ml. of freshly prepared 10% solution of catechol (twice sublimed) following which 4 ml. of a precooled 3:1 solution of sulfuric acid–water were added slowly with gentle agitation in order to avoid excessive premature heating. After a 5 min. period of thermal equilibration the tubes were placed simultaneously in a concentrated sulfuric acid bath of such a capacity and initial temperature that the temperature fell to about 145° when the tubes were immersed in the bath. For the duration of the heating period the temperature was kept at 145 ± 1°. At various intervals tubes were withdrawn, the reaction arrested by immersion in ice-water, and the color intensity read in a Klett–Summerson photoelectric colorimeter or the absorption spectrum determined with a Beckman spectrophotometer.

Results and Discussion

The stepwise character of chromogen formation is shown in Table I in which the results of 10 of 20 such experiments are recorded. Plateau values are shown in bold print and brackets. Over a 10 minute (or more) range two or more steps were observed, each indicating complete cessation of the series of reactions leading to condensation. Patterns determined from different experiments were extremely variable but of similar general character. However, simultaneously withdrawn duplicate and triplicate samples of the same stock generally varied less than 3% in color intensity; furthermore, each plateau value shown represents a separate sample. Therefore, the factors determining the pattern of color development are not randomly distributed among samples of the same solution. In addition, a series of standard glycerol solutions showed color intensities in direct proportion to the concentration,[2a] which suggests that the shape of the curve is also independent of the glycerol concentration. Thus the treppe cannot be explained only as a chain reaction or autocatalysis; the periodic behavior was seemingly determined in some way by fluctuations in the activity of components other than glycerol which were not affected by separation into several tubes. On the other hand, since acrolein reacted to give the chromogen at a much faster rate than did glycerol,[2a] the formation of acrolein from glycerol was the rate-limiting step and rate fluctuations probably involved glycerol transformations. Absorption spectra[2a] of 6, 12 and 18 min. samples varied only in intensity, so that the steps were not a succession of different reactions or polymerizations yielding spectroscopically distinguishable molecular species. When other conditions of glycerol and sulfuric acid concentrations and of temperature obtained, essentially the same type of data was derived.

TABLE I

STEPWISE PHENOMENA IN THE RATE OF COLOR DEVELOPMENT

t, time of heating in minutes; *c*, colorimeter reading with a Klett–Summerson photoelectric colorimeter using a filter of maximum transmission at 540 mμ.

	Experiment																			
	1		2		3		4		5		6		7		8		9		10	
t	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*	*t*	*c*
1	10	*2*	26	*2*	27	*10*	168	*10*	**179**	*10*	**153**	*10*	142	*10*	137	*11*	**151**	*11*	**174**
2	21	*4*	61	*4*	80	*11*	172	*12*	**179**	*12*	**153**	*13*	156	*13*	179	*11½*	**152**	*11½*	**171**
3	41	*6*	99	*6*	**126**	*12*	**185**	*14*	**179**	*16*	206	*16*	**193**	*16*	200	*12*	**151**	*12*	**185**
4	54	*8*	**132**	*8*	**130**	*13*	**186**	*16*	**208**	*18*	**230**	*19*	**194**	*19*	**248**	*12½*	**151**	*12½*	**185**
5	82	*10*	**132**	*10*	**130**	*14*	**186**	*18*	**210**	*22*	**230**	*22*	228	*22*	**248**	*13*	**170**	*13*	**194**
6	94	*12*	**134**	*12*	**130**	*15*	206	*20*	218	*24*	262	*25*	243	*25*	263	*13½*	**170**	*13½*	**194**
7	119	*14*	197	*14*	206	*16*	229	*22*	233	*28*	**302**	*28*	**277**	*28*	**299**	*14*	**170**	*14*	**194**
8	**152**	*16*	229	*16*	222	*17*	237	*24*	253	*30*	**302**	*31*	**277**	*31*	**304**	*14½*	**183**	*14½*	**194**
9	**154**	*18*	247	*18*	232	*18*	242	*26*	262	*34*	**350**	*34*	315	*34*	**340**	*15*	**183**	*15*	**194**
10	**153**		264	*20*	250	*19*	**253**	*28*	289	*36*	**350**	*37*	350	*37*	**345**	*15½*	202	*15½*	214
						20	**254**	*30*	305	*40*	**397**	*40*	365	*40*	**357**			*16*	**226**
						21	**255**	*32*	354	*42*	**397**	*43*	400	*43*	**357**			*16½*	**224**

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(2) (a) S. C. Harvey and V. Higby, *Arch. Biochem.*, **30**, 14 (1951); (b) A. G. Hovey and T. S. Hodgins, *Ind. Eng. Chem., Anal. Ed.*, **9**, 509 (1937).

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