DILATOMETRIC STUDIES IN ENZYME ACTION. PART II. CONTRACTION CONSTANTS OF ENZYME-SUBSTRATE REACTIONS.

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In a previous communication (*Biochem. J.*, 1929, **23**, 975) it was shown that hydrolysis of substrates by their respective enzymes is generally accompanied by measurable changes of volume. At lower concentrations of the substrate, the total volume change brought about by an enzyme-substrate reaction is proportional to the weight of the substrate present in the reaction mixture and is not affected by temperature or concentration of enzyme, hydrogen and other ions, all of which are known to influence the kinetics of the reaction. In the case of crystalloidal substrates whose molecular weight is known, the total contraction is expressed in cubic centimetres per gram-molecule of the substrate and this is a constant for a given enzyme-substrate system. In the case of colloidal substrates, starch, proteins, etc., the contraction constant is expressed in cubic centimetres per 100 grams of the substrate.

The Dilatometer.

The dilatometer employed successfully for time-course studies of enzyme action in previous work was found unsuitable for the present investigation, where we are concerned with the total volume change and not with the rate of reaction at a given moment. In previous studies the enzyme and substrate were mixed outside the dilatometer in a separate vessel at the temperature of the thermostat, after which the apparatus (Fig. I) was filled with the reacting mixture by suction at the capillary end. The whole operation takes 2 to 3 minutes, and, under the most favourable conditions of manipulation, the first reading on the dilatometer can be taken only after 3 minutes. This procedure, therefore, does not bring into consideration the substantial volume change effected (1) on mixing and (2) by reaction during the first three minutes. In some instances the fall is so rapid that nearly 30-35 per cent. of the volume change takes place during this period as shown in Table I.

TABLE I.

Reaction	Volume change occurring after 30 secs.	Volume change occurring after 3 mins.	Total	Percentage of reaction during 3 mins.
Urease-urea ..	1.5	14.3	39.0	36.6
Invertase-sucrose ..	0.0	3.4	12.1	28.1

When the investigation was in progress Rona (*Biochem. Zeit.*, 1931, 235, 214), employing the same dilatometer and procedure as above, obtained values for invertase-sucrose and maltase-maltose systems which unexpectedly coincide with ours derived from the new instrument.

To determine the total volume change, therefore, it is necessary that the substrate and the enzyme should be mixed in the dilatometer itself, after the first reading of the meniscus in the capillary is taken. These conditions have been fulfilled by designing a new type of instrument, which has been made for us by Messrs. Greiner and Friedrichs.

The New Type.

The new type of dilatometer (Fig. II) consists of an inverted Y-shaped vessel, one of the arms being blown into a pear-shaped bulb S, of about 75 c.c. capacity to receive the substrate. The adjacent arm is blown into a smaller bulb E, to contain 10–15 c.c. of the enzyme extract, while the third arm, pointing upwards, ends in a mouth provided with a carefully ground-in glass stopper. A 450 mm. length of capillary of uniform bore (0.4 mm.) is attached at the upper end of the pear-shaped bulb.

EXPERIMENTAL.

The dilatometer, more especially its capillary, is carefully cleaned successively with alcoholic potash, chromic acid, water, alcohol and finally with ether. The capillary is calibrated in the usual way and the observed mean cross-section is used in calculating the volume change accompanying a reaction. The bulbs of the dilatometer S and E are filled with the substrate and the enzyme extract respectively, employing the filler (Fig. II) provided with the instrument; the filler being rinsed each time with 2 c.c. of toluene to wash down substrate or the enzyme extract as the case may be. During the filling great care should be taken to avoid mixing the two components.

The substrate consists of a known weight of the purified substance, usually a twentieth of the gram-molecule dissolved in a suitable buffer at the optimum P_H and diluted to a litre with the same buffer solution. The substrate solution, 40–50 c.c., depending upon the amount of substance dissolved, and 5-10 c.c. of the enzyme extract depending upon its activity, are employed for each experiment. The dilatometer is afterwards stoppered with a rubber cork provided with a T-tube, to one end of which is attached a separating funnel (Fig. III). The other end of the tube is connected to a suction pump by means of pressure-tubing controlled by a screw pinchcock. The capillary end is closed by a short piece of rubber tubing closed at one end by a glass rod, and suction is applied with a filter pump to remove the gases dissolved in the liquids. During the operation, which usually takes 30 minutes, the toluene layer covers the surfaces of the substrate and enzyme extract, facilitating the smooth escape of gas bubbles and preventing foaming or spraying, more particularly, of the colloidal extract. At first great care should be exercised in controlling the suction by means of the screw pinchcock, as otherwise too rapid a removal of the gases may cause spraying and mixing of liquids. When bubbles of gas cease rising in the two liquids, suction is cut off by means of the pinchcock, and high boiling petrol (110—120°), distilled successively over potash and mercury, slowly admitted to the dilatometer (Fig. III) until the remaining air space is filled. A few glass beads are finally added and the apparatus immersed in the thermostat maintained at $30° \pm 0.01°$. After half an hour, the dilatometer is closed with the stopper smeared with a special grease mainly composed of the resin acids of shellac which, by virtue of its insolubility in petrol, prevents the solvent from creeping through the ground glass stopper fastened in position

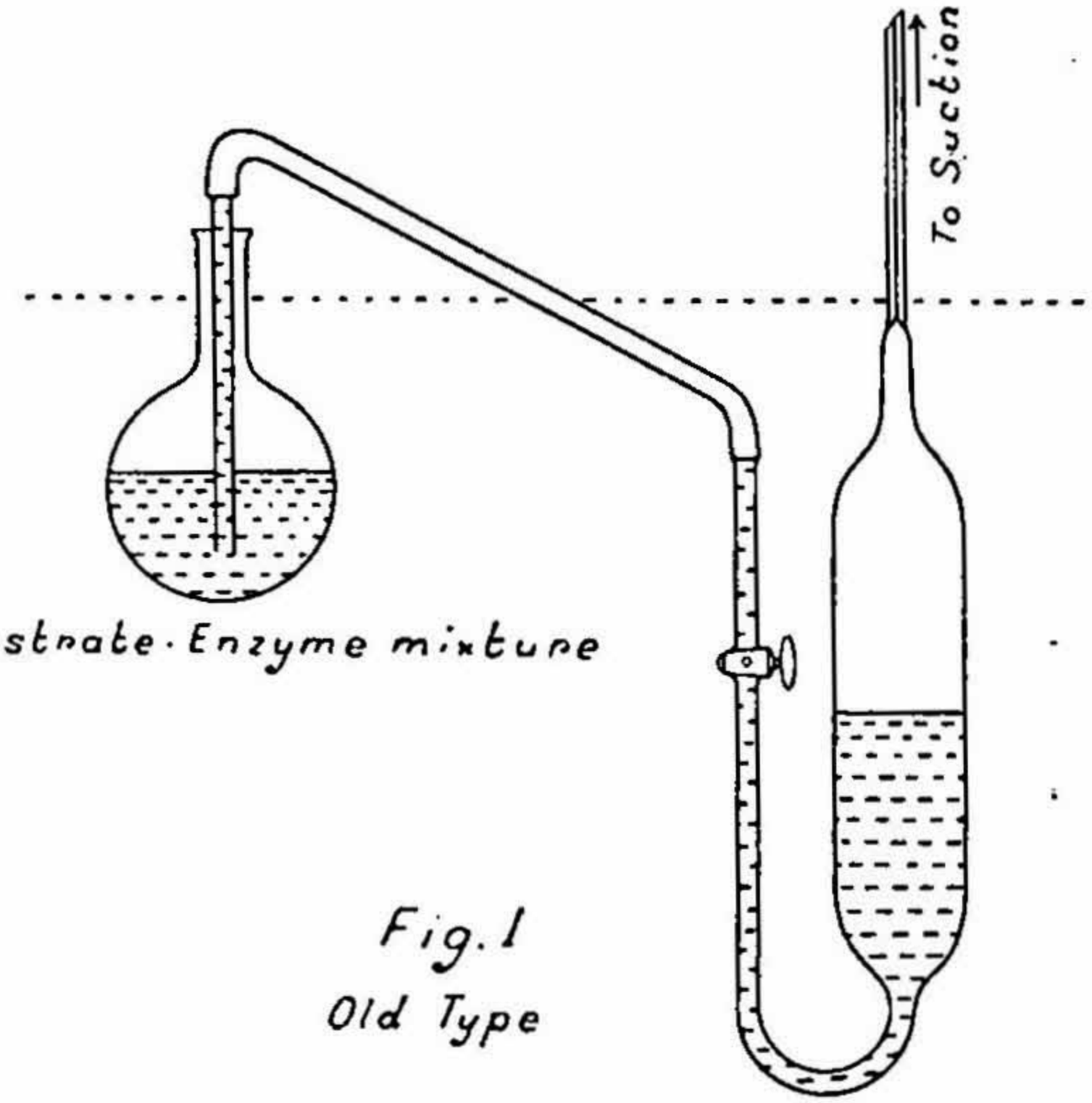

Fig. I
Old Type

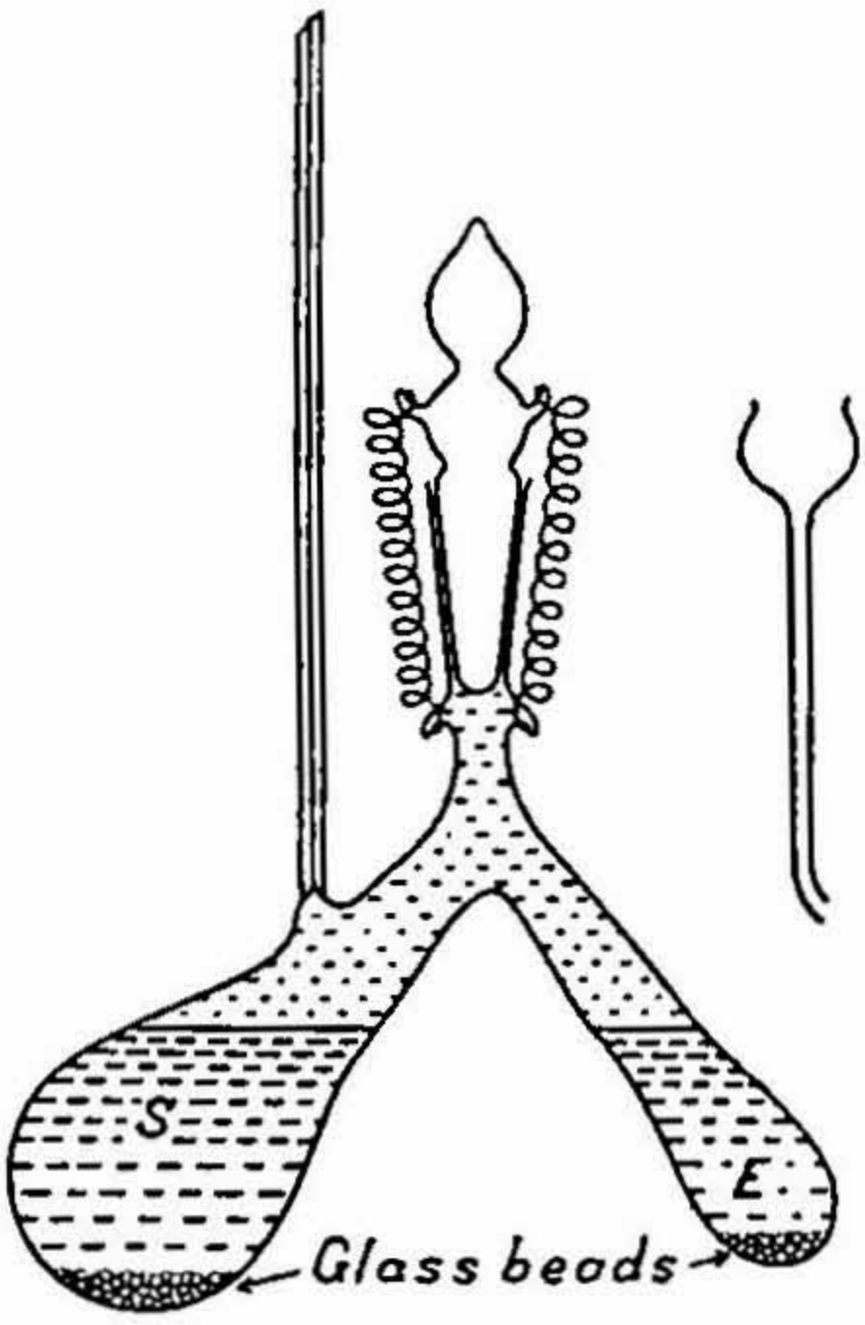

Fig. II

New Type

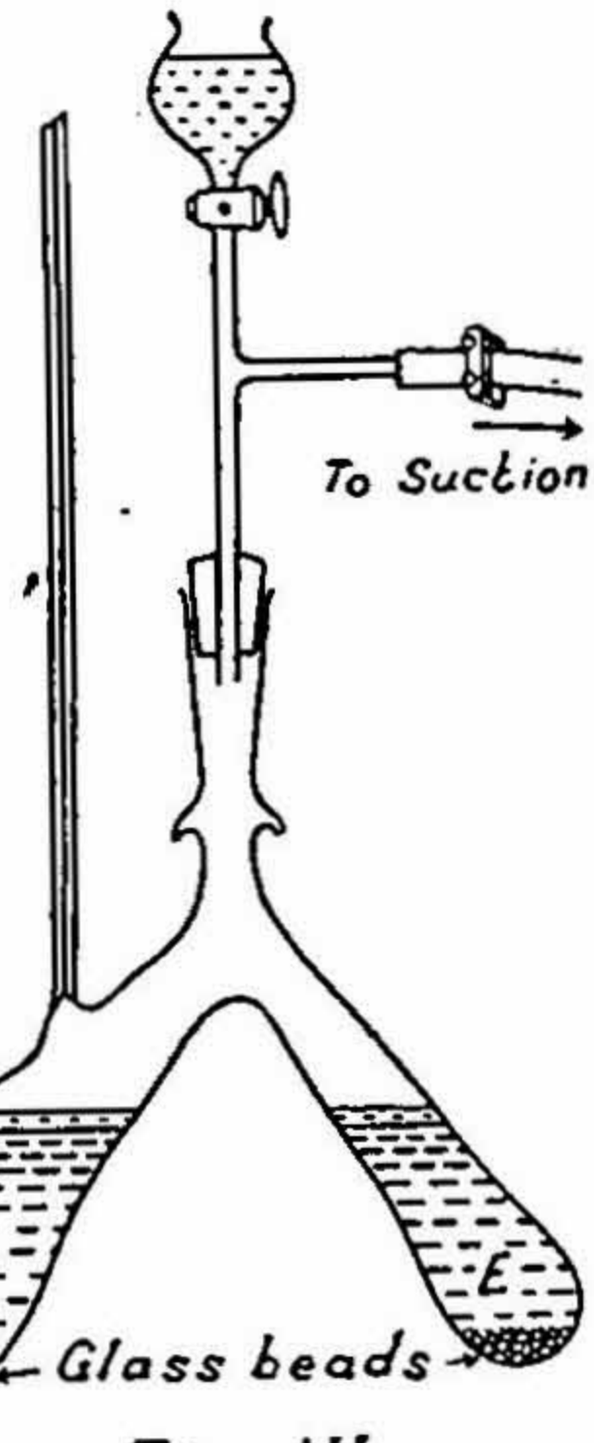

Fig. III

by two springs (Fig. II). A control experiment showed the efficiency of the grease, the meniscus in the capillary varying within the same limits for more than 48 hours. All the dilatometers employed in the investigation were subjected to this test.

The meniscus in the capillary, which usually reaches the top, is adjusted to a convenient lower level by warming the larger bulb by hand, when a portion of the petrol flows out at the capillary end. On regaining the temperature of the thermostat, the meniscus falls to a lower level. The extent of adjustment is to be determined by the nature of the reacting components ; while in some cases there is an almost instantaneous fall in the dilatometric column, a definite and appreciable rise is observed in a few others.

After adjusting the meniscus, the limits of variation of the dilatometric column due to the temperature variations of the thermostat are determined. The combined volumes of the substrate, enzyme and petrol, more particularly on account of petrol possessing a high coefficient of cubical expansion, amounts to nearly 100 c.c. which renders the instrument highly sensitive even to small changes of temperature falling within $\pm 0.01°$; but so long as the variations lie within definite limits, as has been found to be the case, they do not interfere with our experiments. The range of temperature variation, as indicated by the maxima and minima of the meniscus, is determined and agreement between the three sets of consecutive results is an assurance that the entire contents of the dilatometer have attained the temperature of the thermostat. Subsequent readings after mixing are taken either at the maximum or the minimum temperature as indicated by the control dilatometer which is simultaneously maintained in the thermostat, the same quantities of the boiled enzyme, substrate and petrol being employed. Table II shows the readings for successive maxima and minima of the experimental and control dilatometers in a typical experiment.

TABLE II.

		Experimental	Control	Water Control
		cms.	cms.	cms.
Before mixing	max.	5.1	8.8	15.6
,,	min.	4.3	8.2	15.5
,,	max.	5.2	8.9	15.6
,,	min.	4.3	8.2	15.5
,,	max.	5.1	8.9	15.6
,,	min.	4.3	8.2	15.5
,,	max.	5.2	9.0	15.6
,,	min.	4.3	8.2	15.5
24 hrs. after completion.	max.	15.5	9.0	15.6
,,	min.	14.7	8.2	15.5
,,	max.	15.5	9.0	15.6
,,	min.	14.7	8.2	15.5
,,	max.	15.5	9.0	15.6
,,	min.	14.7	8.2	15.5

The reaction components in the dilatometer are then mixed at a definite temperature, the maximum or the minimum shown by the control dilatometer

holding the capillary by means of a cloth and tilting the apparatus underneath the water of the thermostat. The glass beads and the petrol help the mixing which can be effected in about 30 seconds, during which period the meniscus can be easily read. Subsequent readings at known intervals can be taken. Thus it is possible to study not only the kinetics of enzyme action almost from the moment of mixing, but also the total change occurring at the end of reaction. This instrument has the further advantage of revealing to some extent the abnormal and interesting changes occurring in the initial stages of the reaction on mixing. Table III indicates some of the notable changes occurring in a few of the enzyme-substrate systems immediately on mixing.

TABLE III.

	Reaction	Change observed 30 secs. after mixing	
		Experimental	Control
1.	Arginase-arginine	+10 mm.	+8 mm.
2.	Diastase-starch	++	+
3.	Emulsin-amygdalin	0.0 mm.	0.0 mm.
4.	Emulsion-salicin	++++	++
5.	Invertase-sucrose	0.0 mm.	0.0 mm.
6.	Urease-urea	—15 mm.	—5 mm.

+ or — respectively indicates rise or fall in the dilatometric column.

It is difficult, at this stage, to offer any explanation of these initial changes, which are being subjected to a detailed investigation.

The course of hydrolysis can then be followed by taking readings of the dilatometric column at known intervals. The reaction reaches equilibrium in the course of 6–8 hours, after which a final reading of the dilatometric column is recorded and the difference between the first and the final readings gives the total fall in the dilatometric column. From this the total reduction of volume V in cubic centimetres may be calculated. If the weight of the substrate W present in the reaction is known, the contraction constant K is given by the formula,

$$K=\frac{V}{W}M$$

where M represents the molecular weight of the substrate. In the case of colloidal substrates M is replaced by 100.

Sources of Error.—All possible sources of error in these dilatometric estimations have been carefully examined. Errors due to progressive changes of surface tension in the reacting mixture are not taken into consideration in these experiments since the mixture does not come into contact with the capillary containing the high boiling petrol. Evaporation from the capillary meniscus and drained surfaces was found to be negligible. The error caused by fluctuations of atmospheric temperature in the exposed capillary column has been found to have the order of ±0.15 mm. per degree change for an exposed column of 20 cms., which is about the average height attained in our experiments. Sources of error seriously affecting the constant are: (1) purity

of the substrate and (2) percentage of hydrolysis brought about by the enzyme under the conditions of the experiment. The purity of the substrate is ensured by repeated recrystallization and the percentage of purity determined by an independent analysis. The degree of hydrolysis is found by a series of experiments employing a method entirely independent of the dilatometer ; in the case of urease-urea, for example, the aeration method of estimating urea is used. At each concentration of substrate employed, the percentage of hydrolysis was determined, and the correction for complete hydrolysis calculated ; this correction is useful in estimating the substrates of unknown physiological fluids.

The present communication describes the investigation of two closely allied enzyme-substrate systems : (1) urease-urea and (2) arginase-arginine. For a study of system (1) the following materials were employed :—(*a*) The enzyme urease, from Arlco Jack bean preparation in tablet form, and (*b*) Urea (B.D.H.) which, twice recrystallized from alcohol was found to be 99.8 per cent. pure by two independent methods, (1) total nitrogen by Kjeldahl and (2) aeration method of Van Slyke and Cullen (*J. Biol. Chem.*, 1914, **19**, 211).

The reaction mixture consisted of 40 c.c. of a solution prepared by dissolving a known weight of urea in Sorensen's phosphate buffer (P_H 7.0) and 5 c.c. of a 1 per cent. solution of the urease preparation. The experimental procedure before described was closely followed. Table IV gives the results for the urease-urea system while Table V records those of aeration studies carried out under similar conditions to determine the percentage of hydrolysis effected by the system.

TABLE IV.

	Concentration of Substrate	Initial reading		Final reading		Total fall in the dilatometric column	Contraction constant (uncorrected)
		Exptl.	Control	Exptl.	Control		
		mm.	mm.	mm.	mm.	mm.	
1.	*M*/20	10.0	9.0	400.0	9.0	390.0	23.96
2.	*M*/20	9.0	12.5	398.0	12.5	389.0	23.90
3.	*M*/20	12.0	25.0	401.5	25.0	389.5	23.93
4.	*M*/30*	42.0	48.0	365.5	48.0	323.5	23.92
						Average ..	23.93

*50 c.c.

Corrected for purity and percentage of hydrolysis .. 24.13

TABLE V.

	Urea taken in mgms.	Urea found in mgms.	Percentage of hydrolysis
1	30	29.82	99.4
2	30	29.83	99.4
3	15	14.88	99.2
		Average ..	99.3

The following materials were employed for a study of the arginase-arginine reaction. The enzyme was obtained from sheep's liver as the glycerol extract, by the method of Hunter and Daulphinee (*J. Biol. Chem.*, 1929-30, **85**, 627). The substrate was derived from two sources : (1) B. D. H. " arginine", which experiment showed to consist of *d*- and *l*- arginine in equal amounts (see Table VI), and (2) Dr. Theodor Schuchardt " *d*- arginine ". The purity of the two samples of arginine has been ascertained by (1) a determination of total nitrogen by Kjeldahl and (2) the aeration method as described by Hunter and Daulphinee.

TABLE VI.

Percentage of Arginine.

Source	Kjeldahl	Aeration
B.D.H.	101	49.64
Schuchardt ..	100	99.98

The reaction mixture consisted of 50 c.c. of *M*/20 arginine solution prepared by dissolving the requisite amount of arginine in phosphate buffer (P_H 8.04) and 5 c.c. of the enzyme extract. The experimental procedure adopted was the same as described before and the results are given in Table VII.

TABLE VII.

Sample		Initial reading		Final reading		Total depression	Constant (uncorrected)
		Experimental	Control	Experimental	Control		
		mm.	mm.	mm.	mm.	mm.	
B.D.H.	1 ..	31.0	22.0	82.0	22.0	51.0	2.5
	2 ..	45.0	76.0	122.0	76.0	52.0	2.55
						Average ..	2.53
Schuchardt	1 ..	88.5	95.0	191.5	95.0	103	5.06
	2 ..	84.0	71.0	185.0	71.0	101	4.98
	3 ..	72.0	40.0	174.0	40.0	102	5.01
						Average ..	5.02
						Corrected for purity and percentage of hydrolysis ..	5.02

The contraction constants of other enzyme-substrate systems are being determined.

SUMMARY.

1. A new type of dilatometer has been described where the enzyme and the substrate could be mixed in the dilatometer itself, thereby facilitating an investigation of the changes from the beginning of the reaction. The kinetics of the reaction could simultaneously be followed.

2. At lower concentrations of substrate, the total contraction brought about by the system is found to be proportional to the weight of the substrate present in the reaction mixture. The contraction in cubic centimetres due to the enzymic hydrolysis of a gram-molecule of the substrate can be calculated, and represents the contraction constant of that particular enzyme-substrate system.

3. The experimental procedure has been described in full detail ; various sources of error affecting the determination have been carefully examined, and the corrections applied wherever found necessary.

4. The contraction constants of two closely allied enzyme-substrate systems, urease-urea and arginase-arginine, have been found to be 24.13 and 5.02 respectively and the usefulness of these constants in a determination of urea and arginine in unknown physiological fluids and protein hydrolysates is indicated.

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