*MEASUREMENT OF ACTIVITY OF SINGLE MOLECULES OF β-D-GALACTOSIDASE**

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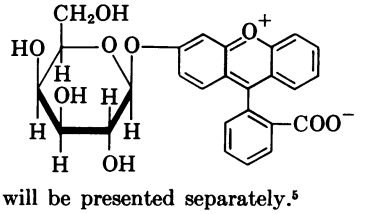
A method capable of measuring the activity of single enzyme molecules could initiate studies of a number of biological problems not yet amenable to experimentation. A technique for measuring β-D-galactosidase in droplets of microscopic size using a new fluorogenic substrate, 6-hydroxyfluoran-β-D-galactopyranoside (6HFG) is described here. Results obtained by this technique indicate that it is capable of detecting individual molecules of the enzyme.

As practical applications, the molecular weight and turnover number of β-D-galactosidase were determined by this method. The values obtained are comparable with those found by conventional techniques. Another application of this method is in determining whether or not an enzyme is composed of a homogeneous molecular species with respect to activity. This is exemplified here by experiments with a genetically altered β-D-galactosidase. We concluded that the altered enzyme is a different species than normal β-galactosidase. In contrast, thermal inactivation leads to a mixture of fully-active and inactive enzyme molecules.

Materials and Methods.—The substrate 6HFG[5] was synthesized by coupling 6-hydroxyfluoran (6HF)[1] and tetraacetyl-β-D-galactopyranosylbromide[2] in anhydrous benzene with silver oxide present.[3] The tetraacetyl galactoside was deacetylated with sodium methylate.[4] The final product, purified by ascendent paper chromatography in a 1-pentanol, 1-propanol, water system (40:11:15), moved with an R_f of 0.17 on Whatman #1 paper at room temperature (22°C). 6HF was located by the strong greenish fluorescence of 6HF liberated after spraying a portion of the paper with β-D-galactosidase. A long wavelength "Mineralight" was used for excitation. UV light of shorter wavelength decomposes 6HFG on paper, forming a compound with bluish fluorescence. This can serve to locate 6HFG on chromatograms.

The purity of 6HFG was established by paper chromatography in three different solvent systems at neutral, acid, and basic pH.[5] Complete hydrolysis of 6HFG by a purified, crystalline preparation of β-D-galactosidase from *Escherichia coli* (kindly supplied by Dr. D. Perrin) with stoichiometric recovery of 6HF and D-galactose indicates that 6HFG has the formula:



Additional proof of structure will be presented separately.[5]

The following properties of 6HF, a deoxy-derivative of fluorescein, explain why 6HF is particularly suitable as a fluorogen: (*a*) like fluorescein, 6HF has a strong greenish fluorescence in *neutral pH* and a quantum yield near 100 per cent;[6] (*b*) it is excited with highest efficiency at 490 mμ, a wavelength which does not generally excite biological material; (*c*) in contrast to fluorescein, 6HF has a single phenolic group; therefore, any 6HF derivative linked through the OH group would be expected to have little or no fluorescence.[7] Accordingly, 6HFG has little fluorescence as compared with 6HF. Furthermore, the two compounds differ markedly in both their excitation and fluorescence spectra. The wavelength for maximum excitation is 495 mμ for 6HFG and 400 mμ for 6HF. 6HFG has a fluorescence peak at 520 mμ, while 6HF has it at 490 mμ. These differences give a fluorescence ratio 6HF/6HFG of about 10^5 for 2.6×10^{-6} *M* solutions in an

Aminco-Bowman spectrophotofluorometer using 480 mμ for excitation and measuring fluorescence at 520 mμ. For macro-assays, 6HF can also be measured by absorption at 490 mμ in a spectrophotometer. This method can be more convenient than fluorescence if dilutions are to be avoided. The optical density was found to be directly proportional to 6HF concentration in the range 10^{-6} M to 10^{-5} M with a molar extinction coefficient of 5.81×10^4.

Crude preparations of β-D-galactosidase were obtained from the constitutive mutant W1317 of *Escherichia coli*, strain K-12.[8] Cultures were grown in mineral medium[9] with 0.4 per cent glycerol with vigorous rotation. About 10^{12} washed cells were suspended in 5 ml 0.02 M sodium phosphate buffer, pH 7.2, and disrupted in a French press.[10] The lysate was spun down at 50,000 × g for 60 min and the supernatant sterilized by filtration through a Millipore HA filter G. β-D-galactosidase activity is expressed as mμmoles of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute under conditions previously described.[8] Hydrolysis of 6HFG by β-D-galactosidase was measured in 0.2 ml of 0.02 M sodium phosphate buffer, pH 7.2, by incubating the reaction mixture at 37° and stopping the enzymatic action with 1.8 ml of the same buffer at 0°. The amount of 6HF liberated was determined within seconds thereafter.

The enzymatic hydrolysis of 1.2×10^{-4} M 6HFG at 37° proceeded at a constant rate for 5 hr at a rate of 120 μμmoles/ml per min until 30 per cent of the substrate was hydrolyzed. The K_m value of the crude β-D-galactosidase extract, as measured with 6HFG under the conditions described above, was 3.2×10^{-4} M and V_{max} was 45 mμmoles/min per ml of extract. With ONPG the K_m was 1.7×10^{-4} M and V_{max} was 1.04 μmoles/min per ml of extract.

Collins' spray method[11] was used to disperse a solution containing the enzyme and the substrate into droplets of 0.1 to 40 μ diameter. In this method, the solution is atomized over a few drops of silicone oil placed on a microscope slide coated with silicone (Siliclad, Clay-Adams). A coverslip, also coated with silicone, is placed over the oil immediately after spraying. A very fine mist is obtained with an atomizer made of a capillary tube of about 20 μ diameter at the spraying tip. Microdroplets rise slowly through the oil and come in contact with the coverslip without apparent change in their spherical shape. This is true only when a silicone oil denser than water is used. For our studies, the following modifications of the Collins method were introduced. To prevent evaporation during prolonged incubation, an oil chamber was devised from a piece of Parafilm (Marathon) 2 cm square with a hole 1.5 cm in diameter perforated in the middle. The Parafilm was then fused to the slide and a coverslip was used as a lid. Five drops of oil were placed in the chamber before spraying. Immediately after spraying, a coverslip with three hanging drops of oil was deposited to seal the chamber. Our atomizer was operated by nitrogen at 2 lb/in.^{2} pressure. Dark field slides (A. Thomas #7038) were selected of a thickness that permitted the dark field condenser to be in focus when in *direct* contact with the slide. This procedure eliminates the subjective operation of focusing the condenser and, therefore, assures a constant illumination for every slide. A silicone oil of lower density than water was used in order that the droplets containing the enzyme-substrate solution would settle to the bottom of the slide instead of rising. The settling time was less than 45 min, and if necessary, this could be shortened by spinning the slides in a swinging bucket centrifuge at 70 × g for 1 min. The slides were fastened to the tube holders with small pieces of modeling clay.

β-D-Galactosidase preparations were diluted in 0.02 M sodium phosphate, pH 7.2, containing 0.2% bovine albumin (Armour, fraction V) and yeast extract[12] prior to spraying. These additions served to protect the enzyme from denaturation. All solutions were sterile. 6HFG was sterilized by filtration through millipore membrane filters. For spraying 0.01 ml of a given enzyme dilution was mixed rapidly with 0.04 ml of 1.2×10^{-4} M 6HFG; the reaction mixture was sucked into the capillary tube of the atomizer (previously tried out with water) and then sprayed on the silicone oil chamber. A pH paper placed near the slide served to guide the spraying. The coverslip, ready with oil, was dropped over the oil chamber immediately after spraying. Until the time of spraying, the solutions were kept cold. After incubation the microdroplets containing the reaction mixture were found evenly distributed at the bottom of the oil chamber and the amount of 6HF liberated by the enzyme in each droplet was determined by microfluorometry.

A Zeiss Universal microscope equipped with a III-Z condenser was used. This condenser permits rapid changes from bright to dark field without changing the focus. The light source was the 6-volt–15-watt tungsten built-in lamp normally supplied with the microscope. For some experiments, it was operated by a 6-volt battery. Monochromatic light was obtained by interposing a

3 mm Zeiss BG-12 blue filter. A 40×planachromatic objective was used in conjunction with a 25× ocular combination (ocular 12.5×optovar 2×) for visual observations. For photometry, the image from the objective was diverted to the monocular tube of the microscope where an Aminco photomultiplier microphotometer equipped with shutter and Wratten #12 filter, to exclude exciting light, was installed. The area measured by the photomultiplier (RCA 1P21) was reduced to about 20 μ diameter by placing a fixed pupil of 2 mm diameter near the point where the image of the object is focused.

The procedure for fluorimetric assay was as follows: (1) the slide was incubated for 15 hr at 36° and then placed under the microscope, (2) a drop of immersion oil was placed on the condenser, (3) the condenser was raised until it came in contact with the slide and then was centered, (4) a given droplet was selected and then its diameter measured using a calibrated ocular micrometer; bright field with green filter was used for this measurement, (5) the droplet was centered in the field by means of cross hairs, (6) the blue filter was placed in position, the condenser changed from bright to dark field, the light directed to the photomultiplier, the green filter removed from the light path and the photometer read, and (7) these last operations were reversed and a new droplet was selected.

About 80 droplets in the range 14–15 μ diameter were measured in each slide. Visual gauging, made possible by using a range of diameters instead of simply one, facilitated the selection of droplets prior to actual measurement. Figure 1 is a photomicrograph of a slide with droplets as used for these experiments.

Controls without enzyme and calibration samples with 6HF were run with every determination.

Results and Conclusions.—Experimental conditions and sensitivity of the method: Figure 2 depicts the relationship between intensity of fluorescence and droplet volume for three concentrations of 6HF. It is seen that the intensity of fluorescence is proportional to 6HF concentration throughout the whole range of droplet volumes. Nevertheless, fluorescence values become confluent below 13 μ diameter and, for this reason, droplets in the range 14–15 μ diameter were selected as optimal for subsequent experiments. Figure 3 shows that fluorescence is proportional to 6HF concentrations in the range $6 \times 10^{-6}M$ to 2×10^{-5} for droplets of 14 and 15 μ diameter. From these results the sensitivity of the method can be calculated. The volume of a droplet 14 μ diameter is 1.4×10^{-9} cm^3. With a 6HF concentration of $2.0 \times 10^{-6}M$, the amount of 6HF detectable is 2.8×10^{-18} moles, equal to 1.68×10^6 molecules. The turnover number of β-galactosidase for 6HFG, calculated from Cohn's turnover number for ONPG (4,000 per sec)[13] and an empirical ratio of (ONPG − 28°)/(6HFG − 37°) = 64.5, is 2.23×10^5 molecules of 6HFG hydrolyzed per hour per molecule of enzyme. Accordingly, our method should detect a molecule of enzyme in less than 10 hr.

Stability of the enzyme during spraying: Experiments were conducted to test if β-D-galactosidase were inactivated during spraying. Enzyme preparations were diluted with 0.2 per cent albumin to give three solutions with activities of 66, 22, and 1.32 mμmoles of ONPG hydrolyzed/min. per ml. Samples of 0.2 ml from each dilution were atomized in long capillary tubes and the mist collected in a beaker with 3.0 ml $0.02M$ phosphate buffer. Other samples were mixed directly with the buffer without spraying. ONPG was added to the final solutions and galactosidase activity was determined. Incubation times ranging from 18 min. to 6 hr were used. The results indicate that less than 2% of the enzyme is inactivated during spraying.

Reconstitution experiments: To determine whether 6HF could diffuse from one droplet to another through the silicone oil, reconstitution experiments were run. Solutions with and without $8.6 \times 10^{-6}M$ 6HF were sprayed consecutively on

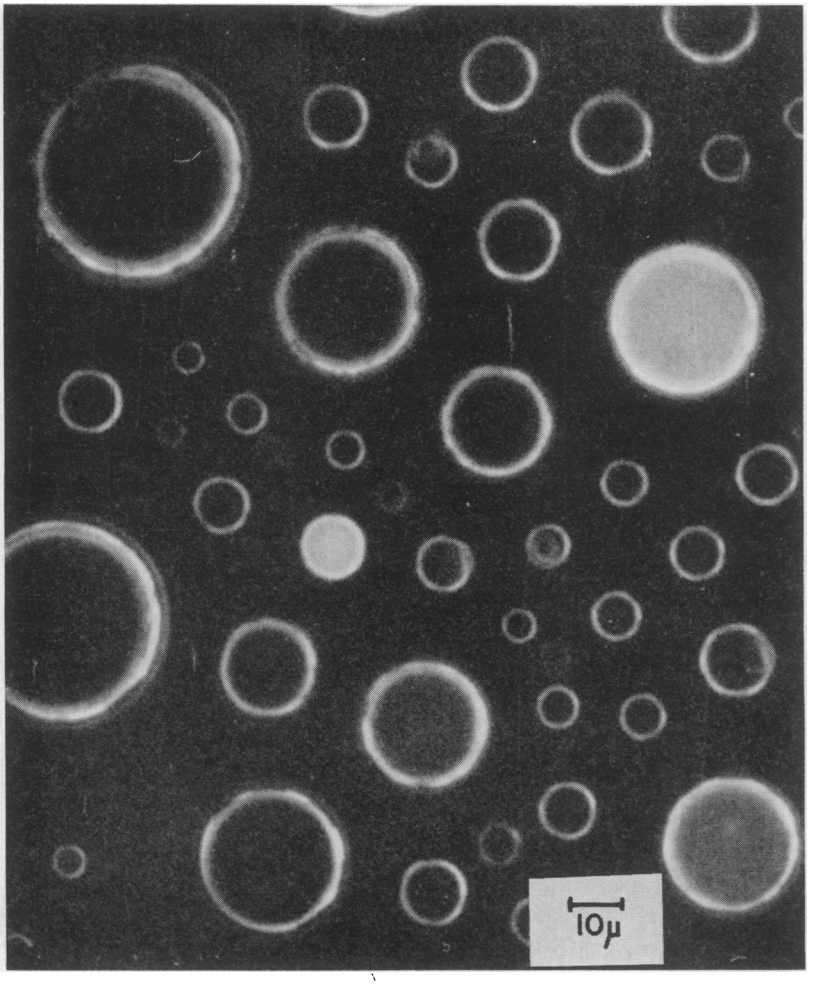


FIG. 1.—Photomicrograph of dispersed droplets of 6HFG in silicone oil taken under dark field with a blue filter on the lamp. Droplets with green fluorescence due to release of 6HF by β-D-galactosidase appear as white circles in the photograph. The intense fluorescence in this slide was caused by single cells of *Escherichia coli*. Note the even distribution of droplets on the slide. This is always observed and is probably associated with electrostatic forces on the droplets.

a slide using two separate atomizers. The interval between the two sprayings was kept within 10 sec. After 15 hr at 36°, fluorescence in droplets of 14–15 μ was measured. As shown in Figure 4, the droplets were distributed in two well-defined groups. One group corresponded to controls without 6HF; fluorescence of the other group was comparable to controls with $8.6 \times 10^{-6} M$ 6HF. No droplets intermediate between these two groups were found in a set of three experiments.

Changes in droplet volume during incubation: The possibility that the volume of the 14–15 μ diameter droplets changes during incubation was examined. To this effect, the diameter of the droplets was measured periodically. The experiments were conducted at 38° in a constant temperature room. Photographs of

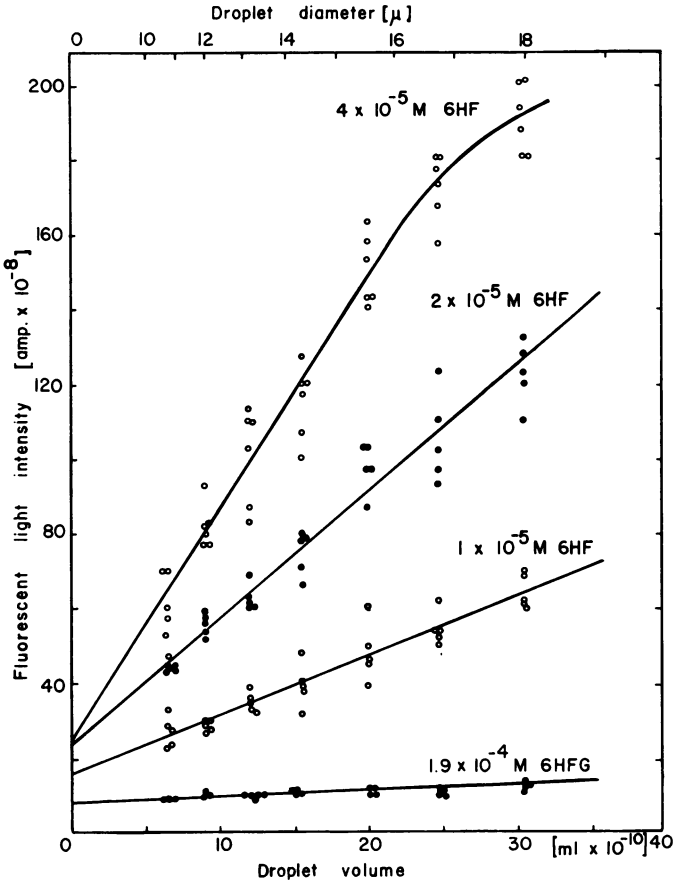


FIG. 2.—Relationship between intensity of fluorescence and droplet volume. The values here were obtained at higher light intensity than that used in other experiments. Droplets of seven different diameters were measured. Small horizontal displacements of the plotted points indicate only that two droplets of the same diameter have similar fluorescence.

the same microscope field were taken with a 35 mm camera at 30–60 min intervals. Diameters of droplets were measured in enlargements made with a fixed amplification factor. Consequently, sequential measurements of several droplets at a time were possible. Taking the initial volume of each droplet as 100, the standard deviation in 27 droplets after 8–15 hr incubation was 1.4 per cent. The range was −2.4 to 5 per cent. These results indicate that no significant changes in volume occurred during incubation.

Determination of molecular weight: If a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability $p(r)$ of finding 0, 1, 2, 3, . . . , r enzyme molecules in a droplet is given by the Poisson law $p(r) = (n^r e^{-n}/r!)$. For example, when the average number of molecules per droplet is 1, the proportion of droplets with 0 and 1 molecules is $e^{-1} = 0.37$; with 2 mole-

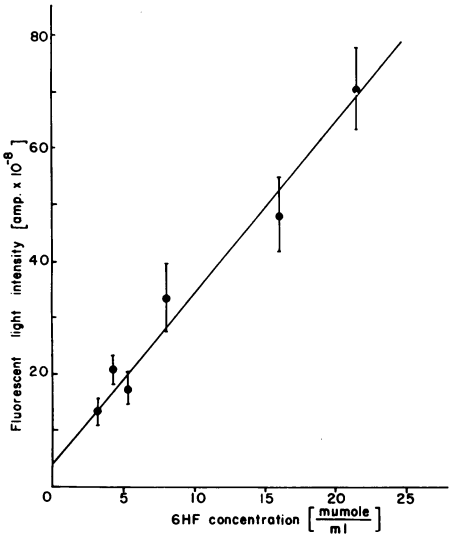


FIG. 3.—Proportionality between fluorescence and 6HF concentration. Each point represents the average fluorescence of 20 droplets 14–15 μ diameter minus a background value of 2. The vertical lines indicate standard deviation.

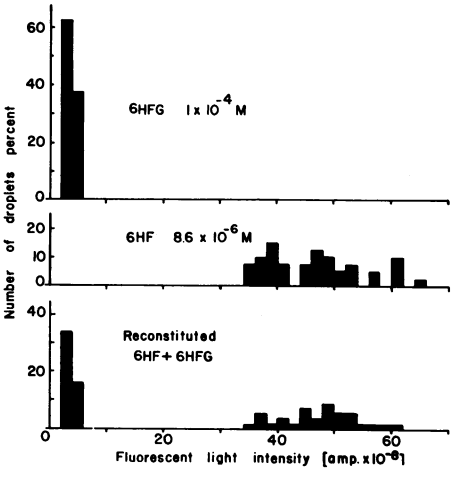


Fig. 4.—Reconstitution experiment to test diffusion of 6HF in silicone oil. Droplets with and without 6HF 8.6 $\times 10^{-6}$ M were sprayed consecutively at 10-sec interval using two separate atomizers.

cules, $e^{-1}/2 = 0.18$; with 3 molecules, $e^{-1}/6 = 0.06$.[14] Experimentally, the average number of enzyme molecules per droplet can be determined from the proportion e^{-n} of droplets without enzyme.[15] From the average number of molecules per droplet and the droplet volume, the molar concentration x of the enzyme solution can be obtained by n/v. The molecular weight of the enzyme can then be calculated as the ratio of the weight concentration and molar concentration of the enzyme solution. The determination of weight concentration requires either a solution of pure enzyme or knowing the specific activity of pure enzyme.

These principles were applied to experiments with β-D-galactosidase. Figure 5 presents the distribution of fluorescence among droplets for a series of dilutions of enzyme, controls without enzyme, and calibrating standards of 6HF. The following conclusions were drawn from the results:

(1) Droplets with measurable fluorescence appeared only when β-galactosidase was present. In 60 experiments without enzyme, involving more than 1,700 droplet measurements, no droplets with significant fluorescence were detected.

(2) The measured distribution of fluorescence among droplets established the presence of four distinct categories: (*a*) droplets without fluorescence, (*b*) droplets with a given amount of fluorescence, (*c*) droplets with about twice the fluorescence of group *b*, and (*d*) droplets with three or more times the fluorescence of group *b*. This differentiation of groups, as well as the proportion of droplets within them, conforms to the expected Poisson distribution of enzyme molecules among the droplets (Table 1). The amount of substrate in the droplet limits the assay when more than two enzyme molecules are in the droplet. For this reason, the probabilities of three, four, and more molecules were added together in Table 1.

(3) The average number of enzyme molecules per droplet was calculated from

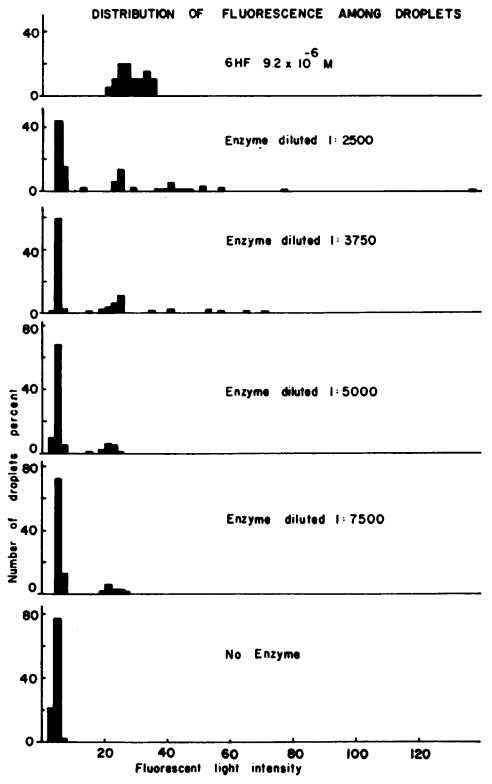


FIG. 5.—Distribution of fluorescence among droplets 14–15 μ diameter for various enzyme dilutions. One ml of the β-D-galactosidase solution before the indicated dilution hydrolyzed 467 mμmoles ONPG per min at 37°.

TABLE 1

THEORETICAL AND OBSERVED DISTRIBUTION OF ENZYME MOLECULES AMONG DROPLETS

Class of Droplet	Percentage observed	Percentage expected	Average fluorescence intensity
Dilution 1:2500			
With 0 molecule	56.3	(56.3)*	5.6
With 1 molecule	25.3	32.0	24.1
With 2 molecules	17.2	9.1	46.1
With 3 or more molecules	1.2	2.0	107.0
Dilution 1:3750			
With 0 molecule	62.7	(62.7)*	5.4
With 1 molecule	27.7	29.5	23.0
With 2 molecules	9.6	6.9	52.4
With 3 or more molecules	0	0.9	...
Dilution 1:5000			
With 0 molecule	82.9	(82.9)*	5.1
With 1 molecule	15.9	15.8	21.5
With 2 molecules	1.2	1.5	36.0
With 3 or more molecules	0	0.3	...
Dilution 1:7500			
With 0 molecule	84.4	(84.4)*	5.5
With 1 molecule	15.6	14.3	23.1
With 2 molecules	0	1.2	...
With 3 or more molecules	0	0.2	...

* The percentage expected was calculated from the proportion of droplets without enzyme using Poisson's law (see text).

the proportion of droplets without enzyme. From this number, the number of enzyme molecules per ml present in the β-galactosidase solution used for spraying was calculated. If this method is correct, the number of enzyme molecules per ml obtained at different enzyme concentrations must be proportional to enzyme concentration measured by standard methods. Figure 6 shows data that conform to this postulate.

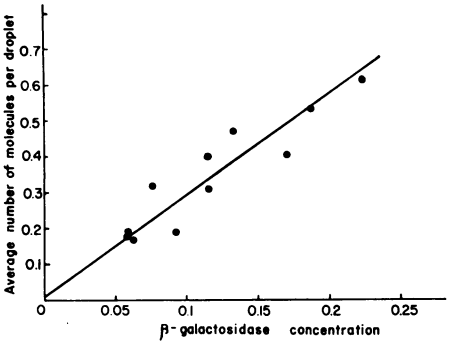


FIG. 6.—Proportionality between enzyme concentration and average number of enzyme molecules per droplet determined by our method. The β-D-galactosidase concentration of the sprayed solution is given in mμmoles ONPG hydrolyzed per min at 37° per ml of enzyme solution.

(4) In addition, the curve in Figure 6, fitted to the points by the least-squares method, provides a better estimate of the average number of enzyme molecules per ml of extract; namely, 8.7×10^{11} molecules/ml equivalent to 1.4×10^{-12} moles/ml. The weight of protein corresponding to β-galactosidase in our extract, 8.9×10^{-7} g/ml, was calculated from the known specific activity of purified enzyme.[13] Dividing this value by 1.4×10^{-12} moles/ml, a molecular weight of 630,000 with an estimated error of ±15 per cent results. The values obtained by conventional methods are 700,000[13] and 750,000.[16]

Kinetics of single enzyme molecules: The rate of 6HFG hydrolysis by single enzyme molecules was measured by the increase in fluorescence of droplets chosen under conditions assuring an 86 per cent probability of selecting droplets with single enzyme molecules (i.e., $n = 0.3$.). A few droplets having detectable fluorescence after 2 or 3 hr incubation at 36° were located on the slide and fluorescence measurements of these droplets made at intervals.

As shown in Figure 7*A*, the rate of hydrolysis was similar in six out of eight droplets. Two droplets exhibited a rate approximately twice that of the others. The conclusion drawn is that the droplets with the lower hydrolysis rate contain a single molecule of enzyme. Accordingly, the average rate for single enzyme molecules is 115 molecules of 6HFG hydrolyzed per sec. The rate for the droplets with faster hydrolysis is 258 6HFG molecules per sec. The possibility that these results are biased because short incubation periods favor selection of droplets containing more than one enzyme molecule was tested by two methods. First, the proportion of droplets without fluorescence was determined in four slides sprayed with the same enzyme dilution and incubated at 36° for different periods of time. Figure 7*B* shows that the proportion of droplets without fluorescence remains constant within experimental error throughout 8 hr of incubation. In another ex-

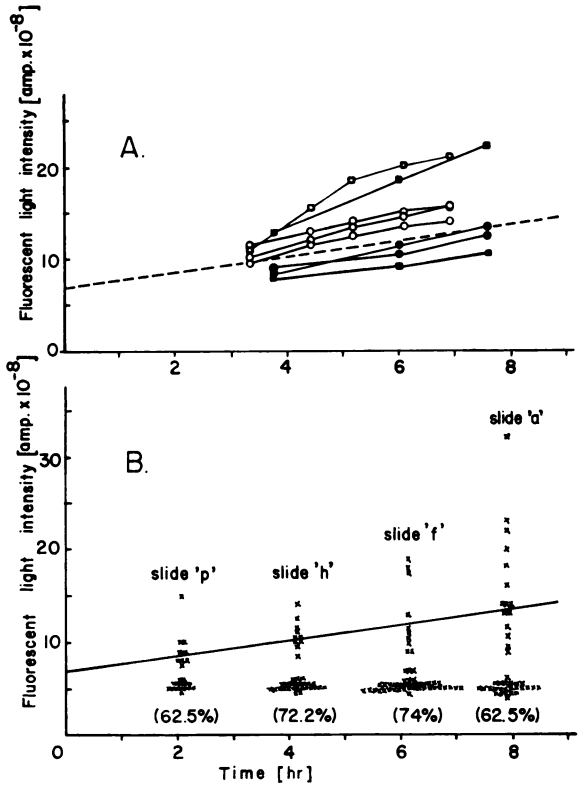


FIG. 7.—Kinetics of single enzyme molecules. *A*: Droplets of 14–15 μ diameter were selected on a slide after 3 hr incubation at 36° and fluorescence measurements in these droplets made at intervals. Open and filled circles indicate two different slides. *B:* Four different slides sprayed with the same enzyme solution were incubated at 36° for 2, 4, 6 and 8 hr. Horizontal displacement of the plotted points indicates only the number of droplets measured. The activity of the sprayed solution was 87.3 $\mu\mu$moles of ONPG per min per ml of enzyme solution.

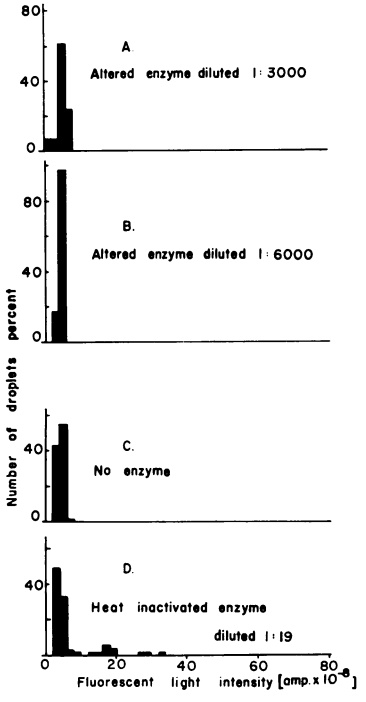


FIG. 8.—Distribution of fluorescence among droplets for a genetically altered β-D-galactosidase. The activities at 37° of the enzymes before dilution were: 264 mμmoles ONPG hydrolyzed per min per ml of altered enzyme solution and 3.1 mμmoles ONPG per min per ml of heat-inactivated enzyme solution.

periment, similar results were obtained with two slides, one incubated for 2 hr and the other for 15 hr. Second, the hydrolysis rate was determined under conditions where probability of measuring droplets with more than one enzyme molecule was negligible. To this effect, the average number of enzyme molecules per droplet was decreased to less than 0.1 using more dilute enzyme solutions for spraying. With average 0.1 the proportion of droplets without enzyme is 90.5 per cent, with one enzyme molecule 9.05 per cent, and with two or more 0.45 per cent; therefore, in these experiments only 4.7 per cent of the droplets *containing enzyme* should have more than one molecule. The hydrolysis rate under these conditions was determined not by single droplet kinetics but by the average fluorescence of 10 to 20 droplets after 15 hr incubation. The rate found, 120 molecules per sec is comparable with 115, the value from kinetic measurements. The conclusion is that the probability of selecting droplets with one enzyme molecule after 2 hr incubation is not influenced by this relatively short incubation.

Turnover number: The prevailing method to obtain the turnover number TN of an enzyme (defined as moles of substrate consumed per mole of enzyme per sec) requires determinations of specific activity a and the molecular weight M of the enzyme: $TN = aM$. In contrast to this method, the turnover of β-D-galactosidase is obtained here directly from the rate of 6HFG hydrolyzed by one enzyme molecule. Furthermore, unlike the conventional method, the single molecule procedure does not require enzyme in pure state.

For purposes of comparison, the turnover of ONPG can be calculated from our 6HFG turnover number applying the ratio between rates of hydrolysis of the two substrates. This ratio, at $10^{-2}M$ ONPG and $10^{-4}M$ 6HFG was found empirically to be 103.5. Thus the turnover number for ONPG is 1.2×10^4 molecules per sec at 36° corresponding to 7,000 molecules per sec at 28°. The difference between the latter value and 4,000 molecules per sec at 28°, calculated by M. Cohn,[13] could not be interpreted by the existing data.

Analysis of Genetically Altered and Heat-Inactivated β-Galactosidase.—The method described above was used also to determine whether an enzyme considered as genetically altered β-D-galactosidase could be a mixture of active and inactive molecules of wild-type β-D-galactosidase or really a different enzyme species. If the activity in the altered enzyme was due to the presence of wild-type enzyme molecules, upon dispersion, the distribution of molecules among droplets should be the same for wild-type and altered enzyme solutions of equal average activity. Altered β-D-galactosidase, purified and crystallized from *Escherichia coli*, strain K-12, 13PO, has a K_m of $10^{-2}M$ and a specific activity of 5.9 μmoles of ONPG hydrolyzed per mg protein per min measured in $M/375M$ ONPG at 28°.[17] By contrast, the wild type enzyme has a K_m of $1.8 \times 10^{-4}M$ and a specific activity of 336 μmoles ONPG per mg protein per min under similar conditions.[13] In our experiments, crystalline altered β-D-galactosidase was dissolved, prior to spraying, to match the ONPG splitting activity of normal enzyme solution previously used (Fig. 5). Altered enzyme solutions were sprayed on slides and fluorescence among droplets of 14–15 μ diameter measured after 15 hr of incubation at 36°.

Figure 8 shows that droplets with altered enzyme do not have significant fluorescence and differ from controls without enzyme only because a slight shift in distribution occurred. This contrasts with the differentiation into groups and the spread found with normal enzyme (Fig. 5). It follows that the altered enzyme does not contain wild-type molecules but is a different molecular species.

Similar experiments were conducted to decide whether heat partially inactivates all molecules or produces a mixture of active and inactive molecules. A solution of wild-type β-D-galactosidase containing 400 times more enzyme than normally used for experiments was inactivated to 0.72 per cent of the original activity by heating at 60° for 6.5 min. Different dilutions, matching ONPG splitting activity of untreated enzyme solutions used in previous experiments, were sprayed on slides and fluorescence in the droplets measured. Comparison between Figures 5 and 8 shows that the distribution pattern of normal and heat inactivated enzyme is similar, and that both differ radically from the pattern of genetically altered enzyme. We concluded that some enzyme molecules retain full activity during heat inactivation while others show no detectable activity.

Discussion.—The results obtained by the fluorogenic method lead us to conclude

that it can measure activity of individual enzyme molecules. This conclusion is based largely on the following points:

(1) The amount of detectable 6HF is consistent with the turnover of a single enzyme molecule calculated by the molecular weight and specific activity of β-D-galactosidase.

(2) The observed distribution of fluorescence among droplets at different β-D-galactosidase concentrations conforms with the predicted Poisson distribution of single enzyme molecules among droplets. The concordance between observed and expected data can be estimated by comparing our figure of 630,000 for the molecular weight to the known values of 700,000–750,000.[13, 16]

(3) The probability that observed distributions occurred by sampling variations alone was estimated from the data and found to be less than 0.8 per cent per experiment. Considering that more than 50 experiments were conducted, this probability is negligible.

The method described here has general applications to problems concerning molecular homogeneity and, at the cellular or subcellular level, determination of enzymatic activity of cells or biological particles.[18]

The β-D-galactosidase assay with 6HFG is a model for other enzyme systems in which 6HF could serve as fluorogen for the substrate. The sensitivity of the method can be increased several fold if fluorescence is enhanced by higher light intensity. However, instability of light sources and fading of fluorescence[19] become problems at certain light levels and would require more complicated instrumentation than that used here.

* This work was initiated at the Enzyme Institute, Wisconsin, and continued at the School of Medicine, University of Chile, and Veterans Administration Hospital, Albany, New York.

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[7] In contrast to 6HFG, a fluorescein-β-D-galactoside synthesized in our laboratory has a strong yellowish fluorescence with peak near that of fluorescein.

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