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C. R. Acad. Sc. Paris, t. 260 p. 2077-2080 (15 February 1965). Group 13.

BIOPHYSICS. -- On a new mode of utilization of analytical centrifugaes (1). Note (*) by MM. RENE COHEN and CHRISTIAN W. HAHN, presented by M. Rene Lucas.

We recall the principle of our method (2), which permits to determine the coefficient of sedimentation s of the complex enzyme-substratum(s) formed by an enzyme that is found in very small quantities (less than 1 µg) being able to be contained even in very impure preperations. We give here a calculus technique, bringing data of experimentation to a value approached by s, we are presenting an experimental example and are discussing briefly its precision.

We recently described a new method of analytical centrifugation applicable to certain enzymes; it permits to determine their coefficient of sedimentation, s, in utilizing only a quantity of the order of enzyme microgram. This last one can even be contained in a very impure preparation where it represents only a weak fraction of the total mass of macromolecules present (2).

In practice, one deposits during centrifugation, in a special cell, a "thin" bed (several microliters) of a solution of the enzyme on another solution (of about 0.5 ml) containing the substratums of the enyzmatic reaction. During centrifugation the bed progresses, carries out the reaction and leaves behind it molecules of products of the latter. Our method is applicable in the case where one of these products, P, absorbs specifically a light of determined color; one then photographs the distribution of product P in the cell of centrifugation with the help of this light.

If this centrifugation technique is simple, the quantitative interpretation of photographs is complex and difficult. It seemed useful to us to present in this Note the procedure that we are using provisionally and that already permits us to obtain an approximate value s^* from the coefficient of sedimentation s of the enzyme studied.

THE PROBLEM. -- The choice of experimental conditions will be presented and discussed in a detailed Memoire; let us say only that the optical density of photographs finds itself always on the interior of the linear region of the sensitometric curve of the utilized emulsion. A photograph taken in cycle t (fig. 1 a) is then translated, with the help of a photodensitometer register, in a curve P (x) (fig. 1 b) representing in cycle t, the distribution of concentration of product P

in function of x (distance to axis of rotation). What is important to us are, in function of cycle, the distributions $E_{(x)}$ (fig. 1 d) of the concentration of the enzyme. These distributions are gaussian (3) but as for what follows, it suffices us to admit that they are symetrical in relationship to their maximum. It is impossible to observe directly these distributions. From experimental curves $P_{(x)}$ we must find again, if not curves $E_{(x)}$, then at least the successive positions $\bar{x}_n(t)$ of their maximum, that is to say of the "center" of the enzyme bed. Knowing the angular speed, the value of g is obtained immediately from values of $\bar{x}_n(t)$.

SIMPLIFIED NUMERICAL INTERPRETATION OF EXPERIMENTAL DATA. -- Conditions of validity. -- 1° The initial concentrations of substrates are such that in the entire zone of the cell and after passage of the enzyme bed, the residual concentrations are still saturants for the enzymatic reaction.

2° The enzyme does not inactivate itself during the duration of the experiment.

Symplifier hypotheses. -- a. Between two successive photographs, the bed sediments without changing form (fig. 1 d); this returns to neglect the radial dilution and the diffusion of the enzyme during the interval of time that separates two successive photographs (2 mn in general).

b. Product P does not sediment and does not diffuse during the interval of time considered the highest.

Determination of a*. -- From two distributions $P_t(x)$ and $P_{t+\tau}(x)$ (fig. 1 b) corresponding to two successive photographs taken respectively at time t and $t+\tau$ (fig. 1 a), it is possible to calculate the differential distribution (4) $D_t^{t+\tau}(x) = P_{t+\tau}(x) - P_t(x)$ (fig. 1 c), representing the variation of the concentration of product P during the interval of time . This variation is due above all to three causes : the first two are sedimentation and diffusion, during the interval of time , of product P already existing at time t; the last one is the enzymatic reaction. Hypothesis b authorizes us to neglect the two first causes (as well as the effect of the sectorial geometry of the cell). We will consider thus that the differential distribution $D_t^{t+\tau}(x)$ is due only to the creation of new molecules of product P by the enzyme between times t and $t+\tau$

In these conditions it is easy to show that the position of the maximum of $D_t^{t+\tau}(x)$ coincides with $\hat{x}\left[t+(\tau/2\right.$, that is to say with the position of the center of the enzyme bed in the middle of the interval of time that separates the two successive photographs : actually the bed is displaced and in all points x the concentration of the enzyme varies with the time; be it then $M_t^{t+\tau}(x)$ the distribution of the average concentration of enzyme between t and $t+\tau$. By another way, if the two conditions enunciated above are respected, the quantity of product P created at a point x at time t is proportional to $E_t(x)$. From all which precedes it evidently results that the total quantity of product created at a point x at times t and $t+\tau$ is proportional to the average concentration of enzyme in x during the same time, which is written:

$$(1) \qquad D_t^{t+\tau}(x) = a\, M_t^{t+\tau}(x), \quad (a = C^{te}).$$

But the distributions $E_t(x)$ not being known, it is not possible to calculate the $M_t^{t+\tau}(x)$; one knows however taken into account properties of symetry and of invariance of form of curves $E(x)$, that distribution $M_t^{t+\tau}(x)$ (fig. 1 e) presents, it too, a maximum in relationship to which it is symetrical and whose position coincides with $\hat{x}\left[t+(\tau/2\right]$, position of the maximum of $E_{t+(\tau/2)}(x)$, that is to say from the center of the bed to the middle of the interval of time τ. There proceeds from it, since the distributions $M(x)$ and $D(x)$ are related [eq. (1)] the following result, already mentioned higher up: the position of the maximum of the

differential distribution $D_{t}^{t+\tau}(x)$ is that of the center of the enzyme bed at time $t+(\tau/2)$.

EXPERIMENTAL RESULTS. — We are giving here an example taken from the study of a commercial preparation (Sigma product) of glucose-6-phosphate dehydrogenation; the bed contained 1 µg of proteins of which only 0.02 µg of enzyme was studied. Several centrifugations were carried out, some at 60,000 t/mn, others at 42,000. In applying the approximate method that we have just presented, we have obtained the following values : $s_{20,w} = 6.30 \pm 0.05$ S to 60,000 t/mn and $s^{*}_{20,w} = 6.32 \pm 0.06$S to 42,000. The detail of these experiments as well as the comparison of these values to those that were determined by other authors will be reported elsewhere.

DISCUSSION. — The approximation utilized here is a priori very open to criticism, the hypotheses adopted being able to appear inacceptable. However very preliminary results for the obtainment of which we have taken into account the diffusion and the sedimentation of the observable product P (here TPNH) as well as the diffusion of the enzyme, seem to indicate, at least for the enzymatic system studied here, that the introduction of simplifier hypotheses involves only a systematic error in the order of per cent. It is besides remarkable that the values of s obtained after centrifugation at two indicated angular speeds, be practically equal: at 42,000 t/mn, the bed sediments two times more slowly than at 60,000, while phenomenons of diffusion (whose omission in our analysis is the essential cause of the systematic error mentioned above) does not depend on the angular speed; to change this latter one comes back again thus to make the relative importance of phenomenons of sedimentation and of diffusion vary; as we are neglecting these latter ones, to change the angular speed comes back thus to cause the relative importance of the preceding error to vary, that is to say, to make s^{*} vary. The absence of an observable variation of s^{*} in the preceding experiments confirms the small value of the indicated error (5).

(*) Seance of February 8, 1965.

(1) This work benefited from the help of the General Delegation to Scientific and Technical Research, and from the Commissariat of Atomic Energy.

(2) R. COHEN, Comptes rendus, 256, 1963, p. 3513.

(3) J. VINOGRAD, R. BRUNNER, R. KENT, and J. WEIGLE, Proc. Nat. Acad. Sc. U.S., 49, 1963, p. 902.

(4) By "differential distribution" one must understand distribution of the difference finie $P_{t+\tau}(x) - P_{t}(x)$.

(5) The collaboration with Miss Annick Guillemont was priceless to us all during this work.

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