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Cellular Esterase Activity: Estimation by Fluorescein Diacetate Hydrolysis

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Quantitative estimation of cellular esterase activity makes it possible to differentiate cells and cell subpopulations. The fluorescence of fluorescein obtained by hydrolysis from fluorescein diacetate is detected and used for measuring the esterase activity. The method described is suitable for the quantitative determination of esterase activity in human lymphocytes.

Estimation of esterase activity in human lymphoid cells has given considerable aid in seperating healthy and sick cells [2, 3, 7, 8]. As substrate, alpha-naphthylesters are mostly used. At a nearly neutral pH after 60—90 min incubation, these release naphthyl which on the addition of diazonium salt develops into an insoluble bright azo stain.

In immunopathological processes, in the essential periods of immune maturation, it is important to differentiate between T, B and O cells [5]. The B cells being esterase negative and the T cells esterase positive, their histochemical separation becomes possible.

Starting from Sontag's results [12, 13] we have attempted to work out a method which would allow the separation of living cells in suspension.

Fluorescein diacetate (FDA) (Fig. 1) as a fluorogenous substrate can be used for characterizing cellular functions [10], for measuring enzyme activity in order to estimate the condition of metabolism as well as for investigating membrane structures, and for differentiating among various morphologically similar cells [11, 12].

The hydrolysis of FDA, just as the enzyme reaction, occurs according to the equation

$$\text{FDA} \longrightarrow \text{FA} + \text{A} \longrightarrow \text{F} \quad 2\text{A}$$

Firs, fluorescein acetate (FA), then fluorescein (F) develops. At alkaline pH the reaction takes place spontaneously, while in the cells it is the result of the esterase effect [13]. The FDA as an apolar material is soluble in acetone and can be given in solution to the examined cells. To cause hydrolysis intracellularly, the FDA must penetrate across the cytoplasmic membrane into the cells; this occurs easily. Then, by means of different measuring methods we may get data for membrane permeability,

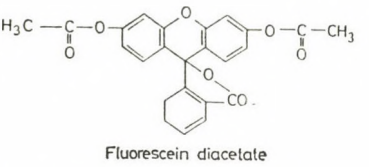

FIG. 1. Chemical structure of fluorescein diacetate

and so the effect of materials which change the permeability, such as for example gramicidin D, can be observed [11]. The active transport of FDA has been assumed to explain its high intracellular concentration which may amount to a hundredfold of that ensured by diffusion.

Intracellularly the FDA is split by esterase, so that the fluorescence of F is a measure of the esterase activity, of which about 95% is of cytoplasmic origin [13]. The F is accumulated in the cell and displays fluorochromasia [10], while the part found extracellularly is due to membrane bound esterase activity, or can be the result of F released from the cytoplasm. Thus, FDA hydrolysis indicates cellular metabolism and if the membrane permeability, the esterase activity and the distribution of F are summed up in the intensity of fluorescence, we obtain a simple method for measuring the cellular metabolism of different cells. In the present case we applied the method for the estimation of esterase activity in human lymphocytes.

MATERIAL AND METHOD

1. *Lymphocyte suspension* [1, 4, 5, 6, 9] Peripheral venous blood was heparinized and sedimented on Ficoll-Uromiro gradient, then washed twice with isotonic phosphate buffer pH 7.4 (PBS) to a cell concentration of 2×10^6/ml. The PBS contained 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.8 g glucose per litre.

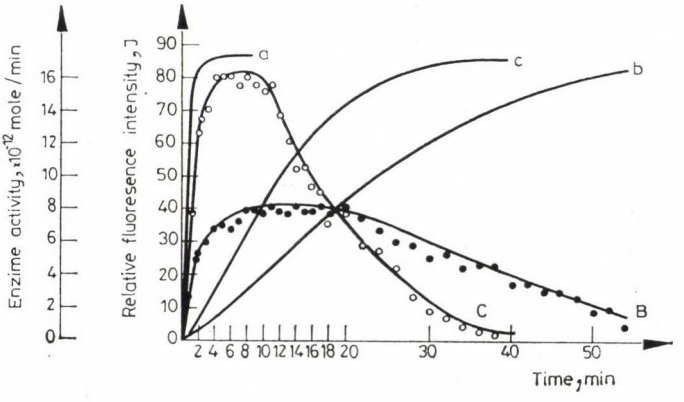

FIG. 2. Fluorescein excitation *(a)* and emission *(b)* curves

2. *Erythrocyte suspension*

Red blood cells washed twice in PBS were diluted to a concentration of 2×10^6 cell/ml.

3. *Fluorescein diacetate* (FDA-Sigma)

A 10^{-4} M stock solution in acetone was prepared and stored in the dark at + 4°C until use.

4. *Estimation*

Fluorescence was measured in a Hitachi MPF-4 spectrophotometer in cylindrical quartz cuvettes of 300 μl capacity (Fig. 2). The spectrum of excitation was estimated at 516 nm; the I maximum was found at 490 nm. The emission spectrum was detected at 490 nm, and the I maximum was found at 516 nm. On the basis of these results, fluorescence intensity was measured at 490 nm (excitation) and 516 nm (emission). Alkaline hydrolysis in PBS of pH 10.2 occurred in every series. The aim was to find the F intensity originating from the split FDA and so to compare the cell populations. The measurements were done at 37°C. Into a 300 μl cuvette was given 297 μl cell suspension with PBS pH 10.2, and at the beginning of registration 3 μl stock solution of FDA was added corresponding to 3×10^{-10} or 10^{-6} M concentration.

Results

The intensity of alkaline hydrolysis and the fluorescence of the cell suspension was obtained in the function of time in relative units (Fig. 3). Hydrolysis took place rapidly and the concentration of F formed in this way did not change, indicating that all the FDA was split (Fig. 3. curve *a*). The fluorescence intensity of 2×10^6 cell/ml lymphocyte suspension is shown in Fig. 3 by curve *b* which can be considered a summation curve of both intra and extracellular F fluorescence. The characteristic feature of the curve is that it becomes linear at a certain height. The differential quotient belonging to the individual points of time is the measure of esterase activity, and its derivative characterizes the temporal changes, as shown by function *B*. Its maximum coincides with the linear interval of the original graph and expresses the peak esterase activity in moles/min (7.94×10^{-12}

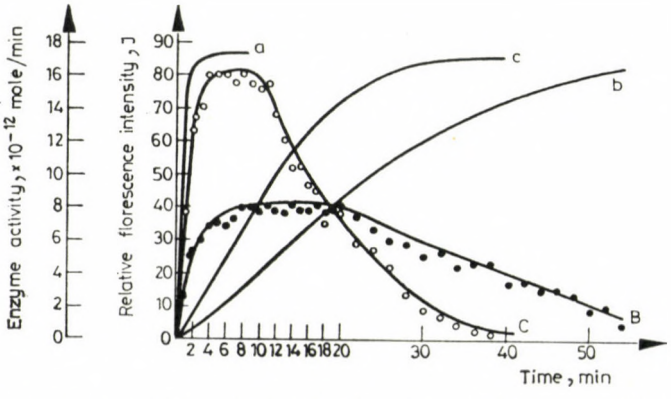

Fig. 3. Relative fluorescence intensity in the function of time *a:* alkaline hydrolysis; *b:* 2×10^6 cell/ml; *c:* 4×10^6 cell/ml lymphocyte concentration. Temporal change of enzyme activity at lymphocyte concentrations of *B:* 2×10^6 cell/ml; and *C:* 4×10^6 cell/ml

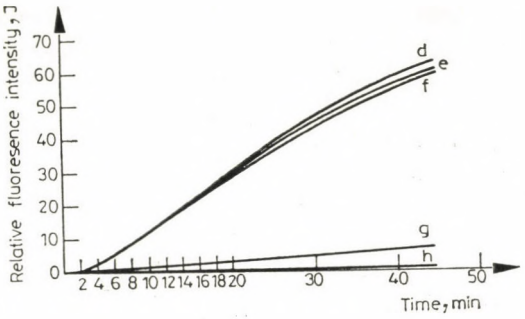

FIG. 4. Relative fluorescence intensity in the case of identical lymphocyte populations; *d, e, f* at 2×10^6 cell/ml concentration; *g:* 2×10^6 red cell/ml lymphocyte; *h:* PBS pH 7.4

moles/min). Curve *c* in Fig. 3 stands for a 4×10^6 cell/ml lymphocyte concentration, and esterase activity is shown by the derivation of function *C*. Its maximum amounts to $15.98 \times \times 10^{-12}$ moles/min, double the previous value.

On the basis of these results it is acceptable that the direction-tangent of the linear section of the curve displaying the increase of fluorescence intensity shows the peak esterase activity (EA).

$$EA = \frac{I_2 - I_1}{t_2 - t_1} \times \frac{3 \times 10^{-10}}{85} \text{ moles/min}$$

The functions *d, e* and *f* in Fig.4 show the lymphocyte EA obtained in different measurements of the same population; the values are $6.22 \times$, $5.94 \times$, and 5.92×10^{-12} moles/min, respectively. Curve *g* shows the activity estimated in a red blood cell suspension of 2×10^6 cell/ml concentration; the value is 7.2×10^{-13} moles/min. The F content of PBS of pH 7.4 is seen from function *h;* it is practically non-perceptible.

Discussion

The method described is suitable for estimating esterase activity in different cells, lymphocyte populations and subpopulations. In the case of human T and B cells it can be used not only for their separation but for quantitative esterase determination as well, and it can be applied for controlling the purity of certain specimens. With the help of some methodological additions it may yield data on enzyme localization and membrane function, too.

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