OBSERVATIONS ON THE ACTIVITY OF ENZYMES AFTER FILTRATION ON (AND THROUGH) A NITROCELLULOSE MEMBRANE

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The ability of nitrocellulose filters to retain specific proteins has been observed and applied to work on codon recognition by Nirenberg and Leder (1964). Applications of this technique have been used for studies on ribosome binding sites in TYMV (Dahlberg and Haselkorn, 1966), and on the thermal stability of the messenger-aminoacyl tRNA-ribosome complex (McLaughlin et al., 1966). It has also been applied to enzyme studies : The two reported examples, RNA polymerase (Jones and Berg, 1966 ; Anthony et al., 1966) and aminoacid synthetase (Yarus and Berg, 1967), show quite a different behavior. In the absence of its DNA template, RNA polymerase is not retained on the filter, not even 1 or 2 μg of proteins; in contrast, with aminoacid synthetase, as much as 100 μg is quantitatively retained. The effect of filtration through or adsorption on nitrocellulose filter on the activity of the enzyme is not clear. The adsorbed aminoacid synthetase seems to be inactivated (Yarus and Berg, 1967) while RNA polymerase appears to have some activity, wether in the filtrate (Jones and Berg, 1966) or adsorbed on the filter (Anthony et al., 1966) in the enzyme-DNA complex state. However, no quantitative data are available as to whether some inactivation occurs in the case of RNA polymerase. There is a possibility that the enzyme filtered through nitrocellulose filters might be damaged or somewhat inactivated, and this is even more likely to occur with enzymes adsorbed on the filter.

However, if these points were clarified, the method could be most useful. In the course of our studies on the mechanism of polynucleotide phosphorylase we also used this technique and checked the effect, on the enzymatic activity, of filtration on and through nitrocellulose membranes. We thought it useful to present some observations we made on the behavior of two enzymes : one,

L-lacticodehydrogenase, looses its activity when adsorbed on the filter but entirely retains its activity in the filtrate; the second, polynucleotide phosphorylase, remains active when adsorbed on a nitrocellulose membrane or when released from it.

The enzyme used in the first case was cristallyzed L-lacticodehydrogenase isolated from yeast (Baudras, 1965). This enzyme was chosen for its purity and the facility of spectrophotometric measures for both quantity and activity. The experiments were carried out as follows :

A series of 4 samples of 3 ml each, containing L-lacticodehydrogenase at a concentration of 302.6 µg/ml were filtered at 4°C on a "millipore" membrane (0.45 mµ, AH Ø20 mm) with slight suction. The first sample was filtered one one membrane, the second successively on two separate membranes, the third on three, and so on, in such a way that the enzyme retained and passed could be quantatively followed as well as the activity remaining in the filtrate. Figure 1 illustrates the almost perfect agreement between quantities and activities passed through the filters. Under experimental conditions, the first slope of the curve seems to indicate a proportional relationship between the amount of enzyme retained and the surface of the membrane. The capacity for retaining L-lacticodehydrogenase is estimated at 230 µg/membrane of Ø20 mm. This value is much lower than for ribosomes which was estimated to be 1200 µg (Nirenberg and Leder, 1964). The filtered enzyme does not seem to be altered, since the molar activity (activity per heme concentration) is the same as that of the unfiltered initial enzyme.

In contrast, L-lacticodehydrogenase retained on the filter does not show any activity. This is demonstrated by direct incubation of filters containing 135 to 230 µg of enzyme in the usual reaction mixture. No reaction can be detected (less than 0.2% of the initial activity). Not only is the enzyme inactivated when it is bound to the filter, but the retention seems irreversible. No significant enzymatic activity could be eluted from the filter by soaking the membranes in either 0.1 M phosphate buffer, pH 7.2, containing 50 mM D-L-lactate, or the same phosphate buffer, containing 20% saturated $(NH_4)_2SO_4$. The elution was followed at 10, 30, and 60 minutes, and at 17 hours.

The second case, where enzyme bound to "millipore" is still active, is illustrated by polynucleotide phosphorylase. When

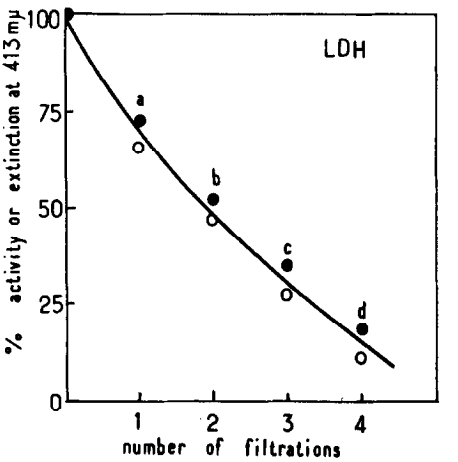

Figure 1

Activity of L-lacticodehydrogenase after successive filtrations through a nitrocellulose membrane

L-lacticodehydrogenase was in a phosphate buffer, 0.1 M, pH 7.2, containing 20 μM EDTA. The concentration, determined at 413 mμ with E = 164 $mM^{-1}cm^{-1}$, was 302.6 μg/ml. Four samples of 3 ml each were filtered : a) on one filter; b) successively on two separate filters; c) successively on three filters; d) successively on four filters.
The activities of the filtrate were followed by the rate of reduction of potassium ferricyanide in a reaction mixture containing : phosphate buffer, pH 7.3, 66 mM; EDTA, 13 μM; D-L-lactate, 16.6 mM; ferricyanide, 0.66 mM.
The procedure and calculation of activity are described elsewhere (Baudras, 1966).
The molar activity is defined as the number of electrons transferred per second per mole of enzyme heme. The initial activity was 252 sec^{-1}.

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a solution of polynucleotide phosphorylase (about 50% pure), 0.2 ml in Tris 0.01 M, pH 8, at a concentration between 1 μg/ml and 50 μg/ml was filtered with slight suction on a "millipore" membrane (0.45 mμ, AH ∅ 18 mm), no detectable activity was found in the filtrate*. The adsorption of polynucleotide phosphorylase on the nitrocellulose membrane does not require divalent ions nor high ionic strength. With increased suction, 3-5% of the activity was found in the filtrate. The activity was checked by ADP-$^{32}PO_4$

* It has been pointed out by Yarus and Berg (1967) that reproducibility of the rentention depends mostly on the membranes used. In our experiments we did not notice maked differences with various batches of "Millipore" (AH 0.45 mμ, during 1965-1967).

exchange, phosphorolysis of poly A, and polymerization of ADP; the first two reactions being the most sensitive as higher specific radioactivy could be obtained. Successive washing with Tris buffer (with or without KCl up to 0.5M) does not increase enzymatic activity in the filtrate which indicates that no active enzyme has passed through.

In contrast, if the washed membrane is incubated in a reaction mixture for either the exchange reaction or the polymerization of ADP, the incorporation of ^{32}P into ADP or the liberation of orthophosphate, according to the reaction chosen, can be followed. The polymerization by enzyme retained on the filter shows a more or less pronounced lag phase which is followed by a reaction proceeding at a rate of about 30% of that of the free enzyme (fig.2)**. The lag phase is dependent on the amount of enzyme bound, and has been observed to be as long as 30 minutes with very small amounts of enzyme. Moreover, the lag phase occurs, even in the presence of high concentrations of oligonucleotides 10^{-4} M of $(pA)_3$, while a concentration of 10^{-6} M normally overcomes the lag of the same preparation of enzyme in solution. The poly A synthesized under these conditions has a high molecular weight, as judged by Sephadex gel filtration.

The question arises as to whether the reaction observed is catalyzed by bound enzyme or by enzyme released into the incubation mixture. The answer is twofold :

Under conditions when the enzyme polymerizes, as shown in fig.2, there is only negligible enzymatic activity released into the incubation mixture; this is illustrated by withdrawing samples at given times, one for phosphate determination and two others for further incubation to determine released enzymatic activity. The comparison of the two slopes in fig.2 indicates that the observed enzymatic activity, when membrane containing enzyme is put into the reaction mixture, cannot be ascribed to the release of the enzyme from the millipore.

** This percentage is the same whether unwashed or abundantly washed membranes are used. It seems, therefore, that this lower rate probably results from a low mobility of the enzyme and a low rate of diffusion of the substrates rather than an inactivation by the detergent present in the filters (Cahn, 1967).

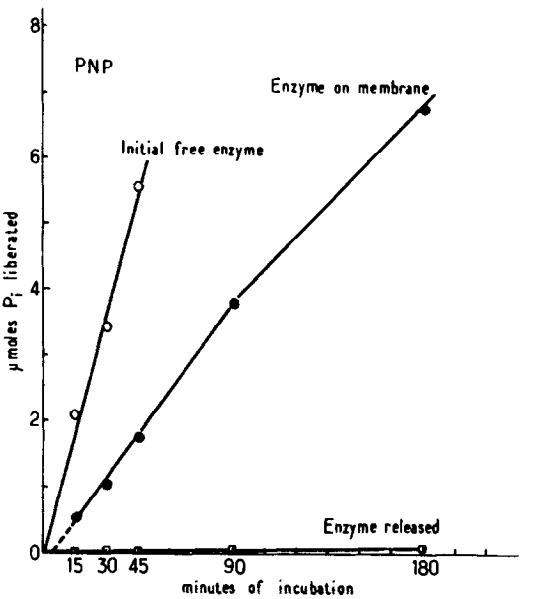

Figure 2

Activity of polynucleotide phosphorylase retained on a nitrocellulose membrane

7 units (3 µg) of purified polynucleotide phosphorylase in 200 µl Tris buffer 10^{-2} mM, pH 8, were filtered onto a nitrocellulose membrane. The filter was then incubated in 3 ml of a reaction mixture containing : Tris, pH 8, 100 mM; ADP, 10 mM; $MgCl_2$, 5 mM; $(pA)_3$, 0.1 mM (in nucleotides); EDTA, 0.1 mM. Incubation temperature 37°C. At indicated times, three identical samples of 100 µl each were withdrawn : one for orthophosphate determination (rate of polymerization by enzyme bound on the filter) ; the two others, after additional incubation respectively for 30 and 60 min were used to determined the rate of polymerization by the enzyme released in the incubation mixture. Only the 60 min incubation with released enzyme is illustrated. The initial free enzyme activity was estimated with 0.35 units of enzyme in the same reaction mixture. For the purpose of comparison the activity was plotted for 7 units of enzyme.

It is important to note that the release of the enzyme occurs only under reaction conditions, i.e. in the presence of all the components required for the polymerization (or for either of the two other reactions). No release could be observed by soaking in the same buffer but in the absence of either ADP or Mg^{++}. Furthermore, the release of the enzyme is very slow. Other experiments, followed by the exchange reaction, indicate that after a lag phase the release is linear with time for one to three hours

and then tends to a plateau asymptotically. Thus, less than 5% of the retained activity is released during the first hour, and about 12-18% after 24 hours incubation. After this long incubation the enzyme retained on the membrane shows about 80-85% of the initial bound activity. The release might depend on the localization of the enzyme inside the pores. Hence, a membrane with retained enzyme can be used numerous times for the synthesis of polymers (fig.3). The decrease of the polymerization rate, beside the very small release of bound enzyme, is solely due to inactivation by time since the maximum rate was the same for a membrane used eleven times over a week and for a control stored at 4°C during the same period, i.e. 40-47% of the ADP present in the mixture was polymerized into poly A. The half-life of the enzyme bound onto filter is about 1 week at 4°C and two months at -20°C.

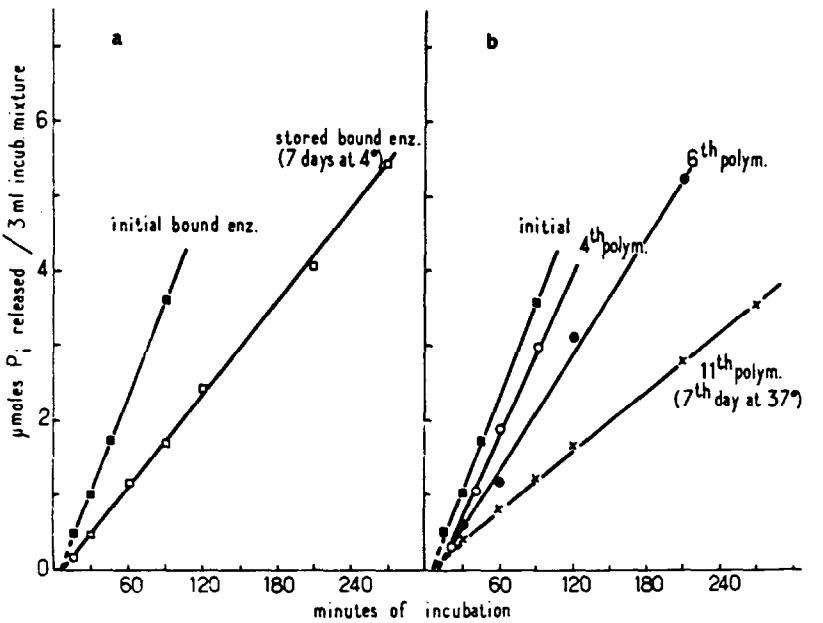

Figure 3

Continuous synthesis of poly A with a membrane containing polynucleotide phosphorylase

Two filters with bound enzyme were prepared as in fig.2. After a first polymerization (as in fig.2) one filter is stored at 4°C. The other is used continuously for the synthesis of poly A for a period of 7 days, being incubated for 7 hours and 17 hours alternately at 37° in 3 ml of reaction mixture each time. The polymer formed at the end of each incubation corresponds to 4-4.65 µmoles/ml. The rate of polymerization was followed at the 4th, 6th and 11th incubation (b). The filter stored at 4°C was used as a control of the stability of the enzymatic activity with time (a).

A second evidence demonstrates that the bound enzyme is active. One can pass a reaction mixture for the polymerization of ADP through a membrane which has retained the enzyme. The filtrate is collected directly into either an alcohol-salt mixture or 5% perchloric acid. The product of the reaction can be seen as a poly A precipitate. Under these conditions it is unlikely that the enzyme could be released into the incubation mixture for synthesizing polynucleotides from ADP.

To summarize, L-lacticodehydrogenase is not inactivated by passing through a millipore filter, and polynucleotide phosphorylase keeps its catalytic properties in a bound state on such a filter. Not all of the polynucleotide phosphorylase is irreversibly bound. The absolute requirement for reaction conditions in order for the enzyme to be released from the membrane seems to indicate that this release is not due to the weakening of the interaction between the enzyme and nitrocellulose, since no elution occurs when any one of the substrate is missing (ADP or Mg). This phenomenon suggests that the conformational state of the enzyme is changed under catalyzing conditions, in such a way that the enzyme can then be freed from the membrane.

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