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MANNER OF ACTION OF LACTIC DEHYDROGENASE IN CONNECTION WITH FLAVINE AND CYTOCHROME SYSTEMS

/Following is a translation of a scientific memo, in the French-language, by Francoise Labeyrie and Piotr P. Slonimski of the Institut de Biologie Physico-Chimique, Service de Biophysique (Physico-Chemical Biology Institute, Biophysics Service), Paris, and the Laboratoire de Genetique Physiologique du C.N.R.S. (Laboratory of Physiological Genetics of the National Scientific Research Center), Gif-sur-Yvette, 66 pages./

The oxidation-reduction equilibrium between lactate and pyruvate is established at a potential of -180 mv /17 /numerals in brackets refer to similarly numbered items in Bibliography/. Therefore, in the scale of oxidation-reductions, it is located among the more negative (-320 mv) NAD-NADH systems, on the same level as the flavines (-180 mv) and considerably below the cytochromes C (+250 mv). Since the classic L-lactic dehydrogenases in the muscle, serum or in bacteria are enzymes linked very closely to the NAD-NADH system, they can, under physiological conditions, only make the reaction develop in the direction of reduction of the pyruvate by the NADH. These enzymes control, therefore, the production of lactate and the reoxidation of the reduced coenzyme; therefore, in reality, they are "pyruvate reductases".

In order to oxidize lactate, enzymes were needed to catalyze the exchange of electrons between this substrate and systems with an equal or higher potential, that is to say, "lactic dehydrogenases", properly speaking. There are three different enzymes of this type that are synthetized by the same yeast cell; all three are flavoenzymes. None of them can exchange electrons with the NAD-NADH system. They

are, as we shall see, more or less specific with regard to the system coupled to the lactate.

It has been rather surprising to observe that they do not all have as substrate the same stereoisomer of lactate; although they all produce pyruvate. One is specific of L-lactate [2,3]; it is the classic L-lactic dehydrogenase of baking yeast (L-LDH) or cytochrome B_2 [5,6,3,7]. A second one, that we demonstrated and then characterized in a yeast cultivated in anaerobiosis, showed itself to be specific of D-lactate (D-LDH) [8,9]. A third, also specific of the isomer D but present, this time, in yeasts cultivated in aerobiosis, was detected and studied later (D-LCR) [10, 11, 12].

These three enzymes are also differentiated, with regard to their catalytic properties, by the acceptors to which they can give electrons from the lactate. The dehydrogenase present in the anaerobic yeast (D-LDH) accepts all the usual electro-active pigments, including the flavines [17], excluding cytochrome C [13, 8]. The two lactic dehydrogenases present in the aerobic yeasts both accept cytochrome C, however, one is closely linked to this single acceptor, it is a D-lactic cytochrome C reductase (D-LCR), while the other, the L-LDH equally exchanges lactate electrons with all the customary acceptors, both monoelectronic and dielectronic. Figure 1 diagramizes all these properties.

As we shall se below, there is, with regard to their structure, a certain analogy between the two specific enzymes of D-lactate. On the other hand, L-LDH appears to be clearly more different structurally. The same does not apply to the regulation of their biosyntheses. L LDH and D-LCR appear to be much closer, since they are both inhibited by fermentation metabolism and are stimulated by oxigen and lactate. The opposite is true of D-LDH which is stimulated by fermentation metabolism and inhibited by respiration [14 to 20].

Most of our results were obtained since 1956 with the colaboration of two laboratories. The following participated, at different phases: Miss C.M. Bacq, Mr. A. Baudras, Miss A. Curdel, Mr. P. Galzy, Miss O. Groudinsky, Mr. A. Isomotor, Mr. M. Iwatsubo, Mrs. Y. Jacquot-Armand, Mr. R. Kattermann, Mrs. B. B. Lippincott, Miss L. Naslin, Mrs. M. Somlon, Miss E. Stachiewicz, Mr. W. Tysarowski.

I. L-Lactic Dehydrogenase or Cytochrome B_2

This enzyme, discovered by Bernheim /4/, has become well-known since the studies by Bach, Dixon and Zerfas /5/ who demonstrated its hemoproteinic nature and who called it cytochrome B_2. It was crystallized as a DNA-protein by Appleby and Morton in 1954 /6/. These authors demonstrated that it not only has a heme, the protoheme, but also a flavine (FMN) in the proportion of one heme and one flavine per 82,000 grams /6, 21, 22/. The molecular unit in solution, as all the hydrodynamic methods show, is in the neighborhood of 183,000 /22/. Therefore it has two hemes and two flavines. As no dissociation of it into sub-units has ever been observed, it is not known whether the 183,000 unit is a homodimer formed by two identical sub-units, or a heterodimer or even a non-dissociable molecule.

With regard to the DNA, it has been observed that it has no bearing at all on the known catalytic properties of the enzyme /23/. The number of fixed molecules on the 183,000 unit is not known; in fact, the different methods utilized to determine its molecular weight have given values varying from 15,000 to 120,000 /23 to 25/.

This lactic dehydrogenase with multiple heads raises very special problems concerning the role of each one. It is necessary to known, in effect, if the prosthetic groups all share in the transfer of electrons from the lactate to the acceptor and in what order. Finally, it is necessary to know that their mutual relationships are. We have, therefore, sought to obtain information on the following points: (a) respective roles of the heme and of the flavine, (b) interactions between the heme-heme and heme-flavine prosthetic groups, (c) protein-heme interactions.

For that purpose, we studied two derivatives of L-lactic dehydrogenase: an apoenzyme obtained by separation of the flavine and a hemoproteinic fragment obtained by tryptic hydrolysis.

1. Apohemoprotein of L-Lactic Dehydrogenase

Preliminary notes on studies made independently by Morton, in Australia /26/ and in our laboratory by A. Baudras /27/ were published a few months apart. Both them, by applying the classic method of Warburg and Christian, prepared a hematinic apoenzyme by separation of the FMN: precipitation

by ammonium sulfate in an acid medium leaves the flavine in solution, in fact; the dissolved residue is a hemoprotein. As Morton demonstrated, the proteinic part itself did not undergo dissociation into sub-units: the hemoprotein that is formed is homogeneous on ultracentrifugation and it forms sediment just as fast as the native enzyme. The value S_{20}, 7.94 S /26/, shows that, like the native enzyme, it has two hemes per molecular unit of about 183,000. Our preparation has the same characteristics (S_{20} = 7.6 S). The apohemoenzyme (apo L-LDH), prepared in this way, still has a weak lactic dehydrogenasic activity. In fact, it is contaminated by FMN which can be eliminated by passage over Sephadex. The original lactic dehydrogenasic activity can be restored in great part by saturating the apohemoenzyme with FMN, as Baudras /27, 28/ has shown. It was thus possible to achieve a specific molecular activity of 12,000 min^{-1} (for one heme) by starting with a freshly crystallized enzyme with a specific activity of 18,000 min^{-1}. The enzyme thus reconstituted could be crystallized like the native enzyme by Morton and Shepley's /29/ method. The specific activity then attains the value of 14,000 min^{-1} for one heme /28/.

An analysis of the saturation of the apohemoenzyme with FMN (Fig. 2) gives a value of K_{FMN} close to 0.1 μM. The graphic representation V_m/v as a function of 1/(FMN) gives a straight line in the area studied which shows that a single FMN intervenes per active site and that the two FMN of the "two hemes" unit have an identical behavior, although they both participate independently in the enzymatic activity.

Reactivation can be obtained, specifically, only with the single FMN. The pure FAD, free from FMN, like the one that could be prepared several times by dissociation of the crystallized D-amino acid oxidase does not produce any activity. The same is true of riboflavin /27, 28/.

Therefore, it was possible for this flavoenzyme, as for most of those known up to now, to dissociate the flavine and to reassociate it by re-establishing almost all the enzymatic activity. However, for a long time it was generally admitted that the dissociation of flavine and L-LDH is not reversible. It was assumed that fixation brought into play a very fragile and auto-oxidizable -SH /30/. In fact, the apo-L-LDH is very stable, more so than the enzyme itself. When it is kept in a concentrated solution, frozen at -10° for 24 hours, it loses only 20% of its reactivation capability.

Baudras /28/ L-LDH reconstituted by saturating apo-L-LDH with FMN is a little different from the native enzyme.

These differences apply, basically, to a decrease in the apparent affinity for FMN (1/K) and an increase in the values K_M and K_i in relation, respectively, to the L-lactate and to the oxalate, a competitive inhibitor (Table 1). Iwatsubo and Di Franco /317 showed, on the other hand, and we shall come back to this point, that the dissociation of the reconstituted flavochemoproteinic complex is more sensitive to salts than that of the native enzyme. All these changes are probably connected with a modification in the tertiary structure of the protein under the influence of the acidity of the medium, during the preparation of the apoenzyme.

An accurate study of the combination of FMN with apo-L-LDH was made by means of a fluorometric method similar to the one first utilized by Theorell and collaborators for the old yellow enzyme and for dehydrogenase alcohol. Iwatsubo and Di Franco /317 analyzed in this way the speed and equilibrium of the association of FMN with apo-L-LDH as well as the dissociation, by dilution of this complex (Fig. 3). The combination isotherm (Fig. 4) is linear in the zone studied. This indicates that two sites of the "two hemes" molecular unit fix FMN independently with the same affinity (K, constant of dissociation = 6.10^{-9}M, 23° in acetate buffer). This result, compared with the one obtained by the kinetic methods (Fig. 2) seems to show that the two flavines of the "two hemes" unit have an identical behavior and have no interaction. That is an indication that the enzyme may really be a homodimer.

Like what had been observed by Theorell for the old yellow enzyme, the fixation of the flavine is very sensative to salts. Certain ions act in a specific manner: phosphate and pyrophosphate competitively inhibit the FMN fixation. An analysis of the experimental data (Fig. 5) gives the value of the K_i constants relative to these salts. They are equal, respectively, to 14 mM and to 8mM. Compared with the value 0.12 μM relative to FMN, in the same medium, they indicate that the phosphoryl group of FMN participates only very little in the total energy of the fixation.

An effect of the salts favoring combination (Fig. 6) was observed with very weak ionic forces. If it really is a question of a non-specific effect, that would indicate the existence of a force of electrostatic repulsion between the flavine and the protein, which is attenuated by the presence of charges.

The carboxylic anions act in a more interesting way by strengthening the flavine-protein connection (Fig. 6 and

Table 2). This is especially important for oxalate, a competitive inhibitor, and for L-lactate itself. Nevertheless, since the flavine carried by the enzyme is reduced in the presence of L-lactate, the interpretation of the lactate effect is ambiguous. It would be interesting to see if the action of the carboxylates is due to a proximity to the fixation sites of the lactate and of the flavine or to a general modification of the tertiary structure.

A comparative study of the properties of the heme carried by this apohemoprotein and by the L-LDH will give indications on the eventual influence exerted by the flavine on the heme. Before examining the results acquired on this subject, we are going to speak of another hemoprotein derived from the L-LDH by hydrolytic division.

2. Hemoproteinic Nucleus of L-Lactic Dehydrogenase /32/

L-LDH is easily hydrolyzed by trypsin, without the need for previous denaturation. Hydrolysis evolves until more than 55 peptidic bonds by "two hemes" unit (Fig. 7). The exact number, in effect, is not given by the potentiometric method utilized to follow the reaction. The factor of proportionality between the number of soda equivalents added to keep the pH constant at 8.5 and the number of peptidic bonds broken depends on the pH /32/. Under our conditions, it is of the order of 0.5 to 1. The maximum number of bonds theoretically hydrolyzable by this protease, the sum of the arginine and lysine residuess is 134 /21/ for the "two hemes" unit.

The fractioning of such a hydrolysate on a molecular sieve (Sephadex G 100) leads to the separation of a single hemoproteinic fraction that we shall call "hemoproteinic nucleus B_2".

This fraction contains all the heme of the original L-LDH. It is homogeneous with regard to the molecular weight. In effect, the diagrams of elution on Sephadex G 75 and G 100 only present a symetrical peak (Fig. 8). The same is true of the analytical ultracentrifugation diagrams.

The molecular weight was estimated, according to the elution volumes on a column of Sephadex G 75 gauged with myoglobin (PM ≅ 17,000) and the cytochrome C from horse heart (PM ≅ 13,000), by Iwatsubo and Curdel's /34/ method. These

authors demonstrated that there is a precise univocal relation between the elution volumes and the molecular weights. Other authors have independently proved the validity of this method /35/. The results (Fig. 8) indicate that the molecular weight is slightly lower than that of the cytochrome C and can be evaluated at about 11,000 to 12,000.

The heme/protein ratios were determined for several preparations of this type, made under quite varied conditions of temperature, of saline medium and of concentration in L-LDH. The preparations are, with one exception, generally those eluted from the G 100, in the region of the maximum of the 413 mμ peak, without additional purification. The values found (Table 3) are little different and give an average of one heme for 16,400 grams. The purified fraction give one heme for 13,400 grams. This estimate is the most valid one. The reproducibility of the results, from one preparation to another, shows that the hemoproteinic nucleus B_2 is not a fleeting and indeterminate compound. It represents an insensitive proteinic fragment, or at least little sensitive to tryptic hydrolysis. When the hydrolysis is less extended, small amounts of non-degraded L-LDH and hemoproteins with intermediary molecular weights are found. The peak measured by extinction at 413 mμ on the elution diagrams on G 100 is then clearly dissymmetrical.

Since it is given that the molecular unit of L-LDH has two hemes, the problem is raised to know if these two hemes are carried, after tryptic hydrolysis, by the same proteinic unit, if they are carried by two distinct but identical units, or by two different units. We are now able to reply that the two hemes are fixed on two distinct units, of the same molecular weight. In effect, we found very close values for the molecular mass (~ 12,000) and the proteinic mass of the hemoproteinic nucleus B_2 carrying one heme (~ 13,400). On the other hand, studies on Sephadex have shown that all the heme is carried by a single homogeneous fraction with regard to the molecular weight. If the homogeneity is confirmed in subsequent studies with other types of fractionings, the fact that the two hemes of native L-LDH, around 180,000, are borne by two identical structures will be a serious argument in favor of the idea that the enzyme is a homodimer.

One of the important problems concerning this L-LDH is the reciprical position of the prosthetic groups. We made an attempt to find out if the flavine can be fixed in proximity to the heme on the hemoproteinic nucleus B_2. For that purpose, we attempted to see if the latter could produce an

extinction of the fluorescence of FMN by combination. The results were negative. It may then be thought that the fixation sites of the heme and of the flavine are remote, from the primary structure point of view. It is possible, nevertheless, that the fixation of the flavine brings into play several close points in the tertiary structure but remote on the polypeptidic chain, so that a single one of these isolated points could not fix it with sufficient affinity for it to be detectable by means of this method.

A small hemoprotein, presenting a certain spectral analogy with the one that we have just described, was extracted from a yeast that was autolyzed for eight days by Yamashita and collaborators [38, 39], who obtained it in a crystalline form. Its molecular weight determined by analytic ultracentrifugation is 22,000. The relations between this hemoprotein with native L-LDH are not known. It could result, as Morton [40] suggests, from a degradation of the L-LDH during the autolysis that leads to its preparation.

3. Comparison of the Properties of the Heme Borne By the L-LDH, the Apo-L-LDH and the Hemoproteinic Nucleus B_2

It is known that the properties of the heme vary considerably from one hemoprotein to another. The factors that determine this variability are: (a) the mode of fixation (number and nature of the iron-protein bonds, existence of porphyrine-protein thio-ether bonds); (b) the nature of the groups sterically close to iron; (c) the nature of the other more or less remote groups whose mode of action is not understood at present.

These factors particularly affect the visible spectrum, the oxidation-reduction properties of the heme (value of E_{m7} of the redox potential), the catalytic properties of the heme.

The spectrum of the heme carried by the apohemoenzyme appears to be identical with that of the heme carried by the L-LDH. Only the loss of the FMN makes spectral differences perceptible [26, 28, 36]. The presence of flavine, therefore, does not alter the hematinic spectrum, as might be expected if they were very close. However, it is not certain that a more accurate spectral study, in a vitreous solvent at a very low temperature, will not subsequently reveal such differences.

The spectral properties of the hemoproteinic nucleus seem to be very close to those of the original L-LDH (Table

3, Fig. 9). The positions of the peaks are identical. Nevertheless, the coefficients of molecular extinction of the heme of the different peaks seem to be systematically lower in the same proportion. Let us note that in all cases, whether it is a question of the native L-LDH, of the apoenzyme or of the hemoproteinic nucleus B_2, we always find a ratio of γ red / γ ox = 1.42 and not 1.71, as Morton and his colleagues always indicated it.

These writers have shown that it is a question of a hemochromogenic type spectrum, since the reduced peak γ (ε = 232 mM^{-1}) is greater than that of the pyridinic hemochromogen. The fixation of the heme therefore appears as identical in the derived hemoproteins and in the original enzyme.

The exact nature of this fixations, in so far as the native flavohemoenzyme is concerned, is not known. The absence of spectral modifications by means of cyanide, carbon monoxide and nitride as well as the hemochromogenic type of spectrum tend to show that the two free coordination valences of the hematinic iron are attached to basic groups of the carrying protein. The bond of the heme to the protein is very strong; the separation, attempted by Morton /267, could not be accomplished except at the cost of a very considerable denaturation of the protein.

On the contrary, with the hemoproteinic nucleus B_2, it is possible to analyze the nature of the heme-protein bond. In effect, we separated the heme and thus obtained a white soluble apoprotein. The addition of hematin to this protein leads to a recombination of the heme with a reconstitution of the B_2 spectrum. The differential variation in optical density shows the saturation curve of the apoprotein by hematin (Fig. 10). It is possible to calculate the coefficient of molar extinction of the heme on the reconstituted hemoprotein. The value found is ε_{413}=126 mM^{-1}. It is known that it is 134 mM^{-1} on the initial hemoproteinic nucleus. A correct value of ε thus determined cannot be expected unless all the heme added is fixed quantitatively on the apoprotein. An equilibrium would lead to an apparently too small ε value. The spectrums of the two containers showing the reformation of the characteristic B_2 spectrum is seen in Figure 10. At the moment when the plateau if reached on the saturation curve, the hemoprotein is then contaminated by about 40% of excess hematin.

Preliminary experiments have indicated that the affinity of the heme for the apoprotein drops between pH 7 and

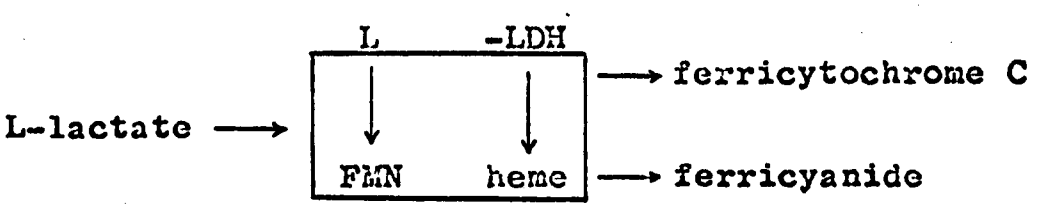
pH 5, suggesting that the two iron bonds are made on two histidine imidazoles, under the basic form, as in the cytochrome C.

The oxidation-reduction properties of heme B_2 are influenced by the proteinic structure, but are not affected by the presence of the flavine associated with the native enzyme. In effect, the native L-LDH, and the reconstituted L-LDH (apo-L-LDH + FMN) have practically the same redox potential value, namely: $E_{m7} = +5$ mv at 30°C. On the other hand, thus hemoproteinic nucleus has an appreciably more negative potential (Fig. 11): $E_{m7} = -$ [illegible] mv at 30°C. This lowering of the redox potential by degradation of the proteinic structure may be compared with that due to the denaturation of hemoglobin. At pH 7, 30°C, the potential then goes from the value of + 140 mv to the value of + 105 mv /43, 44/.

The value that we found for the heme of pure L-LDH is extremely remote from the first evaluations made by Boeri and Cutolo (+ 220 mv) /45/ and Hasegawa and Ogura /46/, who worked with approximately 20% pure enzymatic preparation, containing cytochrome C.

Other determinations by Baudras /28/, made under different experimental conditions, give values that are coherent with the ones given by Fig. 11, taking into account the temperature difference. At 5°C, in fact, Baudras demonstrated that the potential of the heme of L-LDH at pH 7.44 is + 34 mv, while that of the flavine fixed on L-LDH is -18 mv. On the other hand, 1.5 mol of L-lactate are necessary to reduce the heme and the flavine quantitatively. This equivalence, admitted implicitly by all the authors mentioned, had not beenproved experimentally until the present time. It indicates that the flavine passes effectively from the oxidized state to the totally reduced state.

We tackled the problem of the mechanism of transfer of the electrons on the enzyme between the L-lactate and the acceptors. It is a question of comparing the speeds of the various processes in the following diagram:



We attempted, for that purpose, to compare the speeds of the various reactions: reduction of the flavine of the enzyme, reduction of the heme of the enzyme, reduction of the cytochrome C and reduction of the ferricyanide.

Preliminary experiments were performed by Iwatsubo and Di Franco /31/ with the aid of a rapid recording spectrophotometer also utilized for the fluorescence studies previously described. Observation was facilitated by slowing the reaction by means of lowering the temperature to 6° and the pH to 5.8. It was made in the open air. This absence of anaerobic conditions is justified by the slowness of the speed of auto-oxidation of the L-LDH, equal, under optimal conditions, to 0.5 mol or O_2 consumed per second, per L-LDH mol, according to Boeri and Rippa /46/.

The results (Table 4) show clearly that the transfer of the electrons from the enzyme to the acceptor passes primarily, if not exclusively, through the flavine. In fact, the speed of reduction of the heme of the enzyme is much lower than that of the terminal acceptors. Accordingly, the heme does not appear to play a direct role in this transfer. However, it is possible to think that this is not so under optimal conditions of the reaction.

These results are completed with the results of a study of the state of oxidation in a stationary regime of the flavine and of the heme of the enzyme in the simultaneous presence of lactate and acceptors (saturating ferricyanide or cytochrome C). It is possible to see, under these conditions, that, in a stationary state, the flavine is reduced while the heme is oxidized. Masegawa and Ogura /45/ have already observed the oxidized state of the heme under these conditions. The transfer of the electrons from the flavine to the acceptors is slower and the transfer to the heme of the enzyme is still much slower than the process of reduction of the flavine by the lactate.

The flavine is essential to the transfer of the electrons from the lactate to the heme and to the acceptor. The hemoprotein prepared by separation of the flavine and cleared of the traces of flavinic contamination has not catalytic activity. Moreover, its heme is reduced only slowly in the presence of lactate, while the heme of the flavocytochromic enzyme is reduced immediately. The slow reduction is imputable to some traces of residual lactic-dehydrogenase. Therefore, the flavine is essential to the transfer from the lactate to the heme. It is probably the primary receptor of

the lactate's electrons. The manner in which the transfer of this dielectronic flavine to the monoelectronic heme is produced is not known.

To summarize, it seems now that the crystallized enzymatic molecule is, without doubt, a dimer formed by two identical and independent units. The two hemes appear to be borned by equal proteinic structures and the two flavines are fixed independently with the same affinity. On first analysis, according to the spectral data, their appears neighter a heme-heme interaction, nor a heme-flavine interaction, since the flavine does not alter the redox potential of the heme any more. The flavine plays an essential part in the catalytic action of transfer of oxidation-reduction from the lactate to the acceptors. The results suggest the eventuality that the heme does not have a direct part in this transfer.

4. "Physiological" Forms of the L-LDH

The sub-units of the L-LDH mentioned in the foregoing paragraphs are well defined on the molecular level for they come from the crystallized enzyme. Somlo /48, 49/ observed that the L-LDH which is present in the cellular extracts has a certain number of properties that differentiate it from the crystallized L-LDH. This raises the problem of the existence of more "physiological" forms of the dehydrogenase than the ones that an enzymologist normally studies after a crystallization or intensified purification. It is clear that this problem is very general and extremely difficult to solve. There are numerous pitfalls, both methodological and semantic. However, it is fundamental to tackle it, if we want to associate physico-chemical enzymology with cellular physiology.

The L-LDH activity of the extracts, prepared by various suitable methods, is divided between two fractions: namely, one that includes 20% to 30% of the total and is soluble, and one that includes 70% to 80% of the total /48, 50/. The L-LDH bound to the respiratory particles reduces cytochrome C with a slower speed relative to the $K_3Fe(CN)_6$ than the soluble L-LDH and resists the crystallized anti-L-LDH immune serum that inhibits the soluble L-LDH. By detaching the bound L-LDH with a detergent, Tween 80, or by means of mechanical treatment, Somlo /43/ demonstrated that these differences are due to the bond of the enzyme with the particles. The explanation, therefore, is simple: The association of the enzyme to the mitochondrial fragments causes its inacessibility to large molecules like those of cytochrome C or of

the immune serum. The intrinsic properties of L-LDH are, in a first approximation, the same, regardless of whether or not it is bound to the respiratory or soluble particles /48/.

The same is not true when the properties of the L-LDH before and after crystallization are compared /49/. Several constants, like the Km relative to the L-lactate, Ki relative to the D-lactate and to the pyruvate, are increased three or four times after crystallization. On the other hand, the Ki relative to the oxalate remains unchanged and the curves of inhibition in two forms by the crystallized anti-L-LDH immune serum are identical (Fig. 13). It is interesting to note that the "physiological" L-LDH is inhibited by strong concentrations in substrate, while the crystallized L-LDH is not. The inhibition, which has a competitive appearance, is specific of L-lactate (Fig. 12).

A priori, it is possible to explain the differences between the non-purified L-LDH and the crystallized L-LDH in three ways: (a) the molecules of L-LDH are fundamentally identical and the differences observed come from one or several factors of the medium in which the enzyme is before crystallization. (b) Two types of L-LDH can exist within the cell and autolysis with butanol (cf. 6), utilized before crystallization, extracts selectively only one molecular type. (c) There is only one type of L-LDH that undergoes a modification during the purification that leads to crystallization.

It has been possible, by means of reconstitution experiments, to eliminate the first hypothesis. The differences are not due to the dissociable factors that probably affect the L-LDH activity. The second hypothesis is very little likely. In effect, during the successive steps in purification, all the characteristic properties of the "physiological" L-LDH (cf. Fig. 13) remain constant and change abruptly during the step that ends in crystallization. Moreover, the L-LDH that has been purified according to /37/ has the properties of the "physiological" enzyme. A modification of the molecular structure, during crystallization, must, consequently, be retained. This modification may be fotuitous or, on the contrary, it may have a physiological sense. It is premature to reply definitively to this. Let us point out that: (a) The "physiological" form has a sedimentation constant very similar (S20, $\omega \cong 8$, /51/) to that of the crystallized enzyme (cf. above). It is, therefore, not a question of a change in polymerization. (b) There is an analogy

between the general direction of the modifications at the time of passage from the crystallized form to the reconstituted form on the basis of the apoenzyme plus FMN and the modifications at the time of passage from the "physiological" form to the crystallized form (cf. Table 1 and Fig. 13). Nevertheless, the addition of the FMN during the test does not change the properties of the last two forms. Moreover, it is possible to split the "physiological" L-LDH into apoenzyme in accordance with the process used for the crystallized enzyme. By reactivating with the FMN, we obtain the enzyme that has "physiological" constants (Km relative to the L-lactate = 0.3 mM, inhibition by excess of substrate) and not the enzyme having "crystallized" constants. (c) The differences bear primarily on the constants relative to the L- and D-lactates; inhibition by excess of substrate explains perhaps, the fact that the respiration of intact yeast cells is strongly inhibited by high concentrations in L-lactate /52/.

II. D-Lactic Dehydrogenase (Anaerobic Yeast)

Although this enzyme was demonstrated more than six years ago /8/, the preparations obtained have hardly achieved a purity greater than around 10%, and this in spite of the efforts of several laboratories. This low degree of purity obviously excludes any analytical determination of the prosthetic groups. Therefore, it has been necessary to utilize the following indirect ways: (a) Search for methods enabling the prosthetic groups to be dissociated without altering the proteinic part, (b) search for the substance specifically giving reactivation, that is to say, reconstituting the entire functional enzyme. These experiments led to the identification of Zn^{++} and FAD as dissociable prosthetic groups /53, 59/.

Two inactivation methods demonstrate the role of zinc as an essential metal: (a) a precipitation at pH 4 by ammonium sulfate /53/, (b) treatment with a chelator, ethylene-diamine-tetracetate /54, 56, 57/. In both cases, the product obtained, inactive, has the same properties. It can be reactivated only with the zinc, cobalt and manganese cations (Fig. 14), since fifteen other cations, among which Ni^{++}, Cd^{++}, Fe^{++}, etc. do not produce any effect. It must be emphasized that it is necessary to use very pure cations for this kind of study. Therefore, we used spectroscopically pure products. Zn^{++}, Co^{++} and Mn^{++} give three reconstituted enzymes that are distinct with regard to their catalytic characteristics. By comparing the values of the Michaelis constant relative to the lactate of these three reconstitued

enzymes (Fig. 15), it is seen that only the enzyme reactivated by zinc has the same properties as the native enzyme. We shall designate the other two with the term "neoenzyme". Let us note that the properties of the native enzyme are not modified by the presence of Co^{++} and Mn^{++}. Zn^{++} itself is an inhibitor at concentrations greater than 10^{-4} M. The comparison of these neoenzymes bore equally on the values of Vm and Ki in the presence of substrates and inhibitors (Table 5).

It seems that the factor modified by the nature of the metal bound to the apoenzyme is probably principally K_3. The metal probably, without doubt, does not change the affinity of the enzyme for the D-lactate, since it does not change the affinity for the inhibitors that are competitive with it. It has been seen that the values of Ki relative to the oxalate are the same for the various cationic cofactors (Table 5).

Other metals can be fixed competitively with zinc on the apoenzyme, but by giving inactive preparations; this is true of Ni, Cd /55/.

The percentage of zinc that remains attached to the native enzyme by precipitation in ammonium sulfate varies with the pH. This variation indicates a competition between one Zn^{++} and two H^{++} for two basic groups with pK /sic; should probably read pK/ close to 6 /53/. This suggests the bond of zinc to two imidazoles (Fig. 16).

The speed of reactivation by zinc of the apoenzymes prepared either with EDTA or in acid medium, varies with the concentration in zinc (Fig. 17). Very low concentrations of zinc, similar to the ones contained as contaminants in the salts, even if they are of a "pure for analysis" quality, particularly ammonium sulfate, phosphate, lactate, are sufficient to restore the lost activity slowly. Zinc fixed on the other proteins in the preparation or adsorbed on the Sephadex may play the same role. This explains the so-called "spontaneous" reactivations obtained by certain authors /61-64/. By taking special precautions to eliminate these metallic contaminants (multiple recrystallizations in the presence of a chelator then water twice distilled on quartz), we never obtained appreciable spontaneous reactivations (Table 6). We shall return to this question farther on. With regard to the flavinic group, it has been possible to identify it in FAD by means of two methods: (1) FAD completely protects the enzyme from inactivation by quinacrine, while FMN does not protect /66/. (2) It is possible to separate

FAD from the active enzyme by precipitation with ammonium sulfate in a very acid medium. In effect, toward pH 2, an inactive preparation is obtained that cannot be reactivated by the addition of zinc alone, like the preparation obtained at pH 4, but that can be reactivated by zinc plus FAD. Let us note that commercial FAD, as a general rule, is sufficiently contaminated by Zn to give by itself a partial reactivation. FMN does not produce any reactivation /52, 59/

Interactions between the D-lactate substrate fixed on the D-LDH and the prosthetic groups were demonstrated by studying quantitatively the protection exercised by the substrate with regard to the inactivating agents with a progressive action. This method, which was first pointed out by Burton /67/ and has been practically unused, gives very interesting indications. The variation in the speed of inactivation of the enzyme under standard conditions depending on the concentration of the protector (substrate or competitive inhibitor) is measured. In this way, it has been possible to show that the protection afforded by the D-lactate in contrast with EDTA, which extracts the zinc, is complete -- or almost so -- with saturation in lactate, and that the concentration in D-lactate giving semi-protection, identifiable by the constant of dissociation of the enzyme-D-lactate dissociation, is of the order of 6 μM (Fig. 18), or 300 times smaller than the Michaelis /54, 60/ constant. While D-lactate completely protects zinc in contrast with chelators, it has no action on the fixation of zinc to the apoenzyme. It does not alter the speed of combination of the zinc. This situation could be explained if the zinc were necessary to the fixation of the lactate.

By studying the inactivation of the enzyme by quinacrine, a total protection by saturating FAD has been observed, but also a partial protection by saturating D-lactate (Fig. 19). The speed of inactivation of the enzyme-D-lactate complex is around 5 times lower than that of the free enzyme /66/.

The mechanism of these protections is not known: it is a question of a more or less large modification of the reactivity of the inhibitor's attack site either by competitive fixation of the protector, or by a more or less localized change in the proteinic structure, caused by distal fixation of the protector. One of the important applications of this type of study is the determination of the true dissociation constant of the enzyme-substrate complex or protector enzyme. In a general way, we have been able to demonstrate an identity between the constants obtained by this

method and the Ki values of competitive inhibitors /60/.

This D-lactic dehydrogenase was also studied in Boeri's laboratories in Italy /68, 69/, and then by Singer in the United States /61-64/. Since contradictions have occurred between a certain number of results and interpretations presented by these authors and by us, a few words must be said on this subject. The above-mentioned authors believe, in fact, that we have not provided proof of the existence of the essential Zn^{++} cofactor on the D-LDH. They interpret the inhibition of D-LDH by EDTA as a reversible fixation of the EDTA on the enzyme and not as a separation of the metallic cofactor. This interpretation is based solely on two arguments:

(1) In two experiments, described in four publications /61-64/, these authors obtained "spontaneous", slow reactivations after elimination of the EDTA by passing over Sephadex and/or prolonged dialysis in a phosphate buffer. We demonstrated above (Table 6) that this type of experiment has never yielded reactivation when the salts in the medium and the Sephadex itself had been carefully treated to eliminate, previously, the metallic contaminations.

(2) These authors thought that they had determined the values of the concentrations of Zn^{++}, Co^{++}, Mn^{++}, Fe^{++} required to obtain a semi-reactivation, K, as well as the maximum speeds, Vm. The K values found are all very close to 1.6 μM, and the Vm values are respectively 108, 88, 84 and 74 percent of the initial Vm. The analogy of the values of K and of Vm probably eliminates, according to these authors, the possibility of the reactivating agent's being zinc contained as a contaminant in the other metals. It must be emphasized that the value of K, as they were determined, are not valid. They are the concentrations of metals during the test of activity. Now, these authors incubated, prior to the activity test, the inactivated D-LDH in the presence of concentrations of metals one hundred times higher than those reflected by K. Only the concentrations during incubation are significant, if the equilibrium of dissociation of the metal-proteinic complex is not an immediate phenomenon.

All the results presented by Singer and his collaborators can be explained by the presence of Zn^{++} in the state of traces: (a) in the reagents, particularly phosphate and and lactate, Sephadex, etc., that they use, and (b) in their enzymatic preparation, since the metal is fixed partially on the contaminating proteins. Moreover, they admit the presence of zinc with a 6 mμ Mol/mg protein content /63/. The

number of zinc atoms present per molecule of enzyme can be calculated according to the molecular weight of the D-LDH, or 105,000 /70/ and the assumed purity of their preparation, estimated to be 5%. The best preparations that they point out have, in effect, a purity of 10%, as may be calculated according to the differential spectrum of the flavine (E_{ox} - E_{red}) at 450 mμ or 0.1 for a 11 mg/ml proteinic solution /54/. It is found that in their proteinic preparation there are 12 zinc atoms per molecule of enzyme, a sufficient quantity to saturate the enzyme when the preparation is in a concentrated solution. By varying the temperature or adding metals that are inactive by themselves in order to reactivate the D-LDH, the zinc may be displaced from its combination with foreign proteins and combine on the D-LDH by reactivating it.

A more extensive discussion of the manner of action of the EDTA is, moreover, superfluous to demonstrate the fact that the activity of the D-LDH is connected with the presence of a dissociable Zn, as we had made a hypothesis as early as 1959 /54, 57/. In fact, as has been seen above, the treated D-LDH without chelator, but simply precipitated at pH 4 by ammonium sulfate, is also transformed into an inactive form that cannot be anything but the apo-D-LDH /53/. Now, the inactive preparation thus formed has exactly the same properties as that obtained with EDTA: the same behavior in the presence of zinc, cobalt, and manganese, the same properties of the neoenzymes formed with these metals, the same "sontaneous" reactivations in a non-decontaminated medium in relation to the metallic cations.

In conclusion of this study on D-LDH, the similarity of its behavior with that of carboxypeptidase. These two enzymes are characterized by a bond of average strength between the metal and the protein which makes it possible to separate the metal without denaturation of the proteinic part. In that way they are distinguished from a certain number of enzymes bound to NAD, like alcohol dehydrogenase, glutamic dehydrogenase and lactic dehydrogenase of the muscle in which zinc is fixed so strongly that it cannot be separated without an irreversible loss of the activity.

D-LDH, like carboxypeptidase, is not bound in a strictly specific manner to the zinc, since, as we have seen, cobalt and manganese are capable of giving active forms, although to a lesser degree. It is known that, in carboxypeptidase, the substitution of cobalt for zinc produces a neoenzyme that has a different specificity spectrum with regard to the substrates /71/. A detailed study of the modifications

produced by substituting one metal for the other opens the way toward a knowledge of the metal's role.

The least known problem is the one of the physiological role of D-LDH in the metabolism of yeast. The activity per cell of this enzyme may be very high, all the more so since the intensity of the fermentation metabolism is high. On the other hand, as the respiratory metabolism increases, the activity per cell of D-LDH decreases [3, 19, 20, 72]. It is, therefore, highly probable that D-LDH does not serve in the respiratory catabolism of the lactate, in contrast with the other two enzymes, L-LDH and D-LCR, which certainly participate in it. This idea is strengthened by the fact that D-LDH cannot be connected to the terminal respiratory chain, since it does not reduce cytochrome C. Then, for what is it used? We have suggested [97] that D-LDH might possibly participate in the fermentation forms of the metabolism of methylglyoxal in connection with lactoylglutathione. It would be interesting to compare the regulation of the formation of D-LDG and of glyoxalase on the one hand, and the physico-chemical properties of these two enzymes on the other.

III. D-Lactic Cytochrome C Reductase (Aerobic Yeast)

This enzyme was demonstrated by Nygaard [11, 12, 17]. It is a question of a FAD-protein [18] strongly bound to the particles. Gregolin and Singer [73, 74] perfected another method of preparation and confirmed the presence of FAD as a prosthetic group. They give arguments suggesting that it is a question of a zinc-enzyme. The analysis of a very pure, although not crystallized, preparation indicates the presence of three atoms of Zn^{++} per molecule of FAD and per 96,000 g of protein. D-LCR is sensible to chelators: EDTA inactivates it progressively to yield a product that is reactivable by zinc and cobalt [74].

Iwatsubo and Isomoto [65] separated prosthetic zinc by treating the D-LCR, prepared according to Nygaard's method, by precipitation with ammonium sulfate at pH 2.5. When the salts utilized have been specially purified (cf. above), an inactive preparation that is rapidly reactivated by the addition of Zn is obtained in this way (Fig. 20). It is interesting to note that cobalt and manganese also give an almost complete reactivation.

What is possibly the most interesting property of D-LCR is its specificity with regard to various cytochromes C.

Until now, all studies have utilized cytochrome C from mammals (horse or steer) as a "natural" acceptor of the yeast enzymes. Evidently it is not physiological.

Since the recent discovery of isocytochromes C [47], it has been possible to tackle the problem of physiological specificity. It has been demonstrated that the same haploid yeast cell synthetizes two distinct molecular types of cytochrome C, called iso-1 and iso-2, which can be separated on Amberlite XE-64 resin by a very gentle gradient. These two types correspond to two monomers that have identical sedimentation constants and elution volumes on a molecular screen. Each monomer is a polypeptidic chain. The polypeptidic chains of iso-1-cytochrome C and of iso-2-cytochrome C are very similar in their composition in amino acids, their total length and differ only by some substitutions: for example, lysine replaces glumatic acid in C-terminal position, glutamic acid and isoleucine replace valine and leucine in the hemododecapeptide. The absorption spectra and the redox potentials are practically identical (Fig. 21).

The mitochondrial cytochrome oxidase of yeast makes little distinction between iso-1-cytochrome C and iso-2-cytochrome C. The same is true of crystallized or "physiological" L-LDH (Fig. 22), when the activity of the enzyme is followed by the speed of reduction of the cytochrome C as the final acceptor. It is quite different for D-LCR. It is seen in Figure 23 that the washed mitochondrial fragments do not consume oxygen at the expense of the D-lactate except in the presence of added cytochrome C. However, iso-2-cytochrome C is, in an equal concentration, much less effective than iso-1-cytochrome C in catalyzing the transfer of electrons from the lactate to themolecular oxygen. A notable difference is also observed in the maximum speed of reduction of iso-2-cytochrome C as final acceptor of the measured reaction (Fig. 24). It would be interesting to go more into detail on these observations by applying them both to purified enzymes and to more and more organized multienzymatic systems.

The slight molecular activity of iso-2-cytochrome C together with its slight content per cell explain why mutant yeasts, that are deprived of iso-1-cytochrome C breathe poorly and do not grow when the lactate is the sole source of carbon. By taking advantage of this property, we isolated close to two hundred independent lactate$^+$ re versions [75]. Certain reversed strains analyzed to date showed an increased synthesis of iso-2-cytochrome C and perhaps some structural changes in its molecule. It should be possible to

isolate, in the same way, mutations bearing on the structure of the lactic dehydrogenases that probably modify the reactivity of these enzymes in comparison with cytochrome C.

/Table and figures follow/

Table 1

Characteristics of Native and Reconstituted L-LDH Compared. Baudras /28/

	K_M L lactate	K_i D lactate	K_i Oxalate	K_{FMN}
L LDH native [a] + 1	1 mM	6 mM	2 mM	10^{-4} µM +3
L LDH reconstituée [b] + 2	5 mM	25 mM	6 mM	0,12 µM +4;3 1 µM +5

/Legend: a) Native L-LDH; b) Reconstituted L-LDH./

+1 Native L-LDH is crystallized in accordance with /6/.

+2 L-LDH reconstituted by saturation of the apo-LDH with FMN 20 µM.

+3 K_{FMN} estimated in accordance with a fluorometric study (cf. Fig. 4).

+4 K_{FMN} estimated by the concentration of demi-saturation in the kinetic measurements (cf. Fig. 2); Tris-HCl 50 mM buffer; lactate 50 mM; ferricyanide 0.66 mM.

+5 K_{FMN} same conditions, but in phosphate 0.1 M buffer.

Table 2

Action of the Carboxylic Anions on the Fixation Equilibrium of FMN on the Apo-L-LDH (Iwatsubo and Di Franco /31/)

a) Tampon	b) Tris-oxalate 100 mM	c) Tris-acétate 100 mM	d) Tris lactate 100 mM
K	$1,9.10^{-9}$ M	6.10^{-9} M	$0,1.10^{-9}$ M

/Legend: a) Buffer; b) Tri-oxalate; c) Tri-acetate; d) Tri-lactate./

K = constant of equilibrium determined by the fluorometric method (cf. Fig. 4)

Table 3

Characteristics of the Hematinic Spectrum of L-LDH and Its Derivatives /327

	a) Déterminations expérimentales				b) Résultats			
	Extinctions pH 7	Extinctions hémochromogène pyridinique*1	f) Concentration protéique*2	g) Concentration de l'hème*3	ε γ ex	ε β red	ε red / ε ox	h) Masse protéique par hème
	c) bande γ	bande γ red	(Folin)		max	max		
	ex / red	E_{HC}, 419 mμ	mg/ml	mM	$mM^{-1}cm^{-1}$	$mM^{-1}cm^{-1}$		g/mole
L LDH*4	(8,3) 11,85	8,35	3,4 ± 0,5	0,046	(181)	257		74000 ± 10000
	(5,5) 77,7	60,3	23 ± 1,5	0,334	(162)	230		69000 ± 3000
					m = 171	m = 243		m = 71500
Ape-L LDH*4	(39,2) 55,6	44,4	16 ± 2,5	0,246	(159)	226		65000
	(20,9) 29,6	21,9	10,2 ± 1	0,121	(173)	245		83000
					m = 166	m = 235	39 19,5	m = 74000
				λ max	——→ 413 mμ	423 mμ	557mμ 526mμ	
mhl_2*5 de L LDH*5								
n° 6	14,6 (20,8)	24,3	2,34 ± 0,02	0,134	110	154		17500
7	6,90 (9,8)	9,6 ± 0,9	0,82 ± 0,01	0,053	130	185		15600
17	4,6 (6,55)	6,7 ± 0,5	0,75 ± 0,03	0,037	125	177		20200
18	1,30 (1,85)	1,97 ± 0,02	0,18 ± 0,0	0,109	119	169		16500
19	6,06 (8,72)	6,78 ± 0,2	0,56 ± 0,05	0,375	162	233		14900
20	15,42 (21,55)	19 ± 0,5	1,44 ± 0,12	0,105	147	204		13700
					m = 134	m = 197	32 16	m = 16400
				λ max	——→ 413 mμ	423 mμ	557mμ 526mμ	
i) L LDH (MORTON et coll.) (6, 21, 41, 36)					136	232	38,8 16,6	75000
					160			
				λ max	——→ 413mμ	423 mμ	557mμ 528mμ	

/Legend: a) Experimental determinations; b) Results; c) γ band; d) hemochromogen; e) Pyridinic; f) Proteinic concentration; g) Concentration of the heme; h) Proteinic mass per heme; i) (Morton and colleagues) /7

*1: NaOH pyridinic hemochromogen in accordance with /25/

*2: Proteinic concentrations estimated by Folin's method after gauging with recrystallized β lactoglobulin (ε_{280} =0.96 mM^{-1} cm^{-1}) or with human serum albumin (ε_{280} = 0.53 mM^{-1} cm^{-1}), the two gauging curves are practically merged.

*3: For the pyridinic hemochromogen at 419 mμ the value ε = 131 mM^{-1} cm^{-1} calculated on the basis of ε_{557} = 34.8 mM^{-1} (37) was taken with the factor $\varepsilon_{419}/\varepsilon_{557}$ = 3.52 (38), a value verified by us. Determinations under the same conditions on the recrystallized hematin whose concentration is measured in accordance with the extinction of the cyanogenetic derivative (ε_{545}= 11.13 mM^{-1}) gave ε_{419} = 150 mM^{-1}.

*4: L-LDH (crystallized twice) apo-L-LDH prepared in accordance with /27/, on the basis of L-LDH (crystallized once).

*5: Sample corresponding to the tip of the elution peaks at the time of fractioning the hydrolysates of L-LDH on Sephadex G 100, not purified further.

*6: The figure of 75,000 corresponds to the proteinic mass, while the value of 82,000 corresponds to the mass of the nucleoprotein obtained by crystallization.

Table 4

Study of the Comparative Speeds of Reduction by Lactate of the FMN Prosthetic Groups and Heme of the L-LDH and of the Acceptors 6°, pH 5.8 (Iwatsubo and Di Franco /317)

a) Réduction de:	Exp	b) d'observation	k
		mμ	d) électron équivalent /sec./mole L LDH
c) Hème de L LDH (5,4 μM)	1	423	1,3
	2	423	1,3
	3	556	1,2
	4	556	1,3
e) FMN de L LDH $^{+2}$ (5,4 μM)	1	455	20
	2	455	15
Cyt. c (38 μM)			
" par f) L LDH (5,4 μM)	1	550	16
" par L LDH (1,3 μM)	2	550	17
g) Ferricyanure (1 mM) par L LDH (5,4 μM)		423	5,2

/Legend: a) Reduction of; b) from observation; c) Heme of; d) equivalent electron/sec./ mol L-LDH; e) FMN of L-LDH^{+2}; f) by; g) Ferricyanide by L-LDH./

Legend for Table 4

During the few seconds that follow the addition of lactate to the L-LDH or L-LDH + acceptor system, the transmission variation is recorded. L-LDH crystallized in accordance with /67 and then recrystallized in an oxidized state

in accordance with /29/ (TN = 16,000 per minute per heme at 30° pH 8), 200 mM phosphate buffer; 0.5 mM EDTA; 0.2 mM L-lactate.

1: The reaction is really of the order 1 in the time for FMN and the heme of L-LDH. It is of the order zero for ferricyanide 1 mM (saturating concentration) and of the order 1 for the iso-1-cytochrome C of the yeast (cf. 47) 38 µM (non-saturating concentration). The values of k are independent of the concentrations. They are calculated on the basis of the date taken from the graphs in the classic manner.

2: The participation of the heme at 455 mµ is corrected in accordance with the variation in the heme observed at 556 mµ. The calculations are made on the basis of the following values (average of the best experimental estimates) of

For the heme: at 557 mµ , $\Delta = 26.4\ mM^{-1}$; -at 423, 5 mµ: $\Delta = 136\ mM^{-1}$; -at 455 mµ,: $\Delta = 10\ mM^{-1}$.

For fixed FMN: at 455 mµ , $\Delta = 11.5\ mM^{-1}$.

Table 5

Comparison of the Native L-LDH and the Neoenzymes Reconstituted by Adding Zn^{++}, Co^{++}, Mn^{++} to the Apoenzyme

a) METALLOENZYMES	b) SUBSTRATS				c) INHIBITEURS COMPETITIFS	
	D lactate (pH 7,0		D malate (pH 7,2)		Oxalate (pH 7,2	Pyruvate (pH 7,2)
	Vm	Km mM	Vm	Km mM	K_i	K_i mM
D LDH [d] natif	100	1,7	100	3,5	0,007	6
D LDH [d] natif + Co^{++}	100	1,7				
D LDH [e] inactivé partiellement par Chélateur (EDTA)	10	1,8				
Apoenz. ac. + Zn^{++}	100	1,9	100	3,0	0,0045	15
Apoenz. ac. + Co^{++}	28	0,5	12,5	0,17	0,004	15
Apoenz. ac. + Mn^{++}	15	0,5			0,005	
Apoenz. $_{chel.}$ + Zn^{++}	100	2,0				
Apoenz. $_{chel.}$ + Co^{++}	32	0,5				

/Legend: a) Metallic Enzymes; b) Substrates; c) Competitive inhibitors; d) Native D-LDH; e) D-LDH partially inactivated by chelator (EDTA)

The maximum speeds obtained in the presence of Co and Mn are related to the maximum speed of the corresponding apo-D-LDH saturated in Zn. (The specific activity of apoenzyme chelator + 100 μM of Zn^{++} is around 80% of that of the specific activity of apoenzyme chel. ac. + 100 μM of Zn^{++}, varying according to the preparations from 20% to 80% of the native D-LDH + 100 μM of Zn.)

Table 6

Study of the Effect of Contaminants on the "Spontaneous" Reactivations Obtained after Elimination of the EDTA from an Inactivated D-LDH - EDTA Mixture

a) CONDITIONS EXPERIMENTALES b) Mode d'élimination de l'EDTA après inactivation			e) Milieu dialyse			d) ACTIVITES e) Initiale	f) après Inactivation	g) après Réactivation "spontanée"	h) après Réactivation par Zn^{++} 0,1 mM
CREMONA et SINGER [i]	Sephadex puis dialyse (?)	0°	Tp. Phos. 50 mM lactate 0,1 mM	P?		100	0	40 (1h) 100 (2h)	?
A. CURDEL [k]	Dialyse, collodion	0°	[l] Eau bidistillée			100	1	1,1 (2h)	50
M. IWATSUBO [m]	Sephadex puis dialyse, collodion	0°	Tp. Phos. 50 mM lactate 1 mM	P		—	1,6	37 (5h)	100
	idem		idem	PR		—	1,6	13 (5h)	100

P salts quality for analyses

PP idem, but recrystallized, first in presence of EDTA, then twice in twice distilled water on quartz

/Legend on following page/

/Legend: a) EXPERIMENTAL CONDITIONS; b) Method of elimination of the EDTA after inactivation; c) Dialysis medium; d) Activities; e) initial; f) after inactivation; g) after "spontaneous" reactivation; h) after reactivation by Zn^{++} 0.1 mM; i) and; j) Sephadex then dialysis (?); k) Dialysis, collodion; l) Twice distilled water; m) Sephadex then dialysis, collodion idem./7

(The inactivated solution is cleared of EDTA in the manner indicated. The relative activities are given measured in the standard manner, before (initial) inactivation before elimination of EDTA, after elimination of EDTA, then incubation during the time indicated between parentheses at 0°, then eventually after adding Zn^{++} at the same moment.)

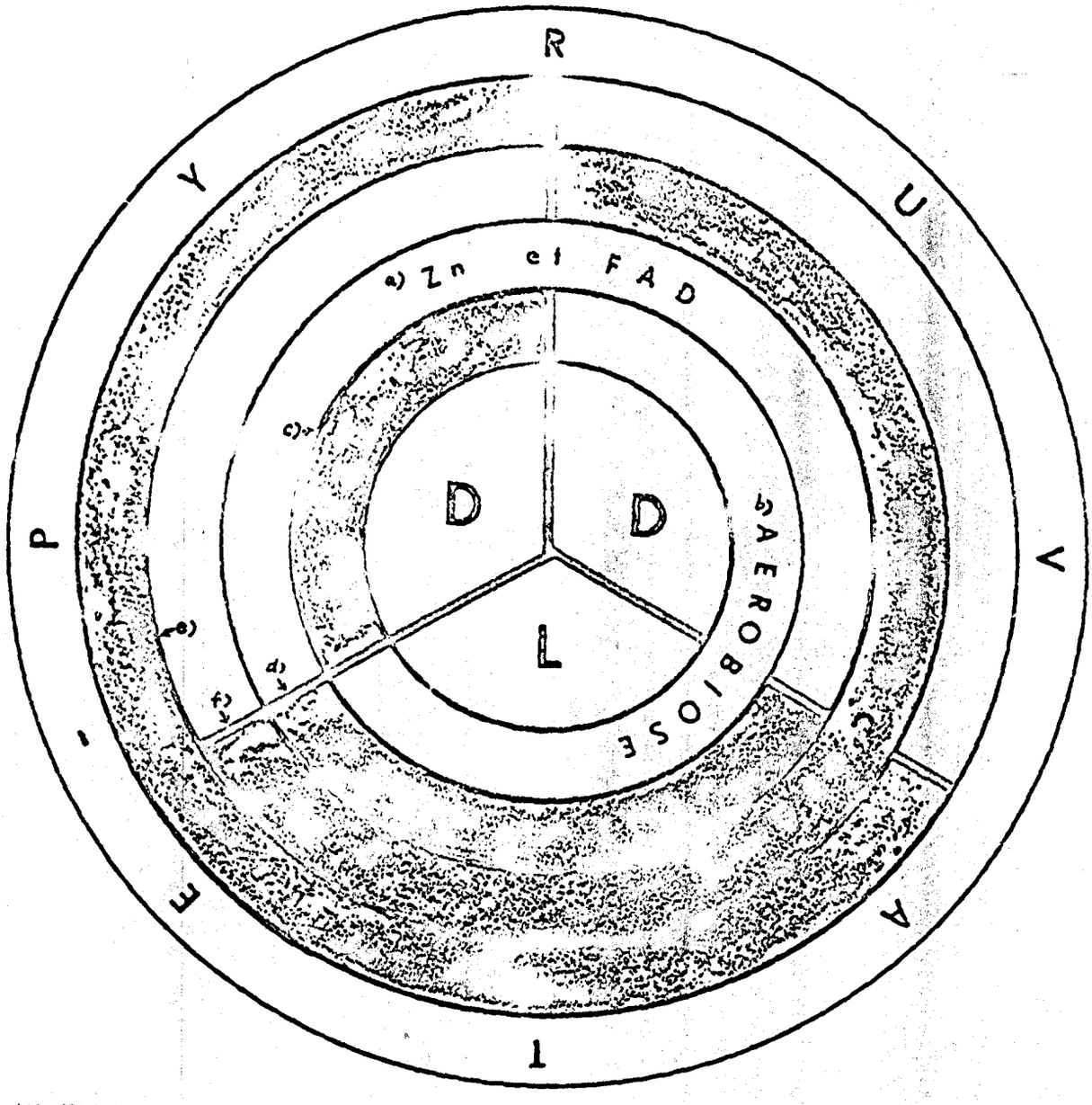


Fig. 1. Diagram of the Principal Characteristics of the Three Lactic Dehydrogenases of Yeast.

/Legend:/ a) Zn and FAD; b) Aerobiosis; c) Anaerobiosis; d) Heme and FMN; e) Ferricyanide; f) Ferricytochrome C.

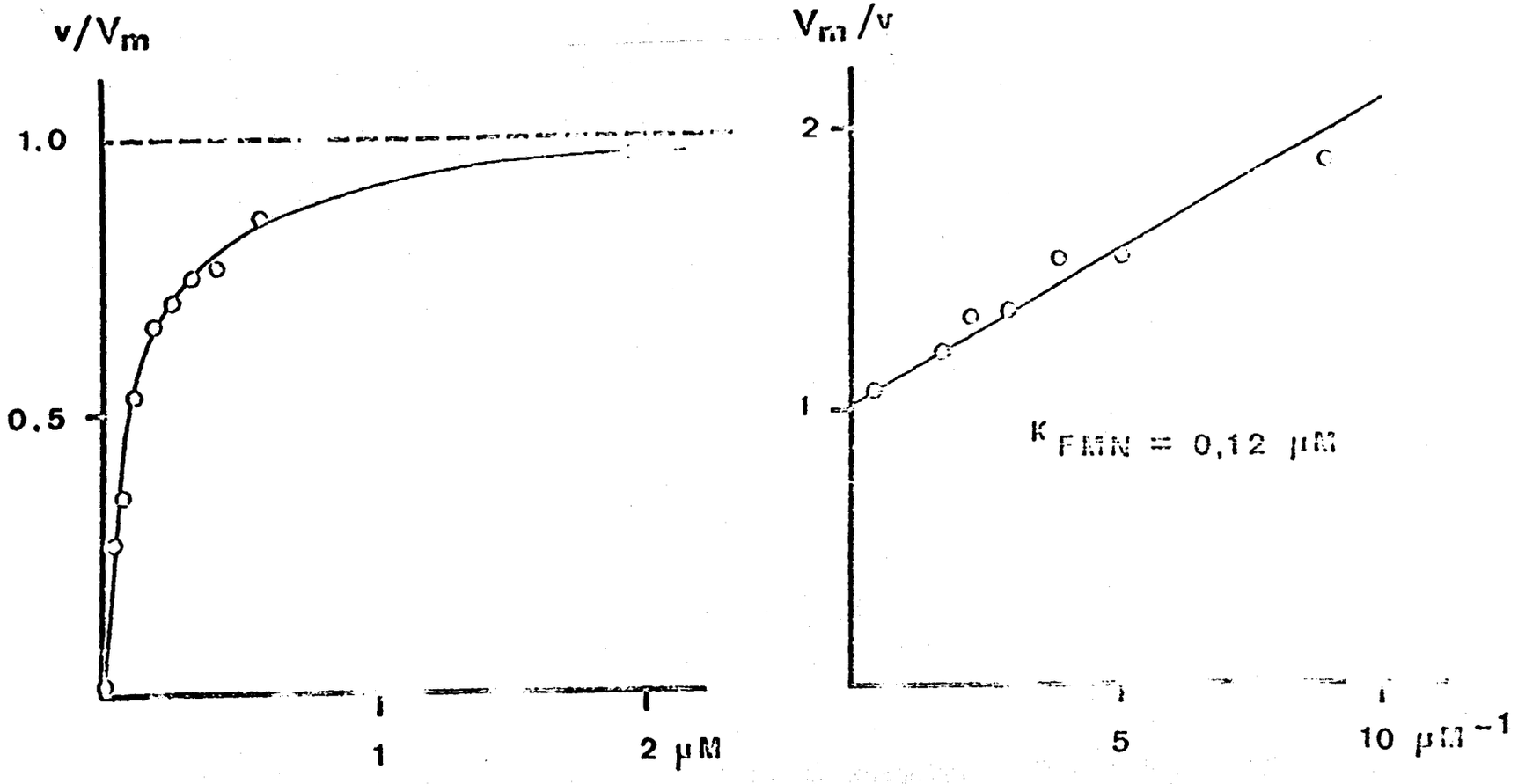


Fig. 2. Reconstitution of the Lactic Dehydrogenasic Activity by Saturating with FMN of the Apo-L-LDH Prepared by Acid Treatment. (Baudras /28/.)

/continued/

Apo-L-LDH prepared in accordance with /27/, passed over Sephadex G 25. The speed of reduction of the ferricyanide, V, is measure at 30° in a spectrophotometric vessel, containing: buffer, Tri-HCl pH 7.3, 50 mM; EDTA 20 μM; ferricyanide 0.66 mM; FMN: variable concentrations on abscissa, apo-L-LDH 20 mμM added last of all to start the reaction. Ordinates show the relationship of the speeds V to the maximum speed V_m for saturating FMN. The maximum specific activity is 80% of that of the initial L-LDH.

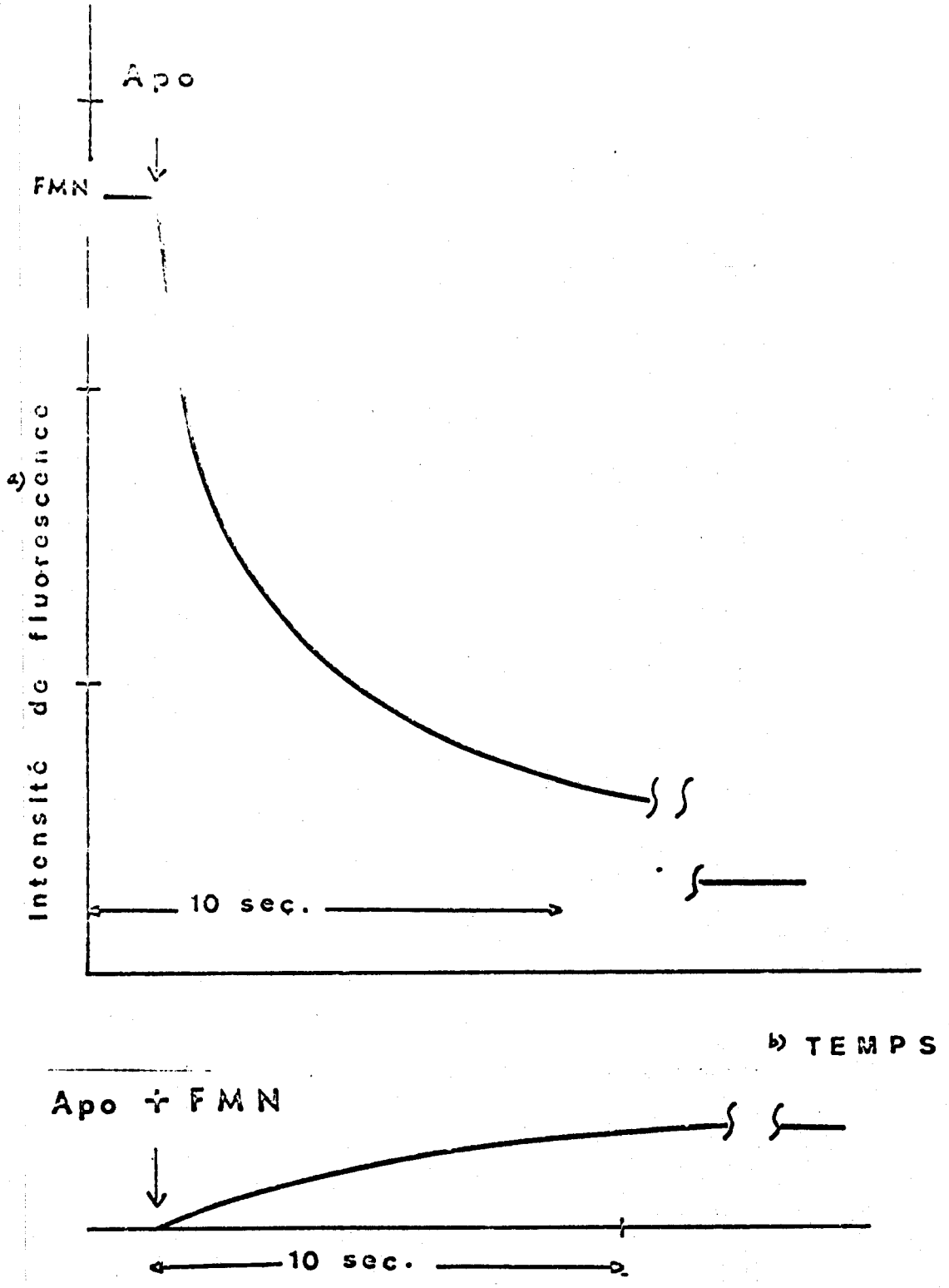


Fig. 3. Fluorometric Study of the Speeds and the Equilibrium of Association of FMN to the Apo-L-LDH. (Iwatsubo and Di Franco [31].)

[Legend on following page]

/Legend:/ a) Intensity of fluorescence;
b) time.

Fixed FMN is not fluorescent. On the left: the addition of apo-L-LDH to FMN is followed by a decrease in the intensity of fluorescence = kinetic and equilibrium of combination. On the right, the addition of an apo-L-LDH + FMN mixture to a vessel containing a buffer is followed by an increase in fluorescence = kinetic and equilibrium of dissociation. FMN 0.30 μM, apo-L-LDH 0.50 μM, Tri-acetate buffer 0.1 M, pH 7.2 at 23° C.

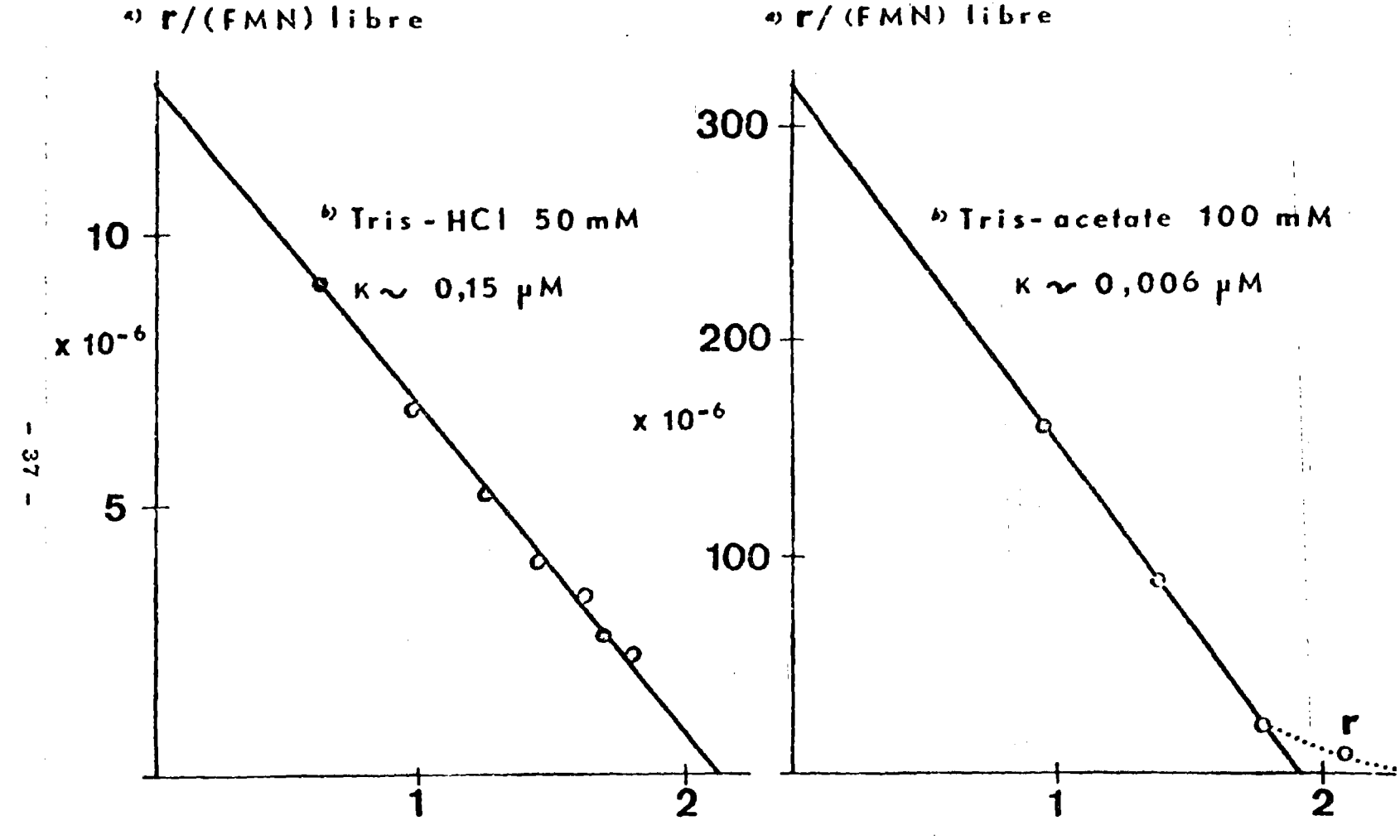


Fig. 4. Determination of the Maximum Number of FMN Molecules Fixed to the L-LDH, n, and of the Constant of Dissociation K. (Iwatsubo and Di Franco /317.)

/Legend:7 a) free; b) Tri-; c) mol FMN fixed by mold of L-LDH.

The points are calculated on the basis of the fluorometric determinations of the ratio of fixed FMN to free FMN for variable concentrations of total FMN and a fixed concentration of apo-L-LDH. n is calculated per molecular unit containing two hemes. K = constant of dissociation = 1/slope.

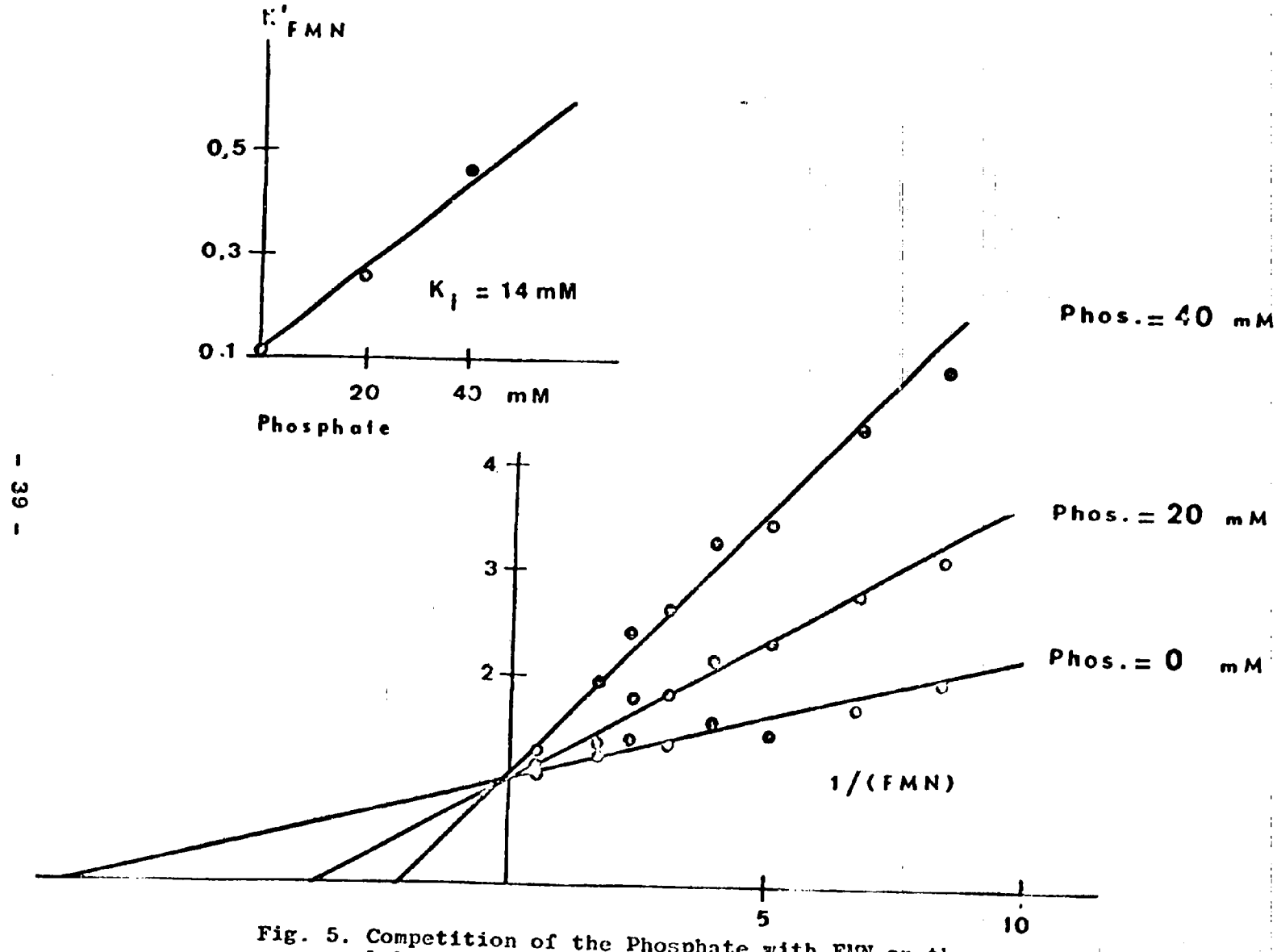


Fig. 5. Competition of the Phosphate with FMN on the L-LDH. (Baudras /28/.)

Study of the variations in the speed of reduction at 30° of the ferricyanide, V, in the presence of apo-L-LDH (fixed concentration), of FMN (variable concentration) and of phosphate (variable concentration). DL lactate 66 mM; ferricyanide 0.66 mM; Tri-50 mM buffer, pH 7.2; EDTA 20 µM. Determination of apparent K_{FMN} for each value of (phosphate); top: determination of k phosphate.

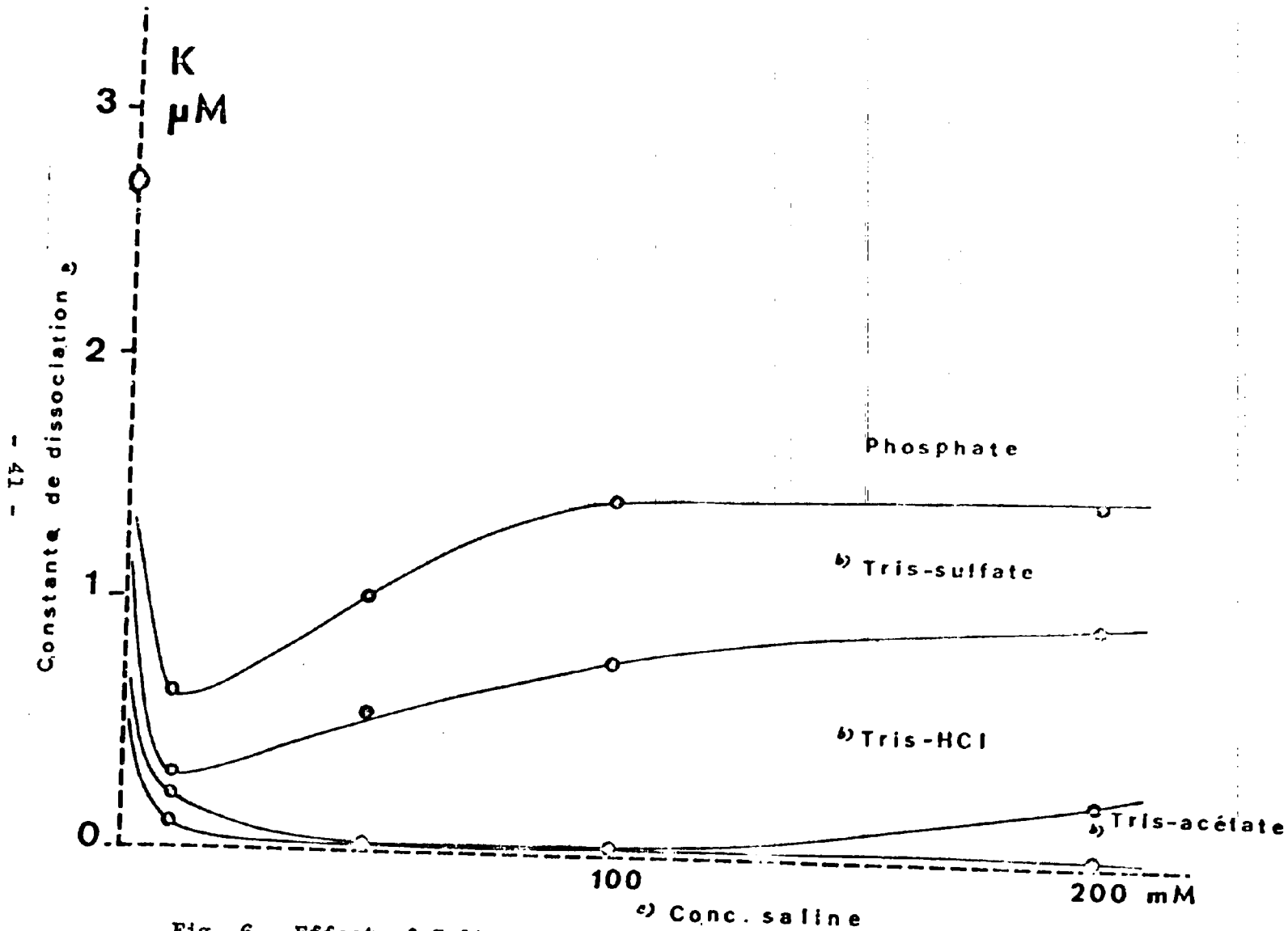


Fig. 6. Effect of Salts on the FDN - apo-L-LDH Combination. (Iwatsubo and Di Franco [31].)

/Legend:7 a) Constant of dissociation;
b) Tri-; c) saline concentration.

On the ordinates, values of K calculated according to the fluorometric data.

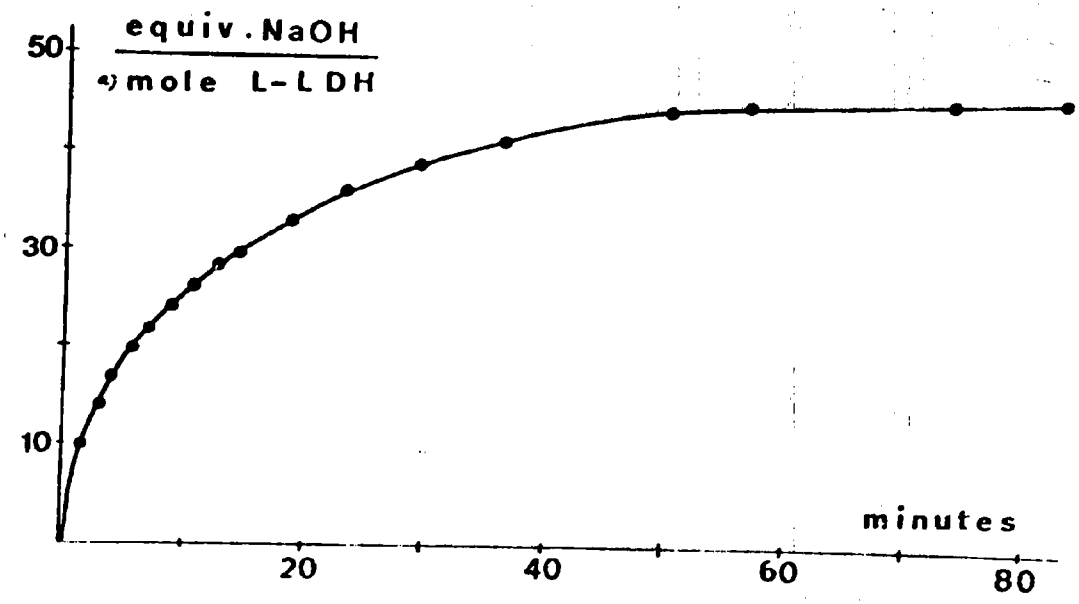


Fig. 7. Hydrolysis of the L-LDH (Labeyrie, Jacquot-Armand, Naslin and Groudinsky [32]), Followed by the Potentiometric Method [33].

[Legend:] a) mol.

On the ordinates, added soda equivalents to maintain the pH at 8.5 per mol of L-LDH (two hemes unit), corrected from the derivative. Trypsin 300 μg/ml added at time zero; L-LDH 40 mg/ml; phosphate buffer 0.1 M, pH 8.5, 30°.

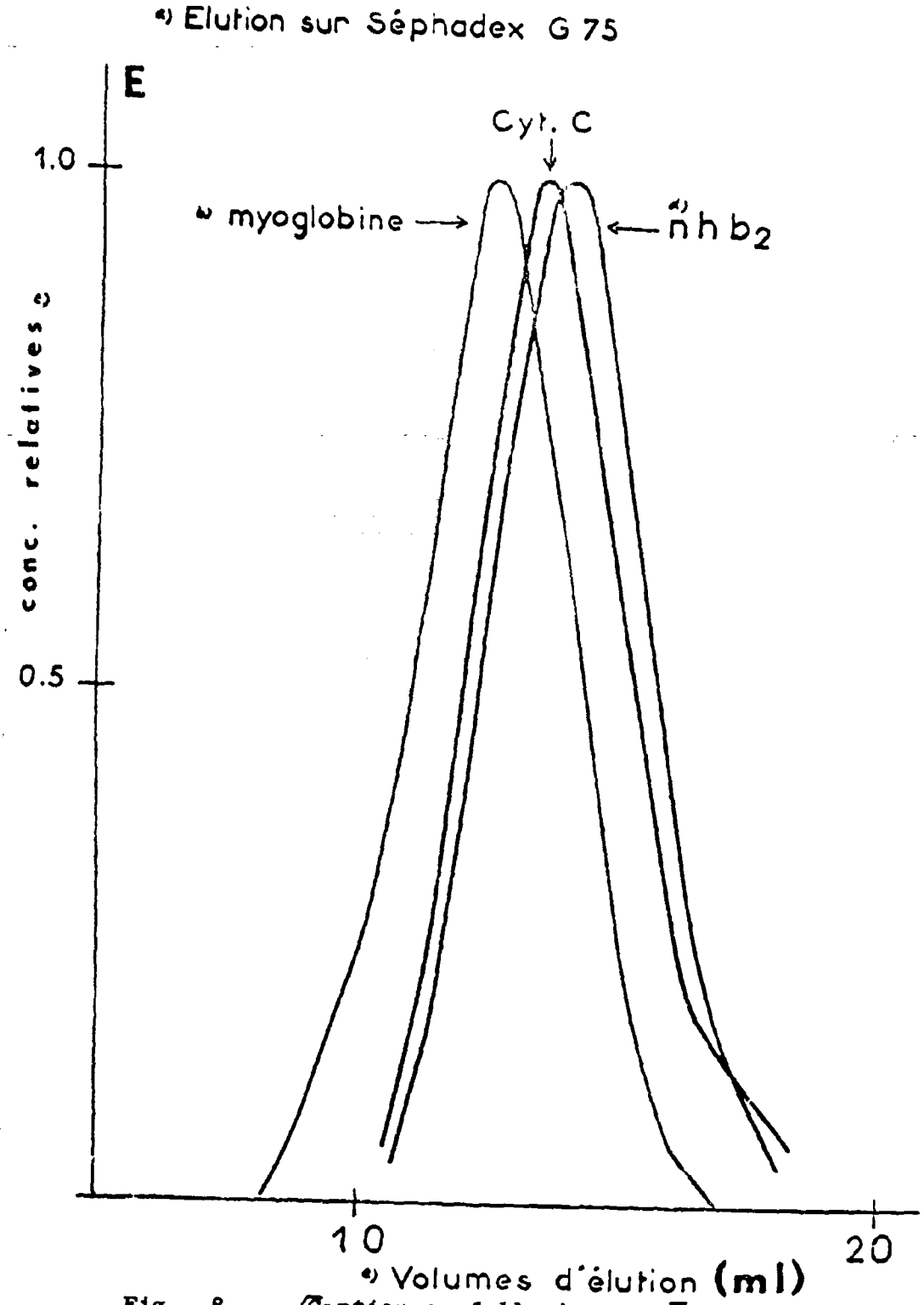


Fig. 8 /Caption on following page/

Fig. 8. Comparison of the Elution Volumes on Sephadex G 75 of the Hemoproteinic Nucleus B_2, of the Cytochrome C from the horse, of Myoglobin. (Labeyrie, Jacquot-Armand, Naslin, Groudinsky [32].)

[Legend:] a) G 75 = Elution on Sephadex G 75; b) myoglobin; c) relative concentrations; d) hnB_2; e) Elution volumes.

One same column (h = 40.5 cm, 0 = 0.8 cm. Tp Phos 0.1 M) serves for the three experiments. A drawing of 0.150 ml of a solution of about 5 mg/ml is placed on the column; collection of 0.70 ml fractions; verification of the volumes by weighing. Ordinates: reactive concentrations in the eluates (determined by the oxidized γ band).

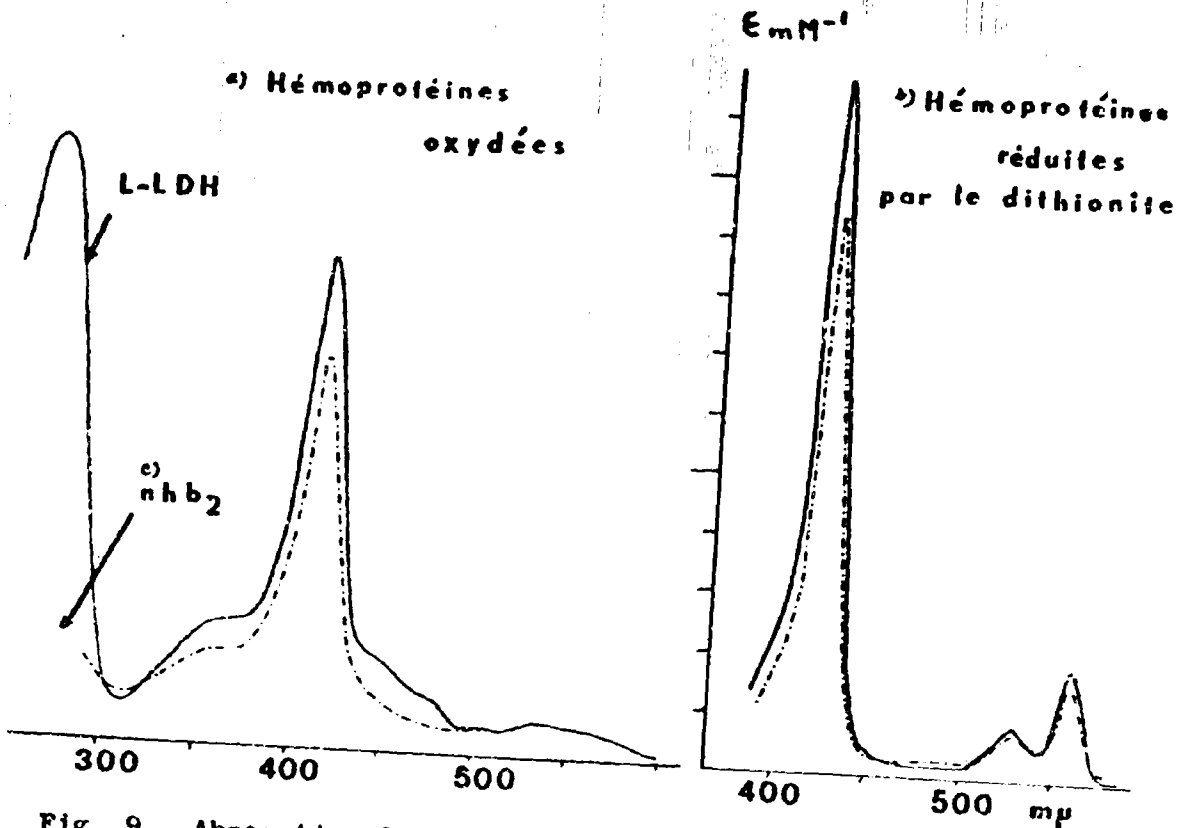


Fig. 9. Absorption Spectra of the Hemoproteinic Nucleus B_2 and of the L-LDH from which It Is Derived. (Labeyrie, Jacquot-Armand, Naslin and Groudinsky /327.)

/Legend on following page/

/Legend:7 a) oxidized hemoproteins; b) Hemoproteins reduced by the dithionite; c) nnB_2.

These spectra have been adjusted for ε_{413} with regard to each ferrihemoprotein, the average value determined elsewhere (Table 3). Spectra reduced by dithionite.

Fig. 10 a

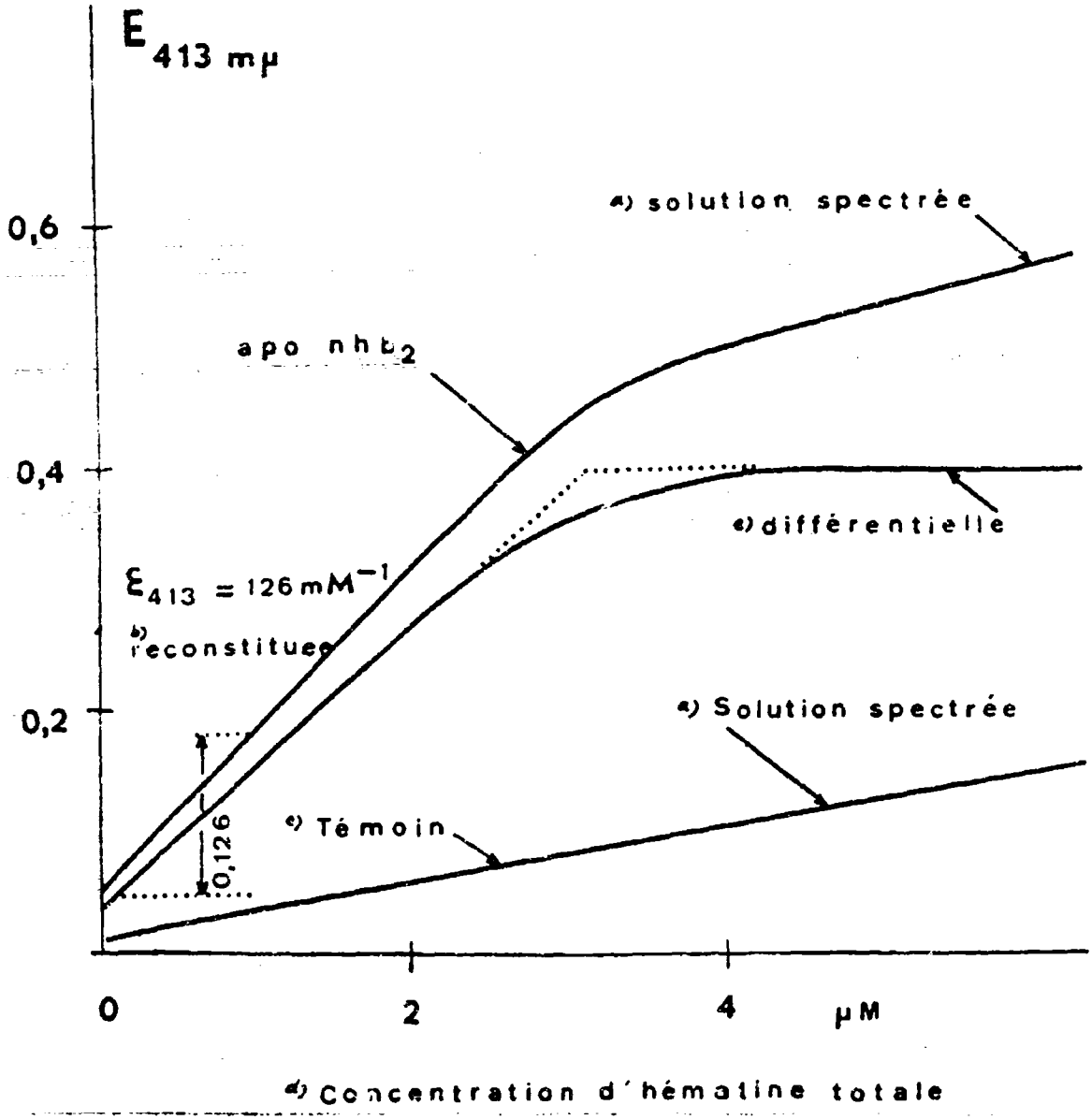
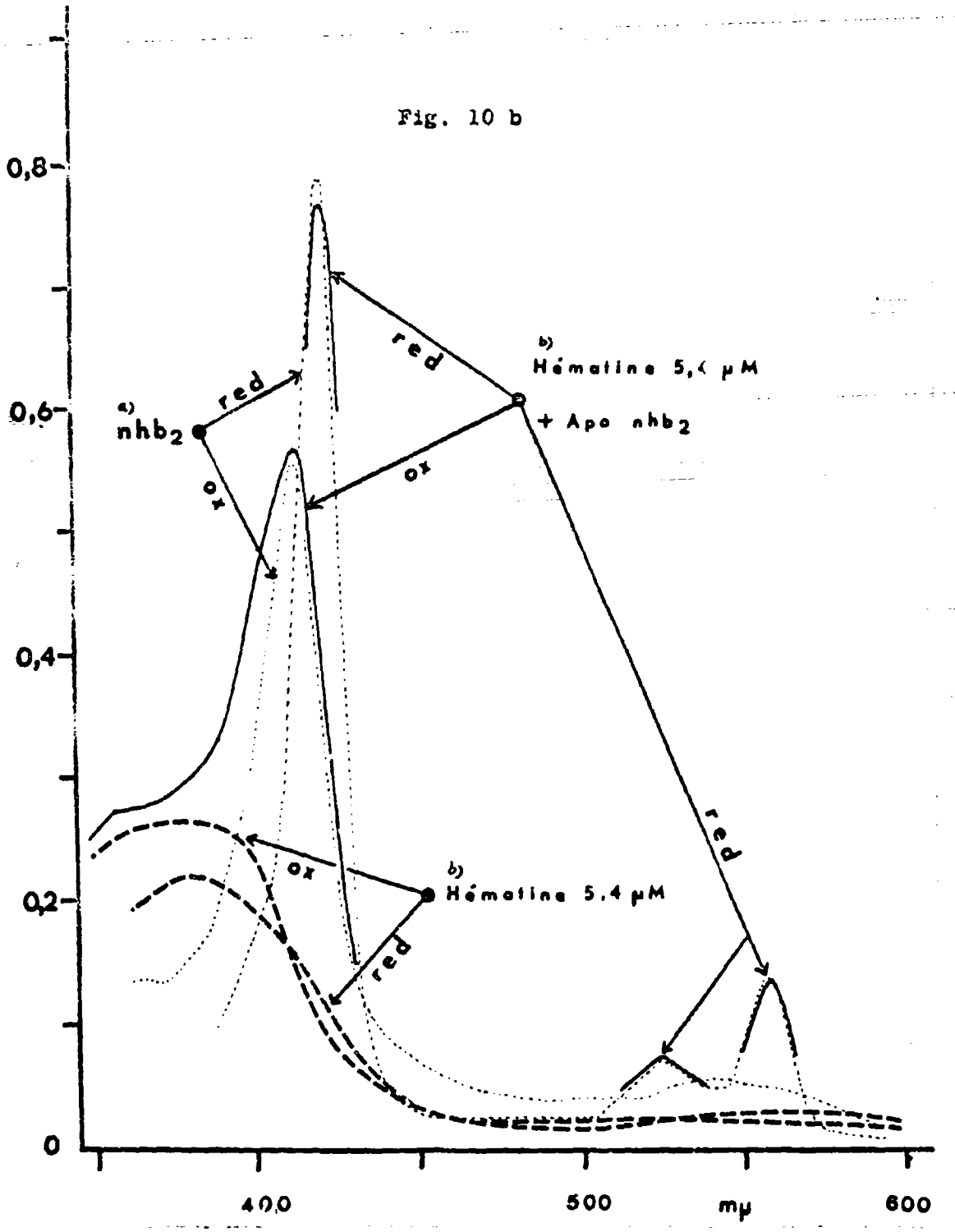


Fig. 10. Reconstruction of the Hemoproteinic Nucleus D_2 by Titration of the Apo-Protein with hematin. (Labeyrie and Baudras /427.) /Legend on page 50/

Fig. 10 b


0,8
0,6
0,4
0,2
0
a)
nhb2
red
ox
b)
Hématine 5,4 µM
+ Apo nhb2
red
ox
b)
Hématine 5,4 µM
ox
red
red
400
500
mµ
600

/Legend: 10a/ a) spectrum solution, hnB_2; b) reconstituted; c) control d) Total hematin concentration; e) differential.

/Legend: 10b/ a) hnB_2; b) hematin.

Sample of hnB_2 containing 13 mμ mols treated at -10° with acetone -ClH(0.006 N) twice. The colorless precipitate formed is collected rapidly and dissolved in 2.5 ml of phosphate buffer 0.1 M, pH 7.2. Addition of hematin (put in solution extemporaneously and titrated in the presence of CNK 0.2 M by spectrophotometry with $\varepsilon_{545} = 11.13\ mM^{-1}$) parallelly in two spectrophotometric vessels containing, one of them this solution (apo-hnB_2), the other one the same volume of the same control (control). Top: variation of ε_{413} with (hematin) and differential curve. Bottom: the outlines of the absorption bands of the two solutions when the total hematin concentration is 5.8 μM; in the vessel containing the apo-hnB_2, there is therefore an excess of 5.8 - 3.2 = 2.6 μM of free hematin. For comparison, a spectrum of hnB_2 is given as a dotted line.

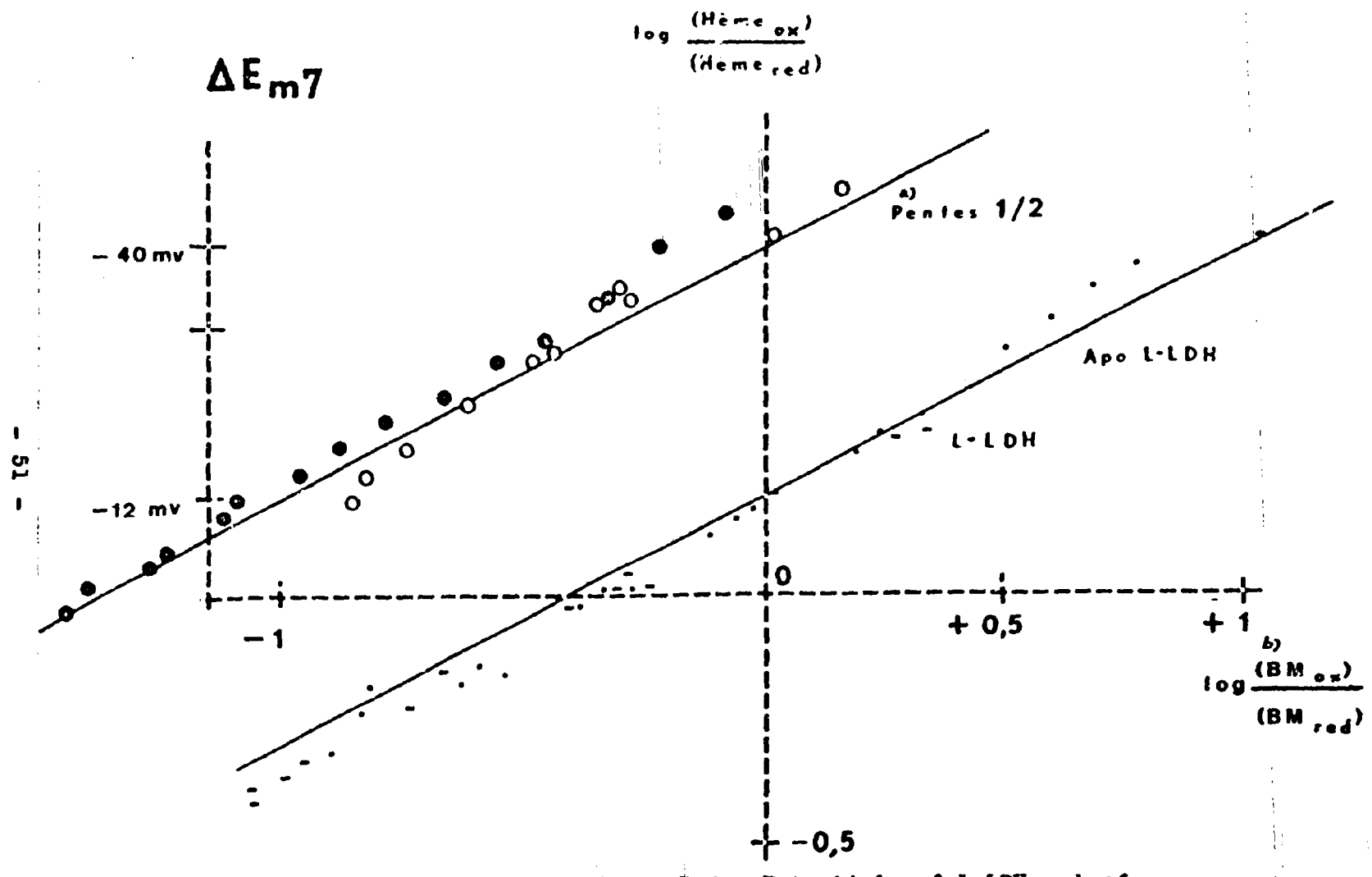


Fig. 11. Determination of t[illegible] Redox Potentials of L-LDH and of Its Derivatives: Hemoproteinic Nucleus B_2 and Apo-L-LDH. (Labeyrie, Jacquot-Armand, Baudras, naslin and Groudinsky /327.)

/Legend:7 a) Slopes; b) methylene blue.

Spectrophotometric study on two wave-lengths (600 mμ, 413 mμ) of the equilibrium between methylene blue (BM) and the hemoprotein. For the hnB_2 and the apo-L-LDH, the reagent medium contains traces of L-LDH and the reduction by the lactate can be followed progressively. For the L-LDH, first of all it is reduced with a small quantity of lactate, then the oxidation obtained by tonometry is followed progressively. Thunberg spectrophotometric tubes under vacuum, 30°, phosphate buffer 0.1 M, pH 7.00. The ordinate at the origin $E_{m7}/_{heme} - E_{m7}/_{BM}$. The slope is n_{heme}/n_{BM}, n: number of electrons involved in the oxidation-reduction.

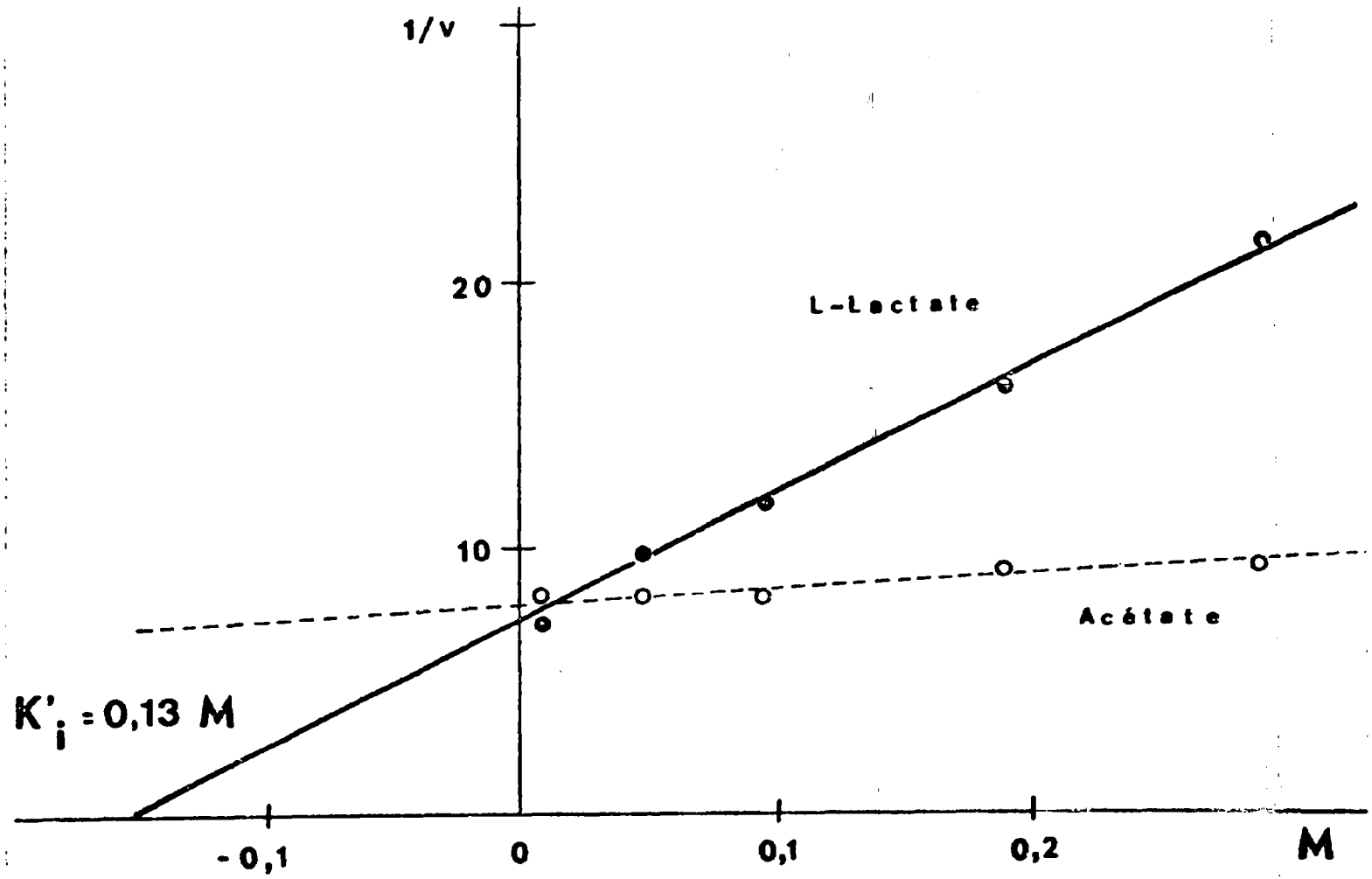


Fig. 12. Inhibition of the "Physiological" L-LDH by the Excess of Substrate. (Somlo and Slonimski [497].)

Test conditions: 28° C; phosphate buffer 0.07 M, pH 7.3; $K_3Fe(CN)_6$ 0.7 mM; L lactate 0.01 M; on the abscissa: concentration in L lactate (or acetate) in addition to the one indicated above; on the ordinates: inverse speed ratio.

	Forme [a] cristallisée	Forme [b] Physiologique
K_M L-Lactate (mM)	1,4 ± 0,2	0,39 ± 0,1
K_i (mM) D-Lactate	6,6 ± 1,4	1,4 ± 0,5
K_i (mM) Pyruvate	7,2	2,6
K_i (mM) Oxalate	0,9	1,0
a) Inhibition par excès de substrat L-Lactate 0,1 M	0 %	50 %
a) Inhibition par l'immunserum anti-L-LDH crist.	—— [c] identique ——	

Fig. 13. Comparison of the Properties of Two Forms of L-LDH. (Somlo and Slonimski /497.)

/Legend:7 a) crystallized form; b) "Physiological" form; c) Inhibition by excess of substrate; d) Inhibition by crystallized anti-L-LDH immune serum; e) identical.

Test conditions as in Figure 12. Inhibition by immune serum tested in accordance with /48/.

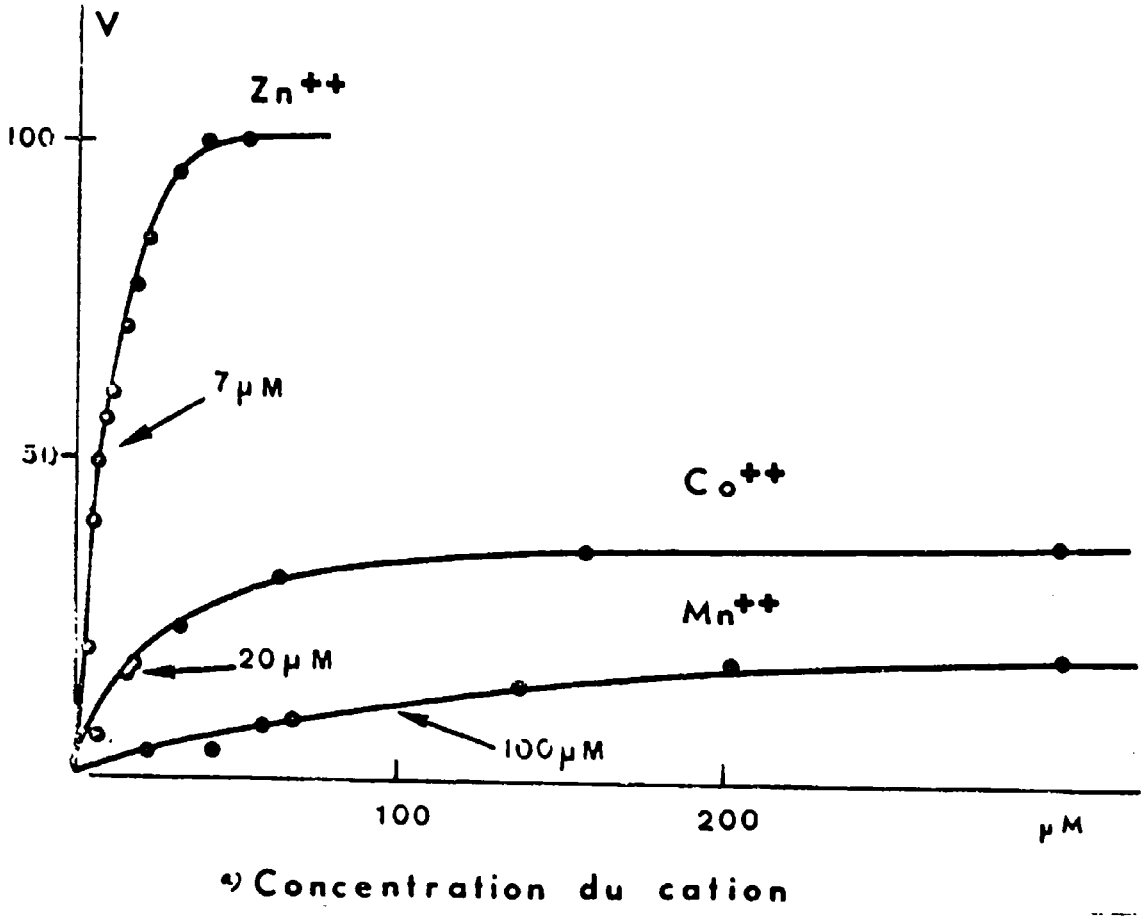


Fig. 14. Saturation of the Apo-D-LDH with Zn^{++}, Co^{++} and Mn^{++}. (Curdel [55].)

[Legend:] a) Concentration of the catio.

Apo-D-LDH prepared by precipitating the D-LDH with ammonium sulfate at pH 4. The vessels utilized to measure the activities at 30° contain: phosphate buffer 66 mM, pH 7.3; 2,6 dichlorophenolindophenol 20 µg/ml; apo-D-LDH (fixed concentration) and metal with variable concentrations (on abscissa). Ten minutes ofter adding the last item, the reaction is started by adding DL-lactate 10 mM (final concentration). On the ordinates: relative speeds in relation to the maximum activity with Zn^{++}. The values of metal concentrations yielding semi-situration are indicated.

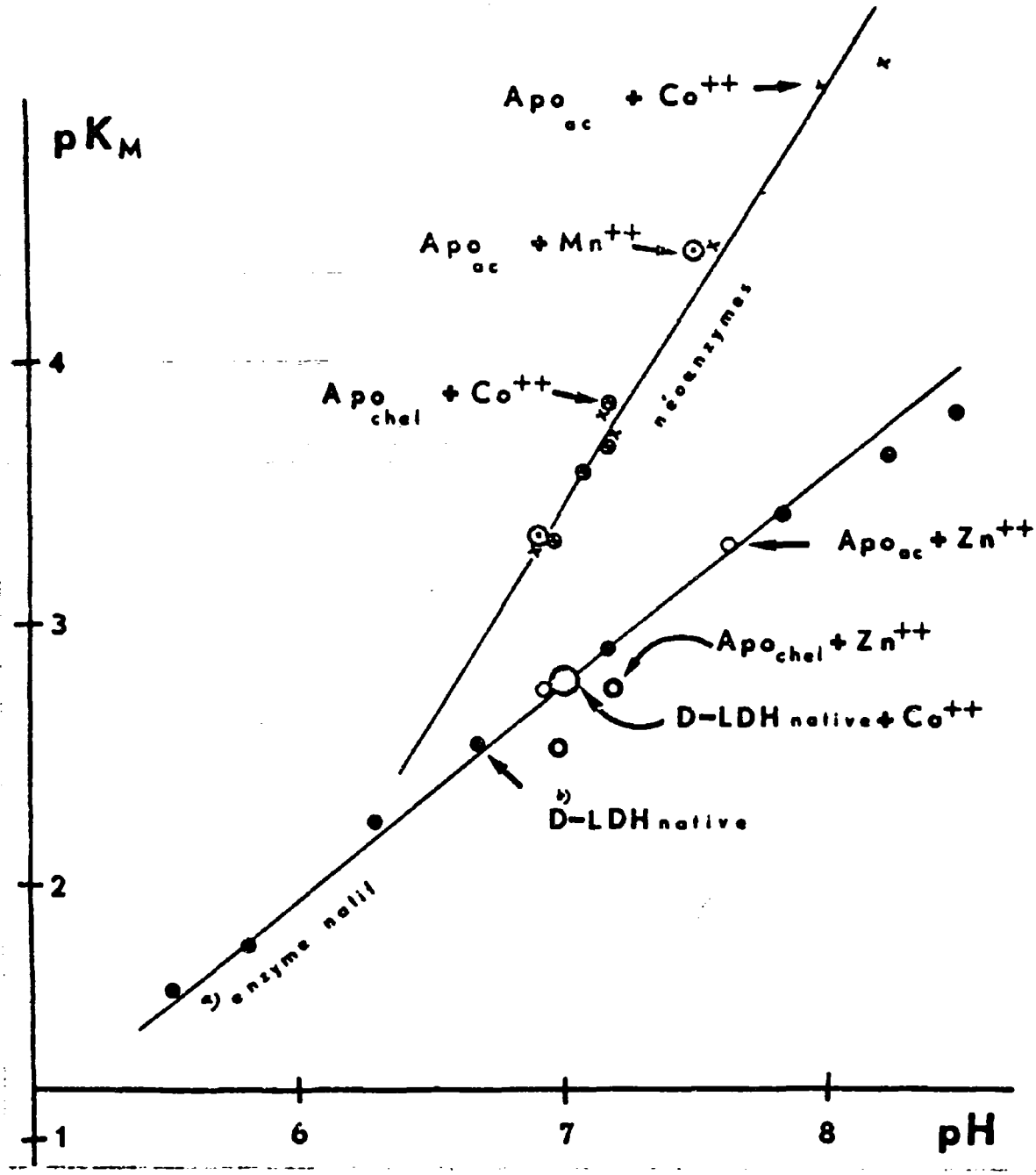


Fig. 15. Comparison of Characteristics of the Native D-LDH and of the D-LDH Reconstituted by Co^{++}, Mn^{++} and Zn^{++}. Study of the Michaelis Constants Relative to the D-lactate Depending on the pH. (Curdel.)

/Legend:7 a) native enzyme.

Preparation of the apo-D-LDH either by precipitating with ammonium sulfate at pH 4 (Apo_{ac}) /517. or by incubating for two hours with EDTA 10-2M, exhaustive dialysis in collodion against twice distilled water (Apo_{chel}). The vessels used for measuring the activity (30°) contain: phosphate buffer 66 mM: 2,6 dichlorophenolindophenol 20 µg/ml; the following is added in succession: Apo-D-LDH or (D-LDH for controls), then immediately the metal (Zn^{++} 10^{-4}M or Co^{++} 1.5 10^{-4}M or Mn^{++} 2 10^{-4}M), then, after 3 minutes: D-lactate (variable concentration) to start the reaction. Final pH on abscissa.

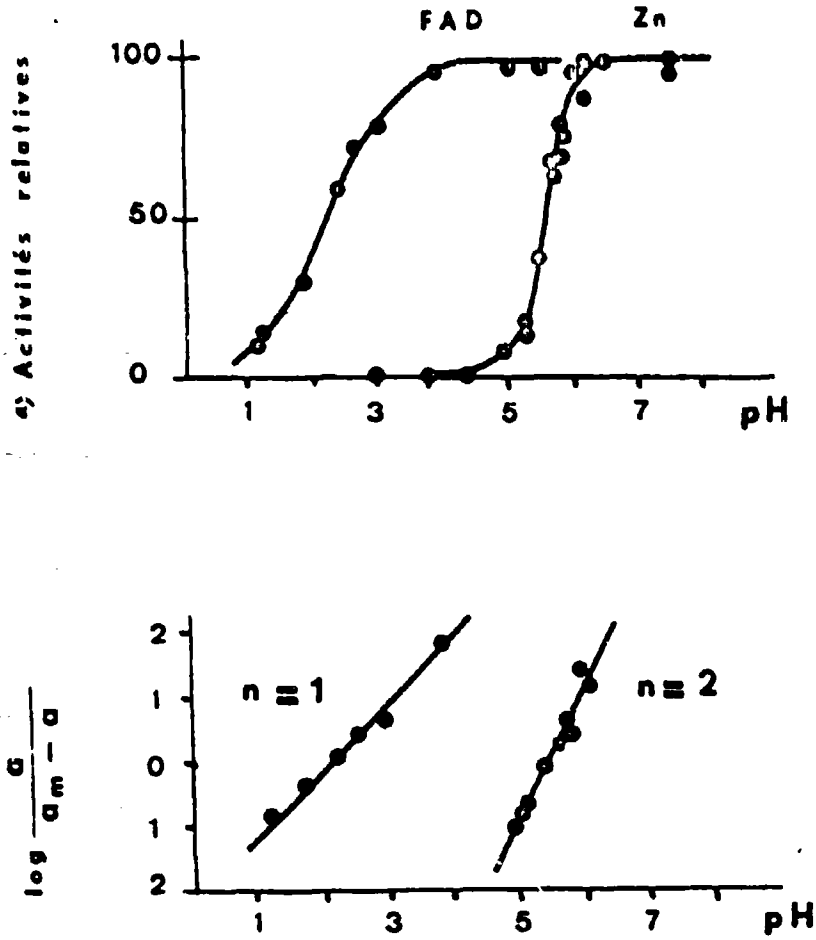


Fig. 16. Effect of the pH of the Precipitation by Ammonium Sulfate on the Dissociation of FAD by Zn^{++} of the D-LDH. (Iwatsubo and Curdel.)

/Legend:/ a) relative activities.

Top: dissociation curves; on the left, dissociation of FAD: a/a_m is the relation of the activities of (treated enzyme + saturating Zn^{++}) to (treated enzyme + saturating Zn^{++} and saturating FAD). On the right, dissociation of Zn: ratio of the activities of (treated enzyme) to (treated enzyme + saturating Zn^{++}). Below: the number of protons brought into play in the dissociations is given by the slope of the logarithmic representation.

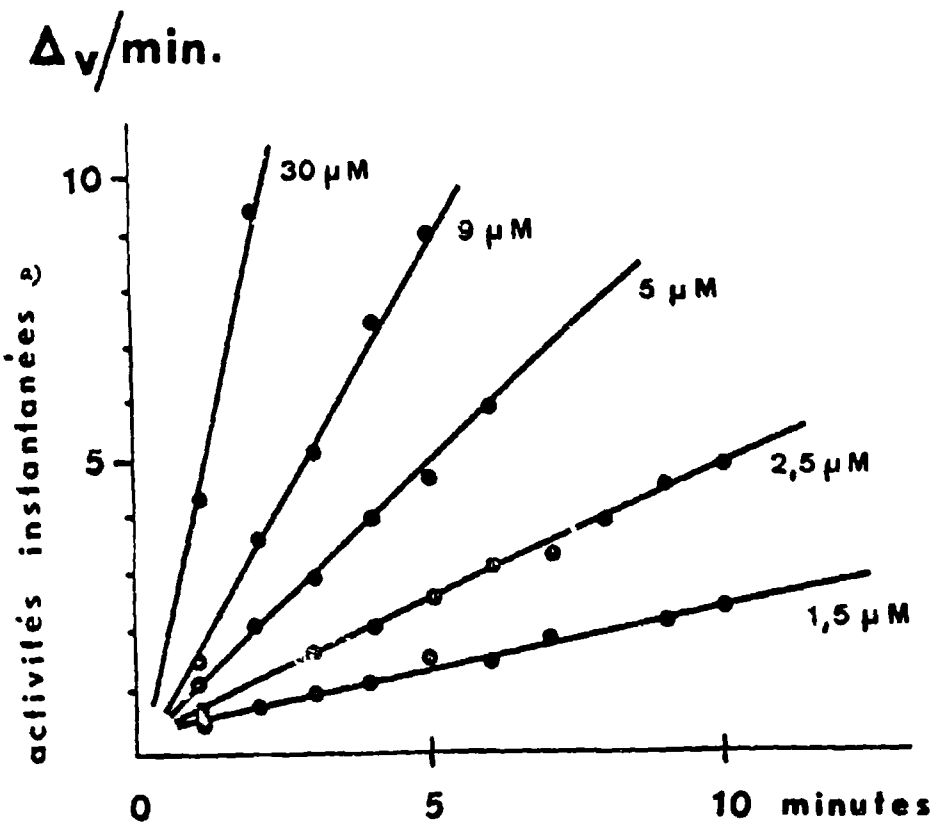


Fig. 17. Study of the Effect of the Zn^{++} concentration Added to the Apo-D-LDH on the Speed of the Catalyzed Reaction. (Iwatsubo.)

/Legend:/ a) instantaneous activities.

The Apo-D-LDH (prepared by acid treatment), fixed concentration, is added, at time zero, to a spectrophotometric vessel containing: ferricyanide 0.66 mM; D-lactate 66 mM, phosphate buffer 50 mM, Zn^{++} in indicated concentrations. The reaction is followed during this time by measuring the E_{420} every 10 seconds. Slope Δv/min. determined at time t on the exponential curve gives the activity at instant t.

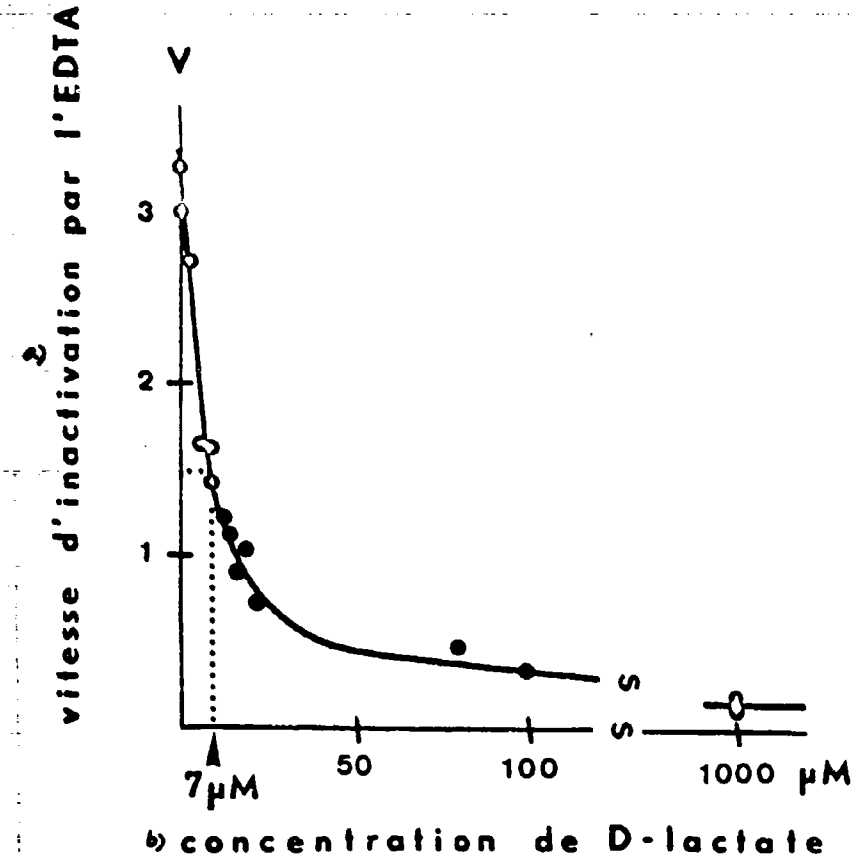


Fig. 18. Protection by D-lactate of the Inactivation of D-LDH by EDTA. (Stachiewicz, Labeyrie, Curdel and Slonimski.) Measurement of the speed of inactivation by EDTA at 30°; vessels contain: phosphate buffer 200 µmols, EDTA 0.3 µmol, D-lactate in variable quantities corresponding to the concentration indicated on the abscissa; D-LDH is added at time zero, volume 2.6 ml. After "t" minutes, addition of DL lactate 30 µmols, ferricyanide 2 µmols, final volume 3 ml, and the residual activity is measured on the basis of which the speed of inactivation V is measure.

/Legend:/ a) speed of inactivation by the EDTA; b) concentration of D-lactate.

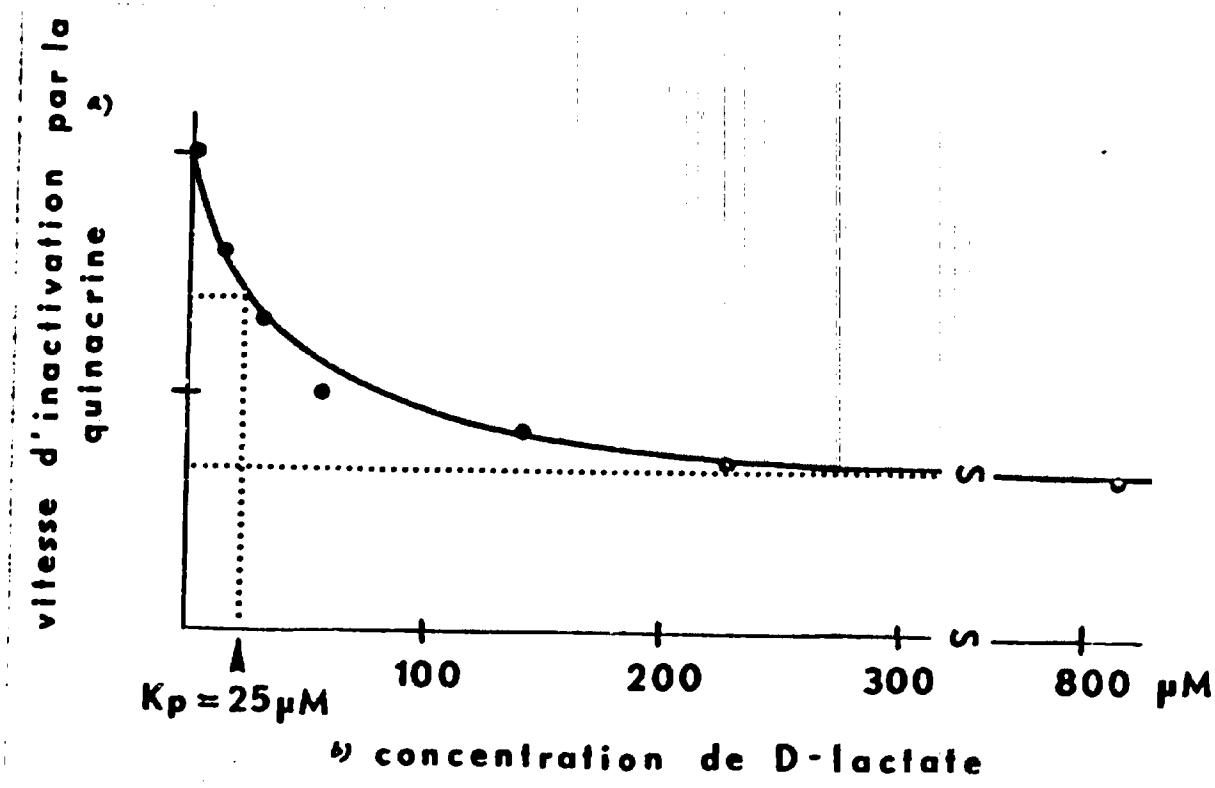


Fig. 19. Protection by D-lactate of the Inactivation of D-LDH by Quinacrine. (Iwatsubo and Labeyrie.)

/Legend:7 a) speed of inactivation by quinacrine; b) concentration of D-lactate.

Measurement of the speed of inactivation by quinacrine at 30°; a vessel contains phosphate buffer 200 μ mols; quinacrine 1 μ mol; D-LDH (fixed concentration); D-lactate in variable quantities; D-LDH added at time zero, volume 1 ml. After time "t", addition of DL lactate 50 μ mols; 2,6 dichlorophenolindophenol 50 μ mols, final volume: 3 ml. The speed of reduction of the pigment measures the resiactivity on the basis of which the speed of inactivation V is calculated.

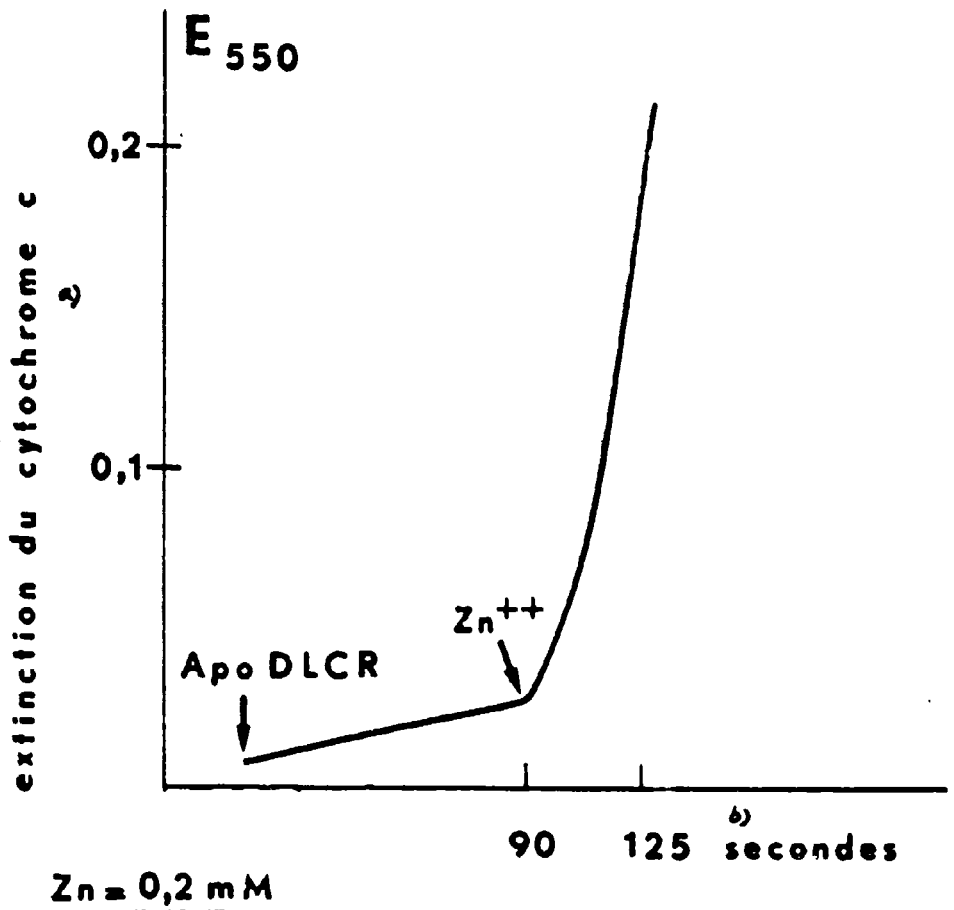


Fig. 20. Reactivation by Zn^{++} of the Apo-D-LCR Prepared by Precipitation in an Acid Medium. (Iwatsubo and Isomoto.) In a vessel containing: phosphate buffer 50 mM, pH 6.4, cytochrome C of yeast (iso 2) 20 μM; D-lactate 10 mM and the apo-D-LCR prepared at pH 2.5, the reduction of the cytochrome C is followed for 80 seconds; then Zn^{++} 0.2 mM is added and we continue to follow the reaction.

/Legend:/ a) extinction of cytochrome C; b) seconds.

	iso-1	iso-2
S_{20}, ultracentrifugation	1,7 ± 0,1	1,7 ± 0,1
a) Nombre résidus aa, analyse	107 ± 2	107 ± 2
ε mM, b) pic α, relatif au Fer	29 ± 1	29 ± 1
λ, pic α, ferrocyt. (mμ)	549,4	548,8
c) Potentiel redox (Em 7)	247 ± 5	247 ± 5
d) Groupe prosthétique	protoheme	
Hémododécapeptide (Δ)	(leu; val)	(ileu; glu)
C-terminal	glu	lys
e) Teneur relative	95 %	5 %

Fig. 21. Comparison of the Properties of the Isocytochromes C of the Same Yeast Cell. (Slonimski, Acher, Pere, Sels and Somlo [47].)

[Legend:] a) Number of a. a. residues, analysis; b) peak, in relation to iron; c) redox potential; d) Prosthetic group; e) relative content.

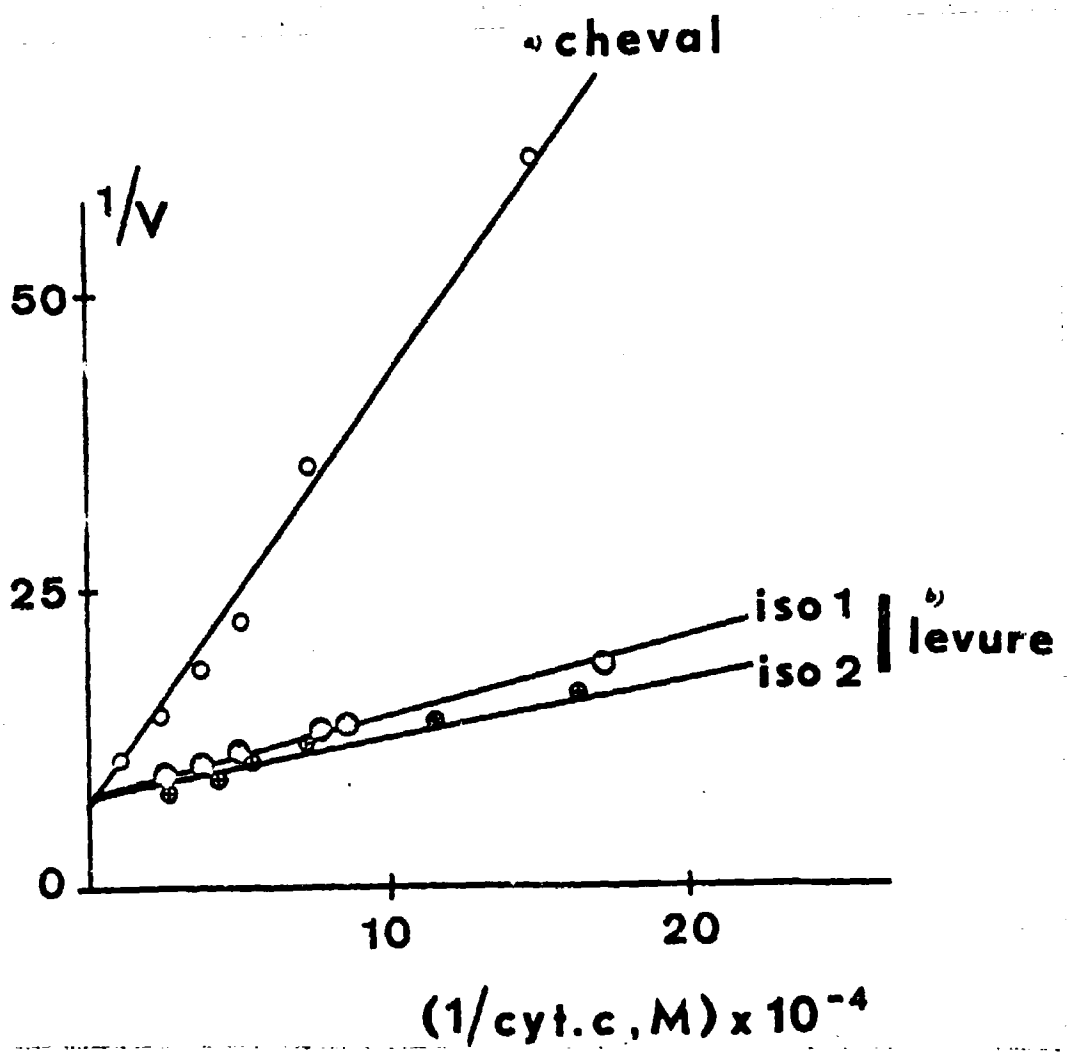


Fig. 22. Speed of Reduction of Two Isocytochromes C by L-LDH. (Slonimski, Acher, Pere, Sels and Somlo /47/.)

/Legend:/ a) horse; b) yeast.

Initial speed of reduction in the presence of L lactate 6.7 mM. By way of comparison, the cytochrome C of horse heart (Sigma, type III).

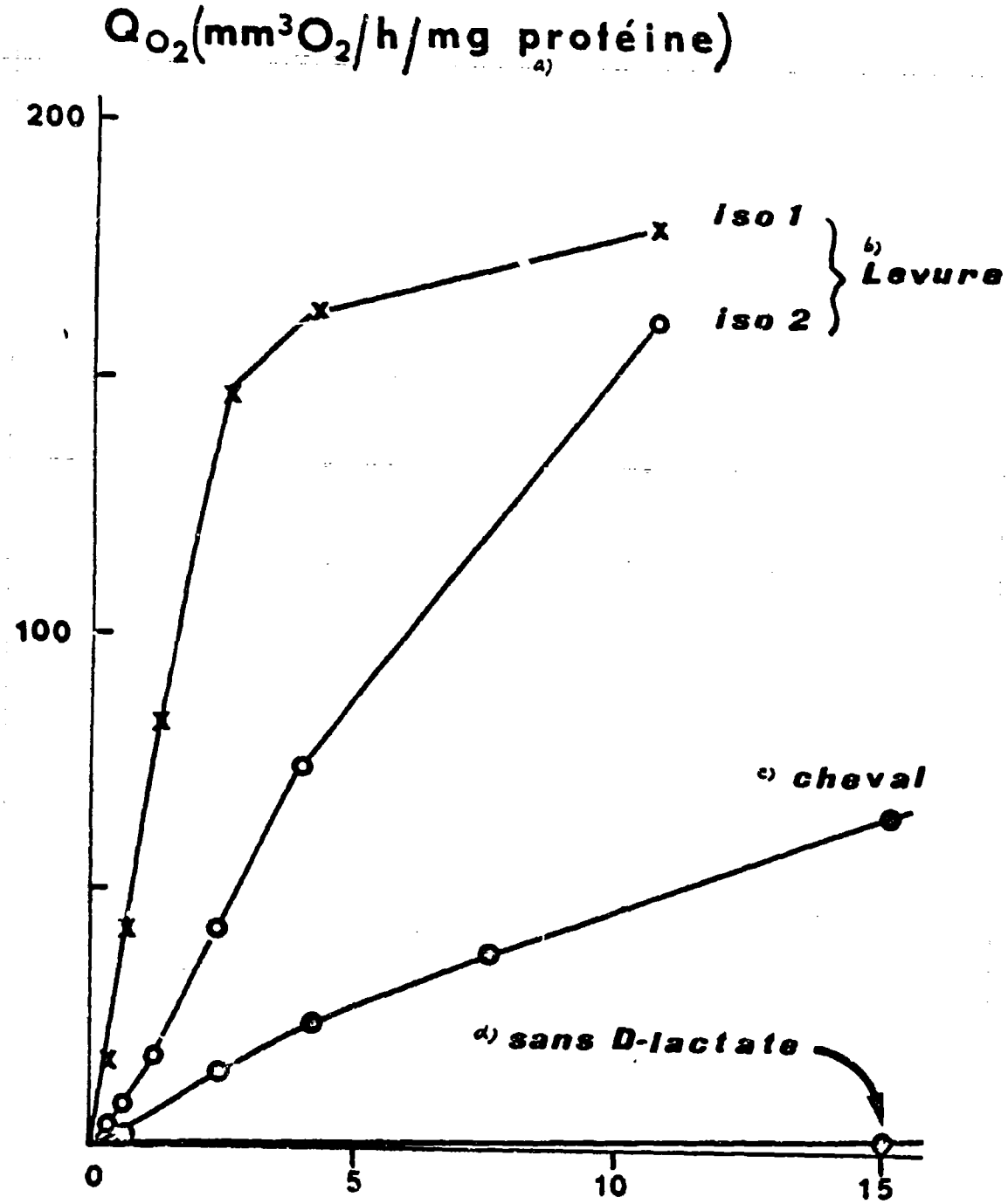

Q_{O_2} ($mm^3 O_2/h/mg$ protéine) a)
200
100
0
5
10
15
iso 1
iso 2
b) Levure
c) cheval
d) sans D-lactate
e) Concentration Cytochrome c
$(10^{-6}$ M)

Fig. 23.

Fig. 23. Catalysis by the Isocytochromes C of the Respiration of the Mitochondrias at the Expense of D Lactate. (Slonimski, Acher, Pere, Sels and Somlo /477.)

/Legend:7 a) protein; b) yeast; c) horse; d) without D-lactate; e) Cytochrome C Concentration;

The mitochondrias obtained by crushing in a Nossal apparatus are suspended at pH 7.2 in Tris 40 mM, kH_2PO_4 5mM, ADP 1 mM, EDTA 0.25 mM, $MgSO_4$ 2 mM, TPP 0.15 mM, DPN 0.2 mM, D lactate 25 mM. The speed of respiration, constant in time, is measured manometrically at 28° C. By way of comparison, the cytochrome C from horse heart (Sigma, type III).

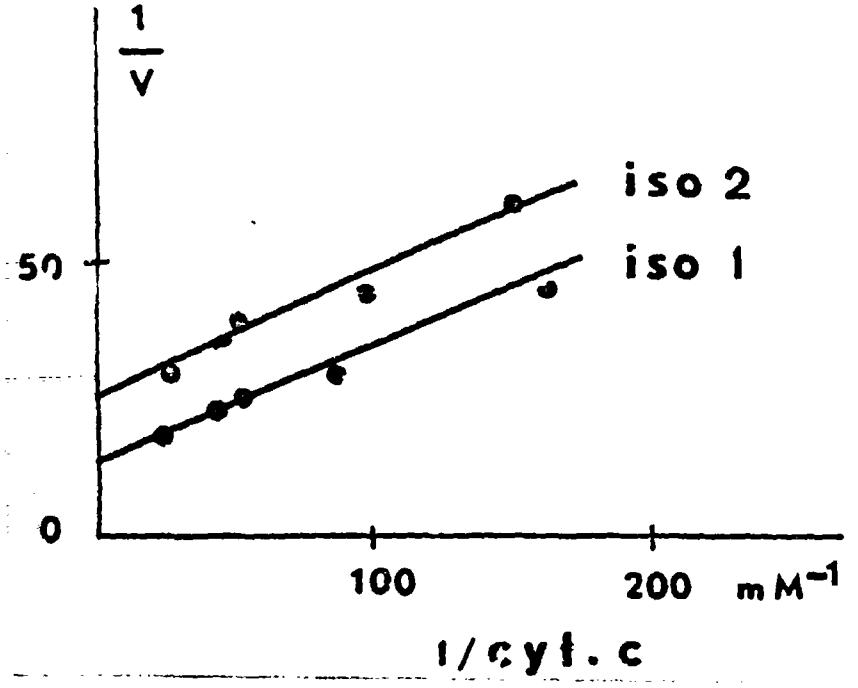


Fig. 24. Speed of Reduction of Two Isocytochromes C by the D-LCR. (Slonimski, Acher, Pere, Sels and Somlo /477/.)

Initial speed of the reaction in the presence of D lactate 6.7 mM.

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