MICRODETERMINATION OF ACID PHOSPHATASE

A STUDY OF THE REACTION OF KING AND ARMSTRONG*

by

RAYMOND G. GOTTSCHALK**

Departments of Biochemistry and of Pathological Anatomy, University of Liège (Belgium)

KING AND ARMSTRONG have introduced the use of disodium phenylphosphate as a substrate for the determination of phosphatase activity[1]; the liberated phenol is measured with the reagent of FOLIN AND CIOCALTEU[2]. The method has gained wide acceptance, and different technical modifications have been proposed[3, 4, 5, 6]. The GUTMANS[7] adapted it to the measurement of the phosphatase with maximum activity at acid reactions. Nearly all the clinical studies on the normal and pathological levels of acid phosphatase have been made with the aid of this method. As the mechanism of the increase of this enzyme in the serum of patients with disseminated prostatic cancer deserves further study, it seems advisable to describe a method permitting the dosage of this enzyme on small samples of sera or on isolated tissue sections with a phenylphosphate substrate. The results can thus be expressed in the units currently used by the clinicians.

Glycerophosphate[8], hexosephosphate[9] and p-nitrophenylphosphate[10] have been used in previously published micromethods for alkaline phosphatase. KING AND DELORY[11] have, however, shown that the rate of enzymic hydrolysis and the optimum pH depend upon the nature of the substrate. The results are also modified by the concentration of the substrate, the incubation time and the nature of the buffer[3, 12]. A simple change of buffer might alter the specificity of the method for phosphatases originating in different cells, as the GUTMANS consider that the acid phosphatase of extraprostatic origin is excluded by the use of citrate rather than of acetate buffer[7]. Slight changes in the method are thus liable to modify the results as well as the unit.

In this micromethod we have thus adapted the original techniques of KING AND ARMSTRONG and of GUTMAN for use with smaller amounts, but without fundamental changes. The conditions of these reactions had however to be studied and precised for proper microchemical application.

PROCEDURE OF MICRODETERMINATION

The reaction is carried out with the microtubes and micropipettes of LINDERSTRØM-LANG AND HOLTER[13]. The preparation of the reagents has been described by KING AND ARMSTRONG[1] and GUTMAN[7].

Ten μl of serum are incubated at 37.5° C with 200 μl of phenylphosphate solution buffered by citrate (or by barbital for alkaline phosphatase). The reaction is stopped after one to three hours by the addition of 90 μl of reagent of FOLIN AND CIOCALTEU diluded 1 to 3. The precipitated proteins are centrifuged off. About 175 μl of the supernatant are transferred to another tube with a micropipette, and an equal volume of a 5% sodium carbonate solution is added with the same micropipette, which is thus rinsed before each transfer. The color developed after 50 to 90 min at 37.5° C, or after 12 to 20 hours at room temperature, is read against water in the 1 cm microcuvette, containing 200 μl, of a Pulfrich spectrophotometer (red filter S 72). The readings are made at time intervals similar to those separating the preparation of the different tubes. Blank and standard solutions are worked up at the same time.

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** Fellow of the Donner Foundation, Cancer Research Division, Philadelphia, Pa.
Present address, Veterans Administration Hospital, Dallas, Texas, U.S.A.

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Values obtained by this method on 10 μl serum do not show more variation than by the usual method of KING AND ARMSTRONG, and they agree closely with it. Results on quadruplicate samples were for instance: 1.53, 1.52, 1.55, and 1.48, average 1.52 micrograms phenol; or 1.27, 1.13, 1.24, 1.15, average 1.20 micrograms phenol for 10 μl serum.

For determinations in tissues, the specimen is frozen with dry ice, and sections, 20 micra in thickness and about 3 mm in width, are cut with a rotary microtome in a freezing chamber at $-20°$ C[14]. The numbered sections are placed in individual microtubes and dessicated in the frozen state over phosphorus pentoxide. Alternate sections are kept for histological study. The weight of the dry sections is measured on a quartz fiber scale[15] or on a microbalance. A single weighed section is introduced into a droplet of 10 μl of water, shortly extracted, and the usual amounts of substrate and of reagents are added to it. For tissues of high enzyme content, larger amounts of substrate and reagents must be used with a single section.

ESTIMATION OF LARGE AMOUNTS OF PHENOL

As the amount of enzyme in tissues varies very widely, and as no sampling is possible in microtubes, it may be necessary to interpret the development of a color falling outside the usual range of colorimetric readings. The color produced per unit of phenol

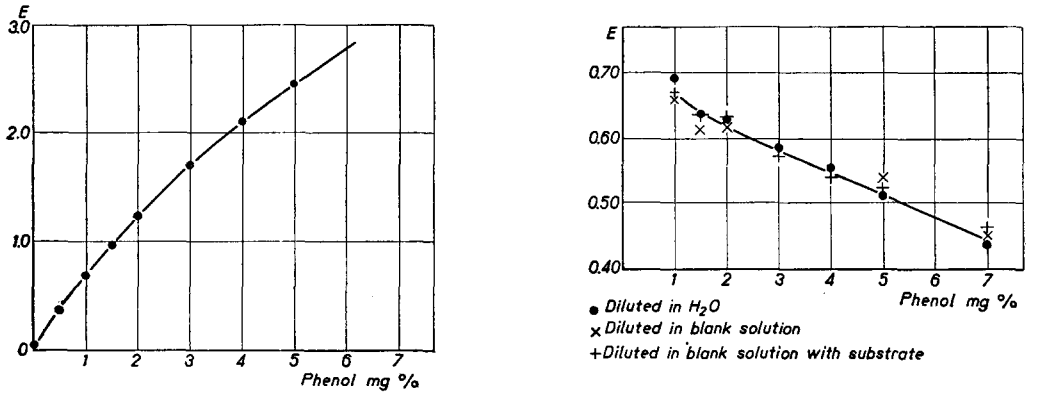

Fig. 1. Color produced by concentrated phenol solutions and reagent. a) *Left:* Extinction values of concentrated solutions (direct readings). 3 ml phenol reagent dil. 1 : 4 + n ml phenol solution 10 mg % + H_2O ad 10 ml; + 10 ml Na_2CO_3 solution. b) *Right:* Extinction values per mg phenol in 100 ml original solution after dilution in water or blank solution.

decreases as its concentration increases (Fig. 1a). This is not only due to the inaccuracy of BEER-LAMBERT's law for strong extinctions, but less of the colored complex is also formed when the proportion of the phenol to the reagent decreases. This is shown by the serial dilutions in water of the highly colored solutions (Fig. 2). All these dilutions follow BEER-LAMBERT's law up to extinction values of 1.2, but a different line describes the dilutions of each concentrated solution.

In the experiment described in Fig. 1b, the colored solutions were diluted with "blank" solutions containing the reagent, sodium carbonate and water, with or without substrate. This did not allow the color to develop beyond the point already reached, and similar values were obtained after dilution in water.

Thus dark solutions should be diluted with water before photometric readings. The extinction value, calculated by multiplying the observed extinction by the dilution coefficient, is corrected by the factor given in Fig. 3. A black precipitate is formed in tubes containing a very high concentration of phenol.

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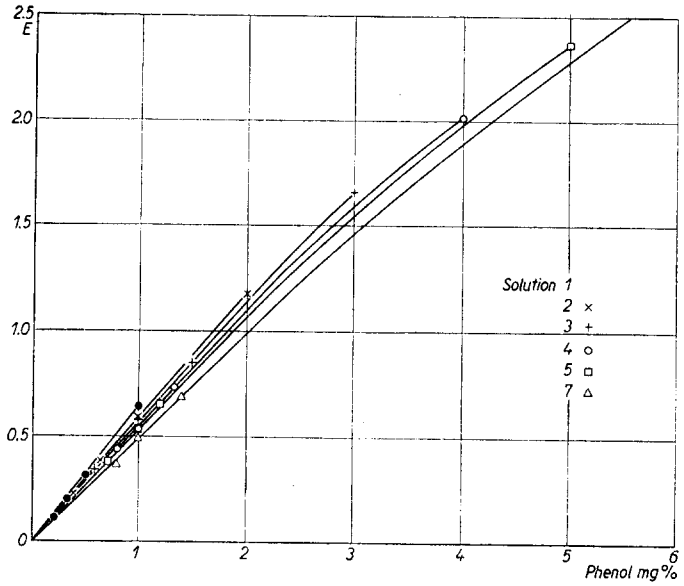

Fig. 2. Extinction values produced by serial dilutions of concentrated phenol solutions. 3 ml phenol reagent dil. 1 : 3 + n ml phenol solution 10 mg % + H_2O ad 10 ml; + 10 ml 5 % Na_2CO_3. The solutions 1,2, etc. are prepared with the corresponding number of ml of phenol solution; serial dilutions are made with water.

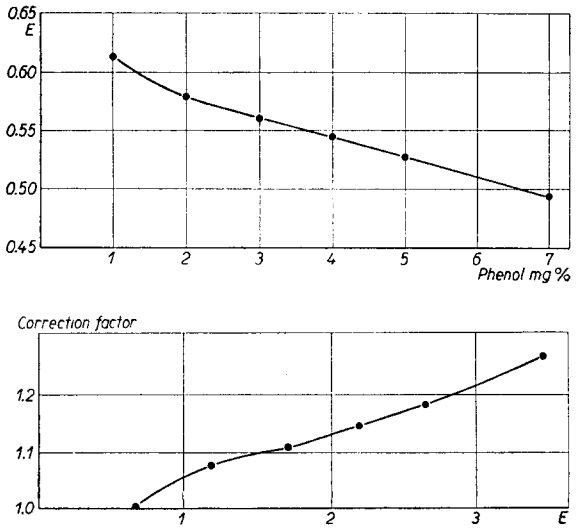

Fig. 3. a) *Above:* Extinction values per mg phenol in 100 ml original solution after dilution in water. Data as in Fig. 2. b) *Below:* Relation between theoretical and observed values of E for high concentrations of phenol in original solution (= correction factor). Data as in Fig. 2 and 3a.

SPEED OF ENZYMIC REACTION

That the results of the tubes with large amounts of phenol need not be discarded is shown by the experiment summarized in Figs 4 and 5. The substrate of p_H 4.9 was incubated in the presence of dilutions of an extract of human prostate gland in saline.

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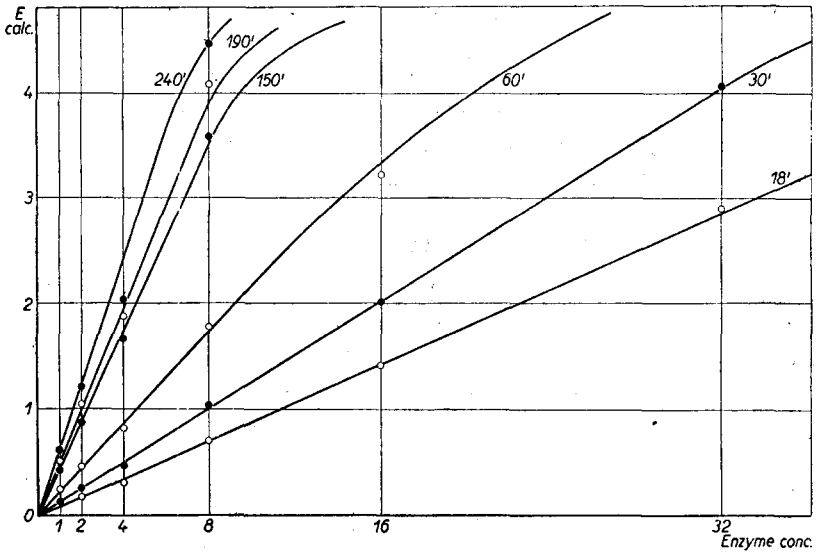

Fig. 4. Relation of phosphatase activity to enzyme concentration. 15 ml substrate pH 4.9 + 0.75 m diluted prostate extract; incubation at 37.5° C; 2 ml sample + 0.858 ml reagent dil. 1 : 3; + 2.858 ml 5% Na_2CO_3. The extinction values have been corrected by the factors for high amounts of phenol given in Fig. 3; the values of the blanks have been subtracted. Enzyme concentrations in arbitrary units. The figures indicate the duration of the incubation. Values of extinction above 4.5 have not been included in the graph.

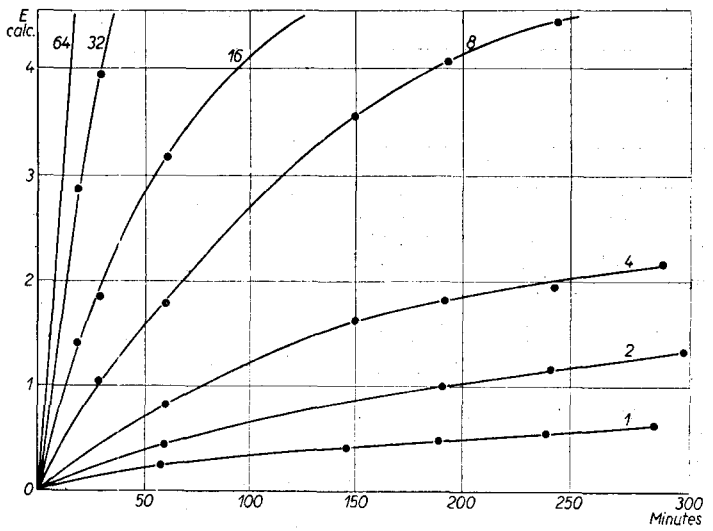

Fig. 5. Relation of phosphatase activity to duration of reaction. Data as in Fig. 4. The figures near the lines refer to the concentration of the enzyme in arbitrary units.

Samples were taken at intervals, and the reactions were allowed to proceed so far as to give highly colored solutions after addition of the reagents. When the extinction values are corrected by the factor given in Fig. 3, the speed of the reaction is proportional to the enzyme concentration up to calculated extinction values of 3.3. The regression lines slope down only at the level where the black precipitate appears in the

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tubes (Fig. 4). About one sixth of the total amount of substrate has been split by the enzyme when these values are reached.

If the amount of phenol liberated is plotted against the time, the lines are curved for the whole length of the reaction; this is observed for low as well as for high enzyme concentrations (Fig. 5). The enzyme is thus partly destroyed as the reaction proceeds. Even at ice box temperature a solution of acid phosphatase deteriorates rapidly.

FIXATION OF THE REAGENT BY PROTEINS

KING AND ARMSTRONG use the phenol reagent to precipitate the proteins and to stop the enzymic hydrolysis. This may appear unorthodox, inasmuch as the color given by phenol varies with the relative concentration of the reagent. In fact the presence of the normal amount of serum causes about a 12% decrease of the colored complex formed by phenol. If three times as much serum is used, all the reagent is fixed on the yellow protein precipitate, and the colorless supernatant fails to become blue upon addition of sodium carbonate (Fig. 6). The phenol remains free in the supernatant, where its presence can be demonstrated by adding more reagent. Inversely the addition of phenol fails to demonstrate any free reagent in the clear supernatant left after protein precipitation. Thus the proteins fix only the reagent, and not a preformed phenol-reagent complex as suggested by BUCH AND BUCH[4].

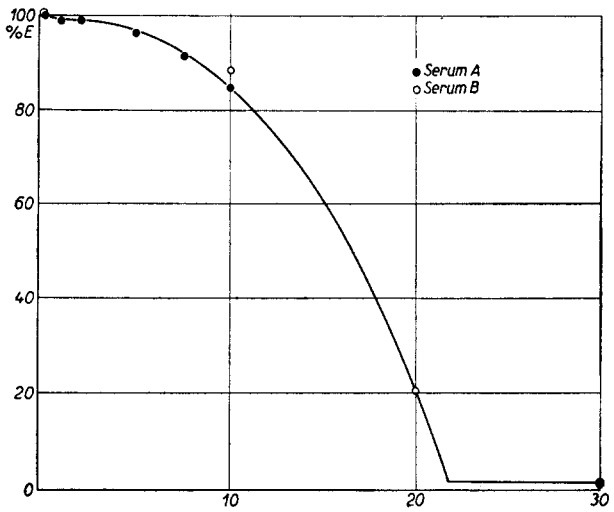

Fig. 6. Ratio of extinction readings to the theoretical values of extinction produced by phenol in the presence of serum. n μl serum + 50 μl phenol solution 10 mg % + 90 μl phenol reagent diluted 1 : 4 + H_2O ad 300 μl; supernatant + equal amount of Na_2CO_3 solution.

The amount of reagent fixed by the protein precipitate is larger if the reagent is added to a solution containing the serum, than if the serum is added to a solution of the reagent.

In the first case the supernatant may have become colorless, when in the second case it is still yellow; the precipitate is bulky for the first sequence, dense and small for the second one. The color given by a mixture of phenol, reagent* and three times the usual amount of serum, is only one fifth of the expected value if the reagent is added to the serum, but reaches 84% after the contrary sequence. The theoretical values are obtained in both cases if an excess of reagent is added to the supernatant. The differences between both sequences would of course be eliminated by proper stirring during the addition of the products; this will seldom be the case, as the reaction is currently made in test tubes.

In the reaction of KING AND ARMSTRONG a different sequence is followed for the incubated and blank specimens. Thus, because of the protein precipitation, there is about a 12% decrease of color formation in the incubated tubes, but the decrease is smaller in the blank specimens.

The importance of these causes of error in the clinical determination of serum phosphatase activity cannot be minimized; they are only partly eliminated by the facts

* Used at dilution 1 : 3 in this experiment.

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that the protein contents of sera do not vary widely, and that the accepted normal and pathological values of serum phosphatase activity have been established by this method.

On the other hand all tissues have a high enzyme activity as compared to their protein content, which thus becomes negligible as a source of error. A linear relationship between enzyme concentration and speed of the reaction was established with dilutions of a prostate extract (Fig. 5), but this would not have been possible by using different amounts of serum.

INFLUENCE OF P_H AND TIME ON COLOR FORMATION

In the methods of KING AND ARMSTRONG and of GUTMAN, the color is read before it has reached its maximum intensity. Considerable errors are introduced by small differences of timing, which are still more difficult to avoid in microchemistry where the tubes must be handled separately. The amount of sodium carbonate and the dilution of the reagent differ also according to the author.

In the reaction described by FOLIN AND CIOCALTEU[2] there is about 4.5 times as much saturated sodium carbonate as reagent left after neutralization of the sulphuric acid. KING AND ARMSTRONG use 2.5 parts of 20% sodium carbonate for 3 parts of reagent diluted 1 : 3, but KOLMER AND BOERNER[16] indicate the same amounts for the reagent diluted 1 : 4. FOLLEY AND KAY[3] advocate the use of about three times as much 14% sodium carbonate as reagent.

In the experiments summarized in Figs 7 and 8, we have attempted to find the conditions fitting best the requirements of microchemistry. As already noted by FOLLEY AND KAY, the color develops more rapidly with increasing amounts of carbonate or at higher temperatures. But the colored complex is also destroyed by large amounts of alkali. Thus high concentrations of carbonate produce a maximum during too short a time for microchemistry. Smaller concentrations give a broader and higher plateau which appears later. With small amounts of carbonate the colored complex is formed extremely slowly.

The optimum amounts seem provided by adding to the mixture containing the reagent diluted 1:3 an equal amount of 5% sodium carbonate solution. This proportion is equivalent to that of KING AND ARMSTRONG, but the readings must be made when the plateau is reached, *i.e.*, after about one hour at 37.5° C, or overnight at room temperature. The color developed in the water

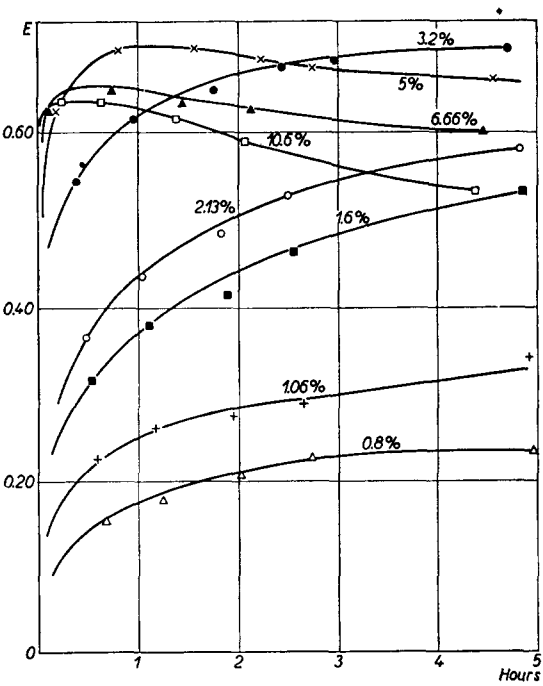

Fig. 7. Color development by sodium carbonate solutions of different concentrations at 37.5° C. 1 ml undiluted phenol reagent + 1 ml phenol 10 mg % + 8 ml H_2O; + 10 ml Na_2CO_3 solution.

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bath remains stable for several hours when the tubes are kept at room temperature.

The speed of the color formation seems to depend upon the final P_H of the solution rather than upon the concentration of carbonate. The curves of P_H and of color development follow each other closely, and they show the same S-shaped outline due to the buffering action of the reagent in the neighbourhood of $p_H 7$ (Fig. 9).

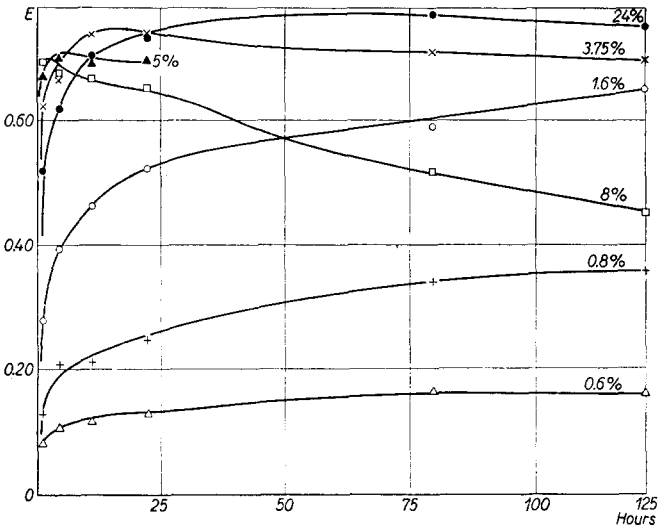

Fig. 8. Color development by sodium carbonate solutions of different concentrations at 20° C. 3 ml phenol reagent diluted 1 : 4 + 1 ml phenol 10 mg % + 6 ml H_2O; + 10 ml Na_2CO_3 solution.

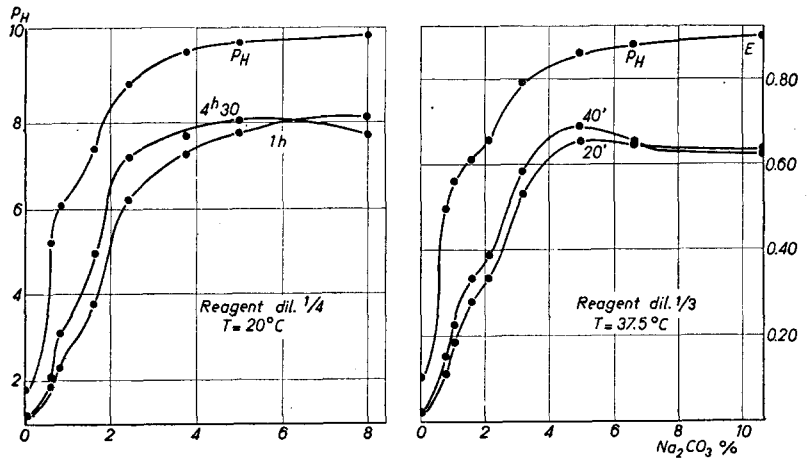

Fig. 9. Color obtained with sodium carbonate solutions of different concentrations; final p_H of these solutions.

APPLICATION TO PROSTATE TUMORS

A prostatic fibroadenoma contained 4200 units of acid phosphatase* per gram dry weight. This activity is so high that the test is run on a single microtome section with

* The unit of acid phosphatase defined by GUTMAN is the amount of enzyme liberating one milligram of phenol per hour (and not per half hour as the KING AND ARMSTRONG unit of alkaline phosphatase).

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four ml of substrate. On the contrary a sarcoma of the prostate devoid of glandular elements contained only 36 units per gram dry weight.

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SUMMARY

A micromethod is described for the determination of acid phosphatase activity with a phenylphosphate substrate on 10 µl of serum or on isolated microtome sections. There is a linear relationship between the concentration of enzyme and the amount of phenol liberated, even for high values of the latter. The relation with the time is not linear. When the reaction of KING AND ARMSTRONG is used for sera, part of the reagent is fixed by the proteins, and this proportion varies in the blanks and in the incubated specimens. The corresponding variations of color formation are an important cause of error in the clinical determinations on sera, but this error is negligible for tissue studies. The rate of color development increases with the pH and the temperature. These factors are discussed for proper application of the micromethod to enzyme determinations in tissue sections.

RÉSUMÉ

Description d'une méthode de dosage de la phosphatase acide avec un substrat de phénylphosphate sur 10 µl de sérum ou sur des coupes isolées de tissu. La quantité de phénol libérée est proportionnelle à la concentration de l'enzyme, même pour de fortes hydrolyses. Par contre, la relation avec le temps n'est pas linéaire. Si la réaction de KING ET ARMSTRONG est utilisée pour des sérums, les protéines fixent une partie du réactif, et cette proportion varie pour les tubes témoins ou incubés. Ceci entraîne des erreurs importantes dans les déterminations cliniques sur les sérums, mais non dans les études sur les tissus. La vitesse de formation du produit coloré augmente avec l'alcalinité et la température.

ZUSAMMENFASSUNG

Beschreibung einer Phosphatasenbestimmungsmethode in 10 µl Serum oder in isolierten histologischen Schnitten. Die Hydrolyse ist, auch bei grösseren Phenolmengen, der Enzymekonzentration, aber nicht der Zeitdauer proportional. Wenn die KING UND ARMSTRONG Reaktion auf Sera angewandt wird, verursacht die Bindung des Reagens an die Proteine grosse Fehler; diese sind bei Gewebestudien unbedeutend. Die Geschwindigkeit der Farbenbildung wird grösser mit pH und Temperatur.

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