A CRITICAL EXAMINATION OF THE HISTOCHEMICAL DEMONSTRATION OF THE ALKALINE PHOSPHOMONOESTERASE

by

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INTRODUCTION

GOMORI[1] has—practically simultaneously with TAKAMATSU[2]—described a method to show the presence of phosphomonoesterase I—the so-called alkaline phosphatase—in tissue sections. The sections are placed in a solution containing a phosphoric acid ester and Ca^{++}ions. The phosphatase liberates PO_4^{---}ions from the ester, which with Ca^{++} ions yield the insoluble calcium phosphate. Hence the precipitate of calcium phosphate is formed in places where the enzyme happens to be present. The calcium phosphate is afterwards rendered visible by transformation into silver phosphate, which substance can be decomposed by light or by transforming it into cobalt phosphate, which can be converted into brown cobalt sulphide.

It is often recommended to let the ferment reaction take place at $p_H = 9.4$ (or still higher), as it is desirable to make use of the optimum p_H of the phosphatase reaction. The solubility of the calcium phosphate that has been formed is also dependent on the p_H. Finally GOMORI[3] recommends addition of $MgSO_4$ as an activator.

The paraffin sections of objects fixed in alcohol or acetone are stretched on warm water in the usual way, placed on a slide and dried in a thermostate or (better) an exsiccator. After deparaffining in xylene, they come, after passing through ethanol (descending concentrations) and distilled water, into the substrate solution at 37° C.

In order to check this process some other sections are put in a solution containing $Ca(NO_3)_2$ instead of substrate, for the rest they are treated analogously. In this manner insoluble Ca-compounds which have not been formed by the action of phosphatase are shown to be present or absent.

An investigation of the localization of the phosphomonoesterase I in the kidney showed that the pictures obtained by this method were not constant. We had the impression that with numerous nephrons the proximal part was incompletely stained and moreover in those parts in which the reaction could be called successful, the localization was mostly diffuse and blurred. The gradation of the blackening in various sections of the same object—also if these sections have at the same time been treated with the same substrate solution—varied considerably. We suppose that errors were already committed before the sections came into the substrate.

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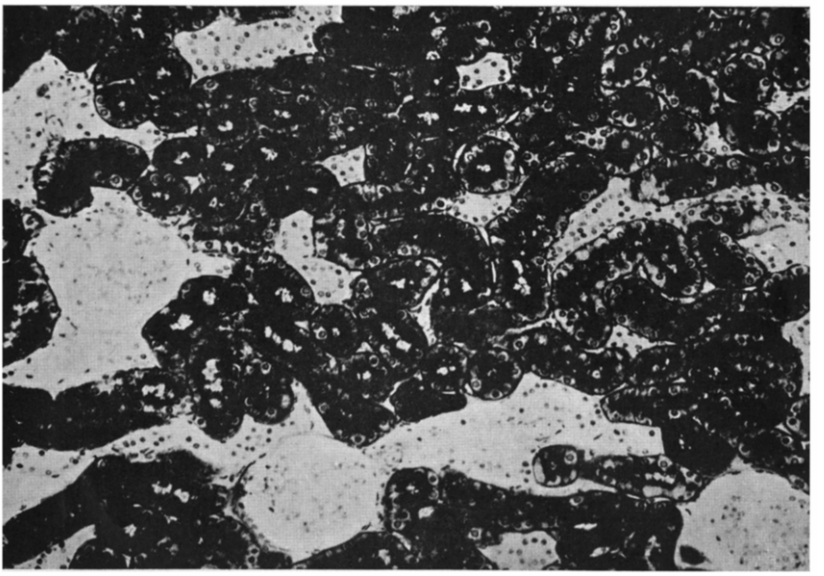

Fig. 1. Kidney of the guinea pig. Section of 6 μ placed on a slide and transferred to the buffered substrate of pH 9.4 at 32° C for 20 hours

EXPERIMENTAL PART

1. *Fixation*

The sections were fixed in alcohol of 80%. This proved to be the best fixative (see also DANIELLI[4]).

2. *Substrate solution*

Most authors choose Na-β-glycerophosphate and $Ca(NO_3)_2$ (GOMORI) for a substrate solution. The p_H is adjusted with a buffer, if necessary.

3. *Enzyme preparation*

To demonstrate that under these circumstances phosphomonoesterase I really reacts as described we carried out the following experiment. From extract of the kidney of a cow we made a purified preparation of alkaline phosphatase in a manner which has been described elsewhere[5]. We obtained the ferment as a white powder, which was washed out with ethanol 96% and subsequently suspended in ethanol. Now celloidin was added and after drying the whole was embedded in paraffin, of which "sections" were cut. As a matter of fact the blackening could be obtained in the way mentioned. A preparation in the control solution remained negative.

4. *Diffusion of the enzyme in the preparation*

The following investigations were carried on with kidneys of the rat, mouse and guinea-pig. The kidney is an organ, the cortex of which, in contrast with the medulla, is rich in alkaline phosphatase, as biochemical research has proved[6, 7]. It has been described histochemically by a number of investigators, who particularly call attention to the localization of the ferment in the proximal part of the nephron.

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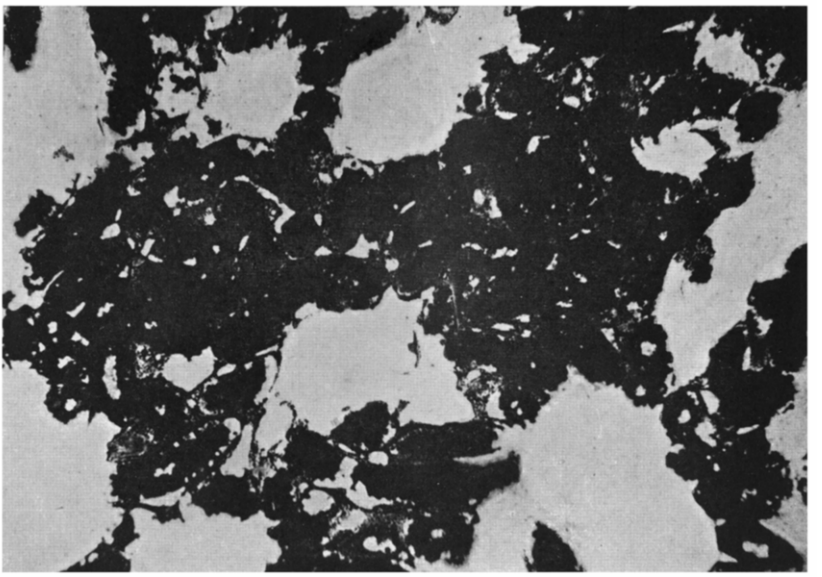

Fig. 2. Technique the same as in Fig. 1. but placed in ethanol 30% for half an hour before transferring to the substrate

Of a number of sections 6 μ thick part were mounted on slides freed of paraffin and again placed in water and part were transferred to the substrate solution at 32° C in a non-deparaffined condition. For these experiments we used an unbuffered solution of Na-β-glycerophosphate and calcium nitrate (cf. GOMORI[1]). The non-deparaffined sections float on the surface of the warm fluid and stretch themselves. In principle this technique was derived from the data given by JACKSON[8], who wanted to reattain the mutual cohesion of the structural parts in a tissue section during the action of trypsin.

After termination of the reaction we allowed the substrate solution to cool down to room temperature. The sections were washed in a calcium nitrate solution (0.5%) and then in distilled water. Subsequently the sections were placed in a 0.5% solution of silver nitrate and irradiated with u.v. light of a mercury lamp till the desired blackening was reached. During all these manipulations the sections float on the surface of the fluid. After termination of the histochemical reaction the sections were mounted on slides and dried. Removal of superfluous $AgNO_3$ and colouring, if any, of the sections took place with the deparaffined sections.

With this modified method we obtained much more constant pictures than with the deparaffined mounted sections treated in accordance with the original method. In addition to the proximal part of the nephron the cells of the basal layers of the pelvis epithelium also show a finely grained blackening.

A more or less identical result is obtained if the sections are deparaffined and mounted, but are then transferred directly from the xylene to the substrate solution, that is to say without letting them pass through the series of descending ethanol concentrations. As is well known the activity of the enzyme is retained after the treatment with ethanol. The transfer of the sections from ethanol 96% to water must be the cause of the varying pictures and atypical localization of the enzymically formed calcium phosphate. The chemical examination had shown that phosphomonoesterase

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Fig. 3. Technique the same as in Fig. 1, but placed in ethanol 30% for 24 hours before transferring to the substrate.

I is readily soluble in dilute ethanol (30–50%); it is more soluble in this medium than in water. If a non-deparaffined section is treated with water for 20 hours this does not influence the reaction. With 30% ethanol the greater part of the ferment can be extracted in 20 hours. By interrupting the reaction after the lapse of different periods one can observe that the solution of the enzyme is accompanied by a diffusion of the latter to the surrounding parts of the tissue. *By taking sections from xylene to water via various ethanol concentrations the true picture of the localization of the enzyme is disturbed.*

5. *Control preparations. Inactivation of the ferment*

A further control was now the inactivation of the phosphatase. If the blackening is indeed exclusively caused by enzyme activity, sections in which this enzyme has been destroyed must be perfectly negative.

a) Destruction by heat. Sections are immersed in boiling distilled water for one minute. After this treatment they are perfectly negative. On shorter heating the activity appears to have strongly decreased.

It was also possible to destroy the phosphatase by heating the sections or thin fragments of tissue of material embedded in paraffin, these being kept dry (temperature about 80° C).

b) Destruction by irradiation. We already know from the chemical examination of organ extracts that by irradiation with u.v.light the enzyme is destroyed and loses its activity. If sections (non-deparaffined) are irradiated for 15 minutes with a mercury lamp at a distance of 50 cm no more blackening is to be seen.

6. *Control preparations. Inhibition of the reaction*

Finally we made the following control experiments:

The ferment reaction is inhibited with KCN-solution. Preparations that had been

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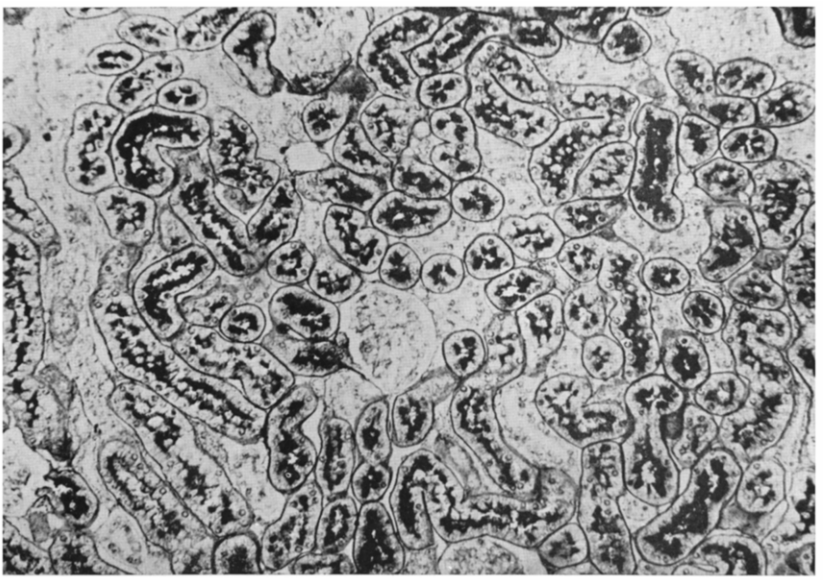

Fig. 4. Non-deparaffined section transferred to the buffered substrate

immersed in a substrate solution containing traces of KCN were practically negative.

7. Control preparations. Suppression of the substrate. The p_H of the reaction

It it obvious that the enzyme reaction should occur at optimum p_H. Accordingly GOMORI[9] recommends a $p_H = 9.4$. Later authors sometimes choose a still higher p_H. The optimum p_H of the alkaline phosphatase of the kidney is (with a high substrate concentration) 9.8. As a matter of fact a number of sections that had reacted at various p_H showed that the blackening at optimum p_H was more intense.

At $p_H = 9.4$ or higher in the non-deparaffined section not only blackening of the proximal part of the nephron had occurred but also of the other tubules both in the cortex and the medulla, of the glomeruli and also of part of the intertubular connective tissue.

The blackening was most intense in the proximal part, while that of the distal part of the nephron and that of the collecting tubules consisted of a thin and finely grained precipitate. It was typical of this reaction that the nuclei were strongly positive.

The picture is different with the mounted deparaffined sections, where the time of the substrate is much reduced. Now the blackening is chiefly localized in the proximal of the nephron: the cells contain a finely grained precipitate, which was diffuse and condensed especially in the neighbourhood of the cuticula and to a lesser degree of the basal part of the cell. In this preparation the nuclei in the cortical region were also positive. The latter not only applies to the epithelial cells, but also to those of the glomeruli and the intertubular cells of the connective tissue and to the epithelium of the pelvis. In the pelvis of the guinea pig only the basal layers of cells show a positive reaction. This bears out the observations of GOMORI[9].

For control purposes sections were placed into a $Ca(NO_3)_2$ solution (0.4%), which was brought to the desired p_H by means of a buffer. For the rest these control sections

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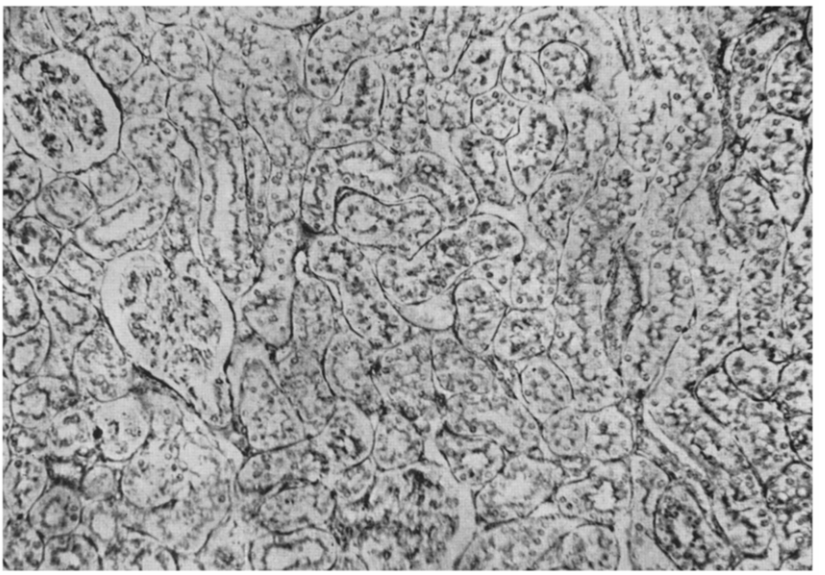

Fig. 5. Non-deparaffined section transferred to a buffered $Ca(NO_3)_2$ solution of p_H 9.4 at 32° C for 20 hours

were treated in perfectly the same way as the sections that came into the substrate. Now non-deparaffined control sections were negative at a p_H = 8.4, but at a p_H = 9.4 pictures were obtained here which were practically identical with the sections that had been treated with substrate at p_H = 9.4. Only the blackening was less intense.

From these experiments it may be inferred that at p_H = 9.4 precipitates of insoluble Ca-compounds are formed, of which it is not known for certain that they are due to phosphatase action. The pictures obtained at p_H = 9.4 do not give an accurate idea of the localization of the alkaline phosphatase.

By washing the sections beforehand in distilled water for 24 hours these disturbing substances could be eliminated. The sections were then only positive in the proximal part of the nephron.

On introducing sections in which the ferment had been destroyed by dry heating in a $Ca(NO_3)_2$ solution at p_H = 9.4 we found a blackening which was perfectly identical with that of the untreated sections. The same picture was obtained by placing the sections in the substrate solution of GOMORI at p_H = 9.4. The enzyme proved to have become inactive as the typical picture of the localization of the enzyme in the proximal part of the nephron is entirely absent. From this it is evident that the blackening which is formed in the non-deparaffined sections that had been in the GOMORI-solution at p_H = 9.4 is not entirely due to phosphatase activity.

8. *Some slight modifications*

As a substrate solution we have substituted Ca-glycerophosphate 0.4% for the mixture of Na-β-glycerophosphate and calcium nitrate. The preparation of the former is simpler and moreover there are no superfluous ions in the solutions.

For rendering visible the Ca-phosphate that had been formed we preferred the silver method to the cobalt method, because sections, both deparaffined and non-

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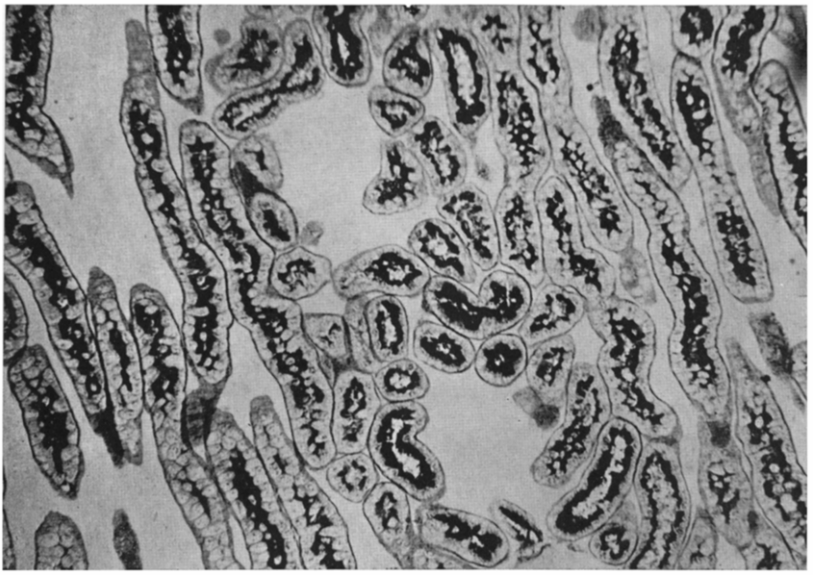

Fig. 6. Non-deparaffined section kept for 20 hours in distilled water at 32° C: subsequently transferred to the buffered substrate

deparaffined, which had been in a $CoCl_2$-solution for some minutes (without any previous treatment) and had afterwards been in an ammonium sulphide solution, turned out not be negative. The nuclei in particular adsorbed Co. LISON[10] also points to this and to the fact that ammonium sulphide transforms many metals (*e.g.*, Fe) into coloured sulphides.

As the conversion of $CaHPO_4$ into Ag_3PO_4 with $AgNO_3$ is not entirely quantitative, owing to the H^+-ions that are formed, we tried to transform the $CaHPO_4$ into Ag_3PO_4 with a saturated solution of silver acetate in 1 m sodium-acetate. The acetate ions present in this solution ensure a quantitative progress of the reaction by their buffering action. The blackening obtained in this way turned out to be more sharply localized as compared to the method that makes use of $AgNO_3$.

CONCLUSION FROM THESE DATA

As a substrate solution a 0.4% solution of Ca-glycerophosphate is very satisfactory.

Deparaffined sections which have been transferred from xylene to water via various ethanol concentrations show a diffusion of the alkaline phosphatase and give blurred pictures.

The localization of the blackening in non-deparaffined sections is very sharp.

Non-deparaffined sections already give a positive reaction at $p_H = 9.4$ on being placed in a $Ca(NO_3)_2$ solution (control sections). The nuclei are also black then.

At $p_H = 8.4$ the control sections are negative. Most nuclei in sections that have been in a substrate solution are negative at $p_H = 8.4$.

Although in the literature it is often stated that many nuclei contain phosphatase, we must doubt if this may be concluded from the histochemical examination.

When the reaction takes place at $p_H = 9.4$ most nuclei show blackening. By

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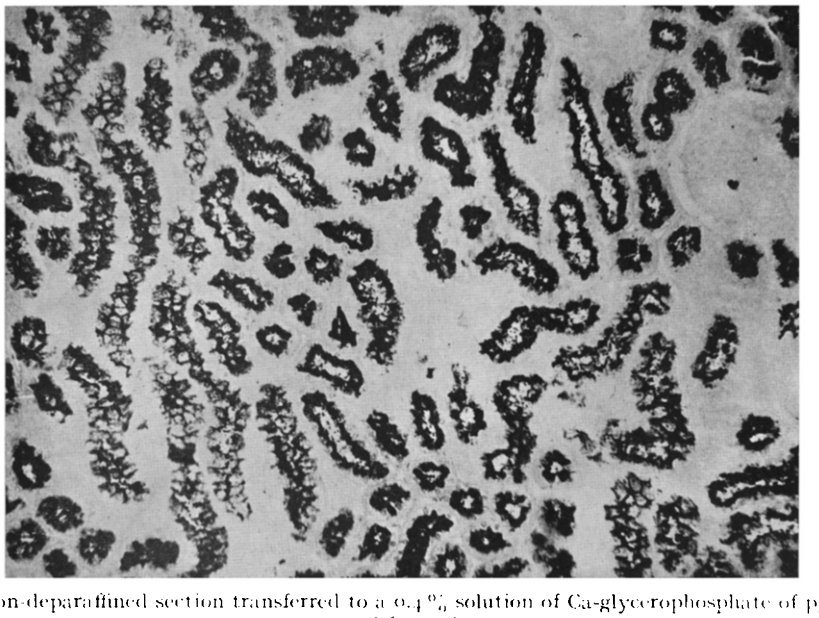

Fig. 7. Non-deparaffined section transferred to a 0.4% solution of Ca-glycerophosphate of p_H = 8.4 at 32° C for 20 hours

treating the sections with distilled water before the reaction a quite different picture is obtained. Now those nuclei are chiefly black which are in cells the cytoplasm of which shows great phosphatase activity. This picture shows great resemblance to that which is obtained by carrying out the reaction at p_H = 8.4.

LISON[10] points to the possibility that the lyo-enzymes may be easily washed out, in contrast to the desmo-enzymes. It might be that the nuclei mainly contained lyo-phosphatase which has been eliminated by the treatment with distilled water. If, however, the phosphatase has been destroyed by dry heating a blackening of the nuclei is still obtained at p_H 9.4 with and without a substrate. We have already observed that with the cobalt method a blackening of the nuclei arises already through adsorption of Co.

If instead of Na-β-glycerophosphate + $Ca(NO_3)_2$ a solution is used containing Na-β-glycerophosphate + $MgSO_4$ and if a NH_4Cl–NH_4OH buffer is used, then it is not calcium phosphate which precipitates but $Mg(NH_4)PO_4$, which may likewise be converted into Ag_3PO_4. With this substrate we can work at p_H 9.4 and even at p_H = 10.0, as the sections with $MgSO_4$ in the NH_4Cl–NH_4OH buffer showed no blackening after washing and treatment with $AgNO_3$ (control experiment). With this method most nuclei were negative.

We may observe that JUNGE, MENTEN, AND GREEN[11] and later DANIELLI[4], using β-naphthol phosphate, phenyl phosphate or phenolphthaleine phosphate as substrate see no positive reactions of the nuclei. The rest of the picture was identical with that which was obtained with Na-β-glycerophosphate as a substrate. LORCH[12, 13], however, thinks that this is due to the above-mentioned reactions being less sensitive.

We believe that one must be very careful when interpreting the pictures. From a blackening of the nuclei it may not be inferred that these contain alkaline phosphatase. An investigation in which amongst other things different substrates are made use of is called for.

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METHOD

We subjoin a description of the method now used by us to demonstrate the presence of alkaline phosphatase in tissue sections.

This fragments of an organ (2–3 mm) are fixed in ethanol 80% for 12–24 hours. The bottom of the vessel is covered with a thin layer of cotton-wool. The 80% ethanol is renewed once during this period. Subsequently the fragments are placed in 2 portions of 96% ethanol. Then in 2 portions of methyl benzoate for 12–24 hours. The fragments are transferred to xylene or toluene for 2–3 hours. From this they pass into 3 portions of molten paraffin (melting-point 54–56° C), for 30–45 minutes in each, and are embedded then.

As during manipulations with loose paraffin sections the organic connection of the tissue constituents generally does not remain intact in sections of this kind, especially during the stay in the heated substrate or control solutions, it is desirable to embed the fixed material in celloidin-paraffin.

When this method of embedding is applied absolute ethanol is substituted for methyl benzoate. From this the fragments pass into a 2% celloidin-solution (equal parts of absolute ethanol and acetone) for 24 hours. Subsequently the superfluous celloidin is allowed to drip off and the fragments are embedded in paraffin via xylene or toluene.

Now sections 6 μ thick are cut and directly placed into a 0.4% solution of Ca-glycerophosphate of $p_H = 8.4$ at 32° C*. This solution is prepared just before use. The sections must float on the surface of the fluid and they stretch themselves through the heat; air-bubbles or creases are removed with a thin glass rod or preparing needle. In this solution the sections remain for 20 hours; thereupon they are allowed to cool down to room temperature and are transferred to distilled water one by one with the aid of a little rod. Afterwards they are washed once more in distilled water and then transferred to a 0.5% $AgNO_3$-solution or a saturated solution of silver acetate in 1 m sodium acetate. This last solution is obtained by dropping a solution of $AgNO_3$ (*e.g.*, 0.5 gramme in 1 ml of water) into 1 m sodium acetate solution until a white precipitate settles out. The fluid is now filtered. When the sections are transferred to one of these fluids they must not be immersed. They remain in this fluid till a distinct blackening appears: in diffuse daylight this will happen after a few hours, the time being much shorter in case of irradiation by a mercury lamp. The latter method, however, has the drawback that there is a possibility of the silver reagent being partly decomposed.

Now the sections are placed on slides after being washed in distilled water, mounted with dilute albumen-glycerine, and dried for some hours in a thermostate at 32° C. The paraffin is now removed by means of xylene and the sections are transferred to distilled water via ethanol. They are washed for 5 seconds in a 1% $Na_2S_2O_3$-solution. After careful washing they can be stained, if this is desired (*e.g.*, haematoxyline, if required in combination with eosin) and embedded in Canada balsam in the usual way.

One of the authors (N) owes many thanks to the ROCKEFELLER FOUNDATION which made it possible to continue this research.

* If the experiment is to be carried out at $p_H = 9.4$ it is absolutely necessary to place the sections in distilled water at 32° C for a few hours beforehand.

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SUMMARY

1. A method has been described to localize the alkaline phosphomonoesterase in paraffin sections. The original method of GOMORI was used with various modifications however.

2. It is shown that a diffuse picture is obtained when the sections placed on a slide are brought into water via ethanol. This picture is formed by the solubility of the enzyme in diluted ethanol. It is recommended to execute this reaction with non-deparaffined sections.

3. It appears from control experiments where the substrate has been replaced by $Ca(NO_3)_2$ that at $p_H = 9.4$ and higher a sediment of unsoluble Ca-compounds appears in the sections which have not been formed by the action of phosphatase. This formation has not been obtained at $p_H = 8.4$. If one would like to execute this reaction at $p_H = 9.4$ then it is necessary to wash out the sections with distilled water first.

4. If the reaction proceeds at $p_H = 8.4$ no blackening is to be perceived in sections in which the enzyme has been destroyed before the reaction either by heating or by u.v. light. This proves that the blackening which otherwise appears at $p_H = 8.4$ must in fact be ascribed to the action of the phosphatase.

At $p_H = 9.4$ a blackening has been formed in sections that have previously been heated with or without substrate. It is identical with that in the control sections (mentioned under 3).

5. From control experiments it has appeared that in the sections the blackening of the nuclei is not due to the phosphatase activity. In an extensive discussion it is pointed out that we may not conclude from the histochemical research that nuclei contain alkaline phosphatase.

RÉSUMÉ

1. Description d'une méthode permettant de localiser la phosphomonoestérase alcaline dans des coupes à la paraffine. Il s'agit de modifications de la méthode originale de GOMORI.

2. Il se produit une image diffuse lorsque les coupes sont placées sur une lame dans l'eau, après avoir été traitées par l'alcool. Ce caractère diffus est dû à la solubilité de l'enzyme dans l'éthanol dilué. Il est recommandé de faire cette réaction avec des coupes non déparaffinisées.

3. Des expériences de contrôle montrent que si l'on remplace le substrat par $Ca(NO_3)_2$, il se forme dans les coupes à p_H 9.4 et aux p_H supérieurs, un dépôt de composés calciques insolubles qui ne correspondent à aucune action de la phosphatase. Cette formation n'a pas lieu à p_H 8.4. Lorsque l'on veut exécuter la réaction à p_H 9.4, il est donc indispensable de laver préalablement les coupes à l'eau distillée.

4. Lorsque l'essai a lieu à p_H 8.4, il n'apparaît aucun noircissement dans les coupes où l'enzyme a été préalablement détruite, soit par chauffage, soit par lumière ultra-violette. Ceci montre que le noircissement qui apparaît à p_H 8.4 dans les conditions normales, est dû à l'action de la phosphatase. A p_H 9.4, il apparaît un noircissement dans les coupes qui ont été préalablement chauffées, et ceci en présence ou en l'absence de substrat. Ce noircissement est identique à celui que l'on observe dans les coupes témoins (voir 3).

5. Des expériences de contrôle ont montré que le noircissement des noyaux dans les coupes n'est pas dû à l'activité de la phosphatase. Une discussion approfondie montre que les recherches histochimiques ne permettent pas de conclure, jusqu'à présent que les noyaux contiennent de la phosphatase alcaline.

ZUSAMMENFASSUNG

1. Es wird eine Arbeitsweise beschrieben, welche die alkalische Phosphomonoesterase in Paraffinschnitten zu lokalisieren erlaubt. Dies ist eine Modifikation der Originalvorschrift von GOMORI.

2. Wenn die Schnitte, nach Behandlung mit Äthanol, in Wasser auf den Objektträger gebracht werden, entsteht ein verschwommenes Bild. Dies ist durch die Löslichkeit des Enzyms in verdünntem Äthanol bedingt. Es wird daher empfohlen, die Schnitte für diese Reaktion nicht von Paraffin zu befreien.

3. Ersetzt man das Substrat durch $Ca(NO_3)_2$, so entstehen in den Schnitten bei p_H 9.4 und bei höheren p_H-Werten, Niederschläge von unlöslichen Calciumverbindungen, die nicht auf Phosphatasewirkung zurückzuführen sind. Diese Niederschläge entstehen nicht bei p_H 8.4. Will man also die Reaktion bei p_H 9.4 ausführen, so muss man die Schnitte vorher mit destilliertem Wasser auswaschen.

4. Wird der Versuch bei p_H 8.4 ausgeführt, so entsteht keine Schwärzung in den Schnitten, in denen das Enzym vorher durch Erwärmen oder durch Ultraviolettstrahlen zerstört wurde. Dies beweist, dass die Schwärzung, die bei p_H 8.4 unter normalen Bedingungen auftritt, in der Tat auf Phosphatasewirkung zurückzuführen ist. Bei p_H 9.4 treten Schwärzungen in Schnitten auf, die vorher mit oder ohne Substrat erhitzt worden waren. Es sind dieselben Schwärzungen wie in den Kontrollversuchen (siehe 3).

5. Kontrollversuche haben gezeigt, dass in den Schnitten die Schwärzung der Kerne nicht auf Phosphataseaktivität zurückzuführen ist. Es wird ausführlich auseinandergesetzt, dass auf Grund von histochemischen Untersuchungen nicht auf das Vorkommen von alkalischer Phosphatase in den Zellkernen geschlossen werden kann.

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Received October 21st, 1948