MICRO PHOSPHATASE DETERMINATION IN 0.01 ml TISSUE EXTRACT OR BLOOD SERUM

by

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In this paper, which is a sequel to our previous article[1], we describe a method which enables us to submit small organs such as the adrenal gland, hypophysis or thyroid gland of cavia, rat or mouse to a chemical phosphatase determination by means of which the p_H-activity curves can be drawn for these organs too.

The procedure is in the main the same as the macro-method described in our previous article, in which we used either 1 ml or 0.1 ml of organ extract. Yet the method of micro-determination requires a few essential alterations, which we wish to set out here.

As it is possible to examine small organs, it follows that small parts of bigger organs can now also be submitted to a phosphatase determination. Thus, for example, we have a means of checking the typical histochemical localisation according to GOMORI* by microchemical determinations, after isolating small parts of the tissue.

In this examination we have dealt with phosphomonoesterases as well as pyrophosphatases.

A discussion follows of some p_H-activity curves of organs of internal secretion of the rat.

A. METHOD

a. The organ extracts

The organ extract should be prepared with particular care. The organ was rinsed in a physiological salt solution and the adhering liquid dried off with hardened filter paper, after which it was at once weighed in a weighing bottle. Next it was homogenised with quartz powder, the few millimetres wide cavity in the bottom surface of a glass stopper serving as mortar, and a glass stick with frosted glass knob serving as pestle.

We then took a quantity distilled water equal to 20 times the weight of the organ. By sucking into a small glass tube with a rubber tube attached, the contents of the cavity, after they had become homogeneous, were transferred to a tapering micro-centrifuge tube. After some practice it was found possible to transfer the whole quantity. We always allowed the organ extract to autolyse for 24 hours at room temperature, after adding a small crystal of thymol. To give an idea of the volumes concerned we may take as an example the adrenal gland of the rat: The gland weighs about 14 mg, to which can be added 280 μl of distilled water. After centrifuging — which is, however, often unneces-

* A modification of the histochemical method of GOMORI, worked out in our laboratory by J. H. C. RUYTER, will shortly be published.

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sary, the quartz powder settling sufficiently — more than 200 μl organ extract can easily be obtained. As 10 μl only is required for each separate determination, this quantity is sufficient for a p_H-activity curve of 20 points. We took about 10 mg as minimum quantity of the organ for a p_H-activity curve. Only in the case of very active organs was it feasible to start with a smaller quantity of the tissue, it then often being necessary to dilute the organ extract a little in order to bring it into the most favourable measure range for photometrics. It is often desirable to determine this factor of dilution first for the maximum p_H by means of a separate preliminary test. For this see[1], page 188.

b. The buffers

For the micro-method the same buffers were used as mentioned in our previous paper[1]: for the acid region veronal-acetate-HCl buffers (from p_H 2.1–p_H 9), and for the alkaline region ammonia-ammoniumchloride buffers (from p_H 8.3–p_H 11.2).

c. The substrate

We used the sodium salt of β-glycerophosphoric acid at a concentration of 40 mg per ml as substrate for the phosphomonoesterases, and sodium pyrophosphate at a concentration of 15 mg per ml as a substrate for the pyrophosphatases.

d. Method of measurement

For reaction vessels we used small flasks, 2.2 cm high, conically shaped at the bottom and with ground-in stopper, as shown in Fig. 1. These flasks were placed with their points in a layer of plasticine on the bottom of a drawer which could be placed in a hermetically closed brass box (Fig. 2). After having been heated to 37° C this box was suspended completely under water in a thermostat at 37° C. After a few minutes the contents of the flasks had reached the desired temperature.

The order in which the various liquids are put into the flasks is important. In contradistinction to the order employed in the macro-method, this time we filled the flasks as follows:

1. 0.01 ml organ extract,
2. 0.01 ml substrate solution.

Fig. 1. Flask for the micro-determination (height 2.2 cm, diam. at the top 1.5 cm, diam. at the bottom 0.3 cm)

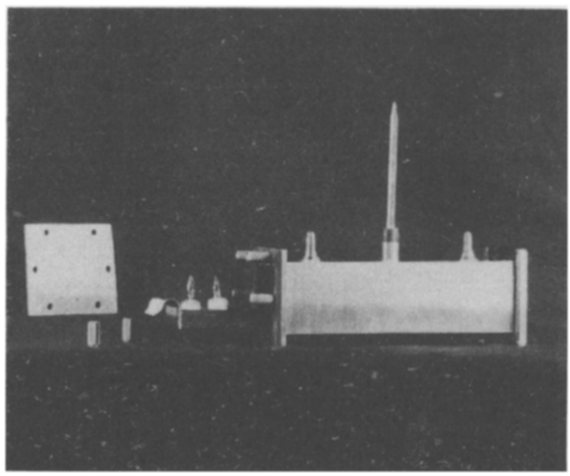

Fig. 2. Brass box (21 × 7 × 5.5 cm) in which the flasks are put into the thermostat.

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As these two little drops often stick to the side of the flasks we added as a last item, for mixing:

3. 0.05 ml buffer.

It must be emphasized that this order cannot be adhered to with the most acid buffers up to p_H 4.0. With these the buffer and the substrate should be put into the vessel first and the organ extract last. The addition of the substrate solution to the most acid buffers shifts the p_H by about 1 p_H unit towards the alkaline side. Now the peculiarly steep slope of the p_H-activity curve at very low and very high p_H is due to destruction of the phosphatases. In the order mentioned above there is a possibility with the most acid buffers of the little drop of organ extract coming into contact with the very acid buffer before a complete mixture with the substrate solution has been attained. In this case there is every chance of the enzyme being destroyed completely. No activity will then be found, whereas the same extract, added to the mixture of buffer and substrate, shows a clearly evident activity.

After remaining in the thermostat at 37° C for one hour, the proteins were precipitated and the enzyme reaction was stopped with 0.1 ml of 10 % trichloracetic acid. (With extracts containing much protein and with serum 0.1 ml of 20 % trichloracetic acid was added in order to obtain a clear filtrate). After 10 minutes the mixture was filtered through ashfree filter paper in a funnel of diameter 1.5 cm. For rinsing the flasks and the filter another 0.4 ml trichloracetic acid 10 % (or 20 %) was used.

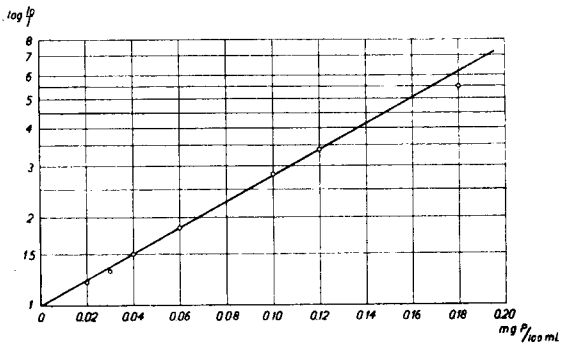

Fig. 3. Standard curve with KH_2PO_4 (analytical reagent)

There were then added:

1. 0.05 ml* ammonium molybdate, 5 % in 15 vol. % H_2SO_4, and, after well mixing,

2. 0.1 ml* of a fresh 25 % solution of ferrous sulphate[2], to which a few drops of trichloracetic acid had been added.

The total volume was then made up to, for example, 2.5 or 5 ml.

e. Accuracy and reproducibility of the method

The phosphor concentration can be measured with our photo-electric apparatus to an accuracy of 1 μg P per 100 ml for small values and an accuracy of 2 μg P per 100 ml for higher values. To test the reproducibility of the enzyme reaction in the organ extracts we used the same organ extract for about 10 determinations. With these the error was less than 4 μg P per 100 ml for small activity and less than 8 μg P per 100 ml for high activity.

f. Comparison of the micro- and macro-method

Since the ratio buffer-substrate-enzyme of the micro-method was the same as that found to be the most suitable with the macro-method, the two may be compared directly. The activities were expressed in Bodansky units, i.e., as the number of milligrams P

* These quantities were fixed by experiment. They gave a very good standard curve, as shown in Fig. 3, for which KH_2PO_4 (analytical reagent) was used. A tube containing only the reagents served as a blank.

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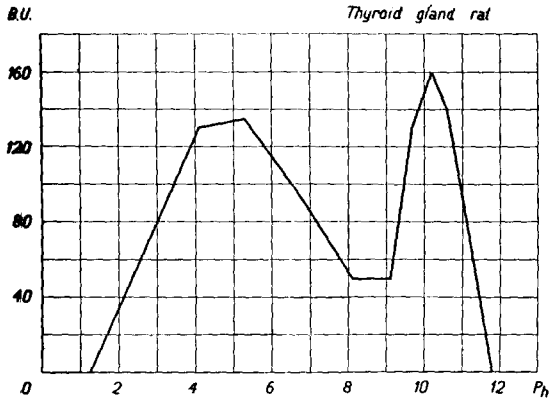

Fig. 4. pH-activity curve of the phosphomonoesterases of the thyroid gland (B.U. = Bodansky Units; see text)

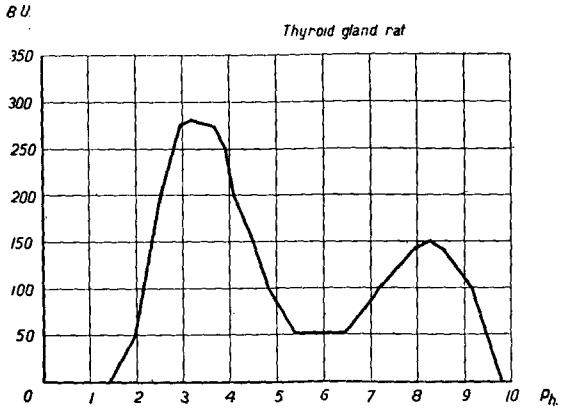

Fig. 5. pH-activity curve of pyrophosphatases of the thyroid gland

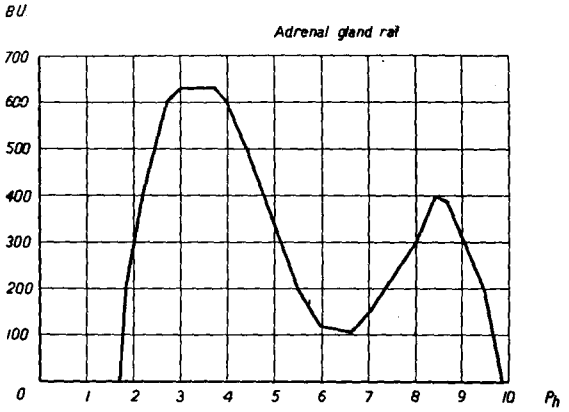

Fig. 6. pH-activity curve of the pyrophosphatases of the adrenal gland

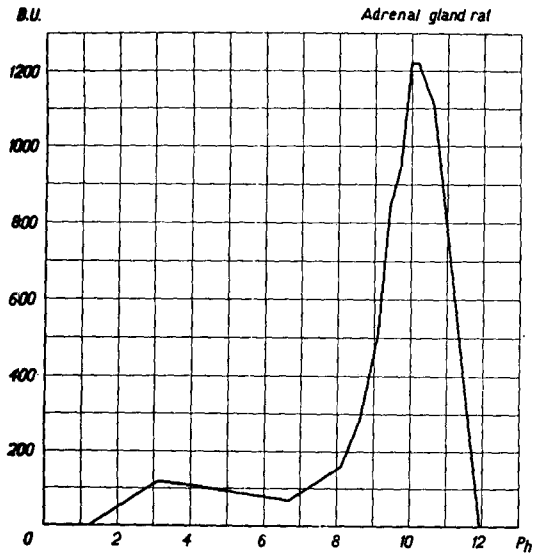

Fig. 7. pH-activity curve of the phosphomonoesterases of the adrenal gland

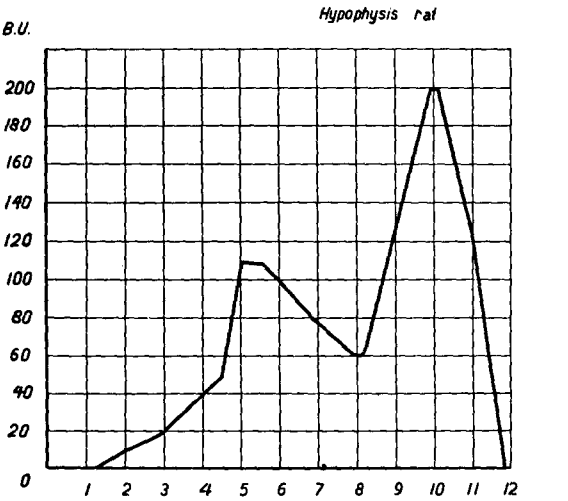

Fig. 8. pH-activity curve of the phosphomonoesterases of the hypophysis

per 100 g organ liberated from the substrate in one hour by the phosphatases at 37° C (B.U. in the graphs Fig. 4–8). There was always satisfactory agreement between the values found by the micro- and macro-methods. The difference never exceeded 10 %.

B. A FEW p_H-ACTIVITY CURVES OF THE PHOSPHOMONOESTERASES AND PYROPHOSPHATASES OF SMALL ORGANS (THYROID GLAND, ADRENAL GLAND AND HYPOPHYSIS OF THE RAT)

Without entering here into the biological problems involved in the phosphatase examination of these organs, we give some p_H-activity curves of organs of internal secretion of the rat.

With the phosphomonoesterases as well as with the pyrophosphatases we find a p_H-activity curve with two peaks, a flatter one on the acid side and a sharper one at about p_H 10 on the alkaline side.

The absolute values of the maxima may vary with different physiological conditions, but their mutual relation proves again to be characteristic of the organ concerned. This holds good also for the pyrophosphatases, which show, however, a shape of their own, completely different from the curves of the phosphomonoesterases (Cf. Figs. 4–8).

We wish to express our thanks to the Rockefeller Foundation for helping us to carry out this research.

SUMMARY

1. A description is given of a micro-method of obtaining the p_H-activity curves of very small organs or very small parts of larger organs with very good reproducibility.

2. At the same time this method offers the possibility of p_H-determination in 0.01 ml serum. Thus the blood of smaller animals can also be examined.

3. The small organs of internal secretion proved each to have a characteristic p_H-activity curve both for the phosphomonoesterases and for the pyrophosphatases. Some of the curves are reproduced.

4. The curves of the phosphomonoesterases and those of the pyrophosphatases each have their own typical shape, suggesting an independence of the two enzymes in the cell metabolism.

RÉSUMÉ

1. Description d'une microméthode permettant d'obtenir, d'une façon parfaitement reproductible, des courbes d'activité-p_H de très petits organes ou de très petites fractions d'organes.

2. Cette méthode permet en même temps la détermination du p_H dans 0.01 ml de sérum, ce qui permet d'étudier à ce point de vue le sang de très petits animaux.

3. Les petits organes à secrétion interne présentent chacun une courbe caractéristique d'activité-p_H, pour les phosphomonoestérases et pour les pyrophosphatases. Quelques-unes de ces courbes sont reproduites.

4. Les courbes correspondant à ces deux groupes d'enzymes ont chacune une forme caractéristique, suggérant l'indépendance de fonctionnement des phosphomonoestérases et des pyrophosphatases dans le métabolisme de la cellule.

ZUSAMMENFASSUNG

1. Eine Mikromethode wird beschrieben, die es uns ermöglicht, die p_H-Aktivitätskurven sehr kleiner Organe oder sehr kleiner Teile von grösseren Organen mit guter Reproduzierbarkeit zu bestimmen.

2. Diese Methode bietet gleichzeitig eine Möglichkeit zur p_H-Bestimmung in 0.01 ml Serum. Dadurch kann auch das Blut kleinerer Tiere untersucht werden.

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3. Die kleinen Organe mit innerer Sekretion haben alle, wie bewiesen wurde, eine besondere p_H-Aktivitätskurve, und zwar sowohl für die Phosphomonoesterasen als auch für die Pyrophosphatasen. Einige dieser Kurven werden abgebildet.

4. Die Kurven der Phosphomonoesterasen und die der Pyrophosphatasen haben immer ihre eigene, typische Form. Dies könnte auf eine voneinander unabhängige Funktion der beiden Enzyme im Zellstoffwechsel hinweisen.

REFERENCES

[1] D. B. KROON, H. NEUMANN, AND W. J. TH. A. KRAYENHOFF SLOOT, *Enzymologia*, XI (1943–45) 186.
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