Since spontaneous activity is deducted for calculating $CO_2$ production during the last hour of incubation, the lower it is the greater will be the method's sensitivity. On the other hand, variations in time elapsed between feeding and animal sacrifice modifies epididymal fat sensitivity to different concentration of insulin. Thus it seems to be better that animals should be fed 5 h before fat incubation, which gives the added advantage of less dispersion of results[13].

*Résumé.* Les auteurs ont verifié l'influence du temps écoulé depuis le dernier repas sur la sensibilité de la graisse de l'épididyme du rat à l'insuline *in vitro*, en utilisant la méthode manométrique. Des groupes d'animaux ont été alimentés 24, 5 et $2^1/_2$ h avant d'être sacrifiés. Le délai de 5 h semble être préférable parce qu'il offre la meilleure base et des résultats plus constants.

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[13] *Acknowledgment.* The authors are indebted to the Rockefeller Foundation and the Conselho Nacional de Pesquisas of Brazil for their contributions and to Mr. P. DOS SANTOS for his technical assistance.

## Double pH Optima of Potato Invertase

Enzyme systems required for both the biosynthesis and hydrolysis of sucrose have been shown to be present in protein preparations from the potato tuber[1]. Further study on the effect of pH on the invertase activity of these preparations from cold-stored potatoes, has revealed the presence of at least one optimum at pH 6.0 and one pH minimum at 4.7 as shown in Figure 1. Acid hydrolysis of sucrose controls (complete assay system less enzyme) below pH 3.5 vitiated attempts to obtain accurate rate measurements. It is reasonable to assume, however, that the second optimum exists below pH 3.5. Similar results have been obtained using buffers other than citrate. This double pH phenomenon is therefore not due to the effects of differing ionic species of buffer on the enzyme.

Figure 1 shows that potato invertase also hydrolyzes raffinose, but at a slower rate than sucrose, and that this hydrolysis also exhibits the double pH phenomenon. No free galactose was released during the hydrolysis of raffinose. Since the disaccharides, maltose, melibiose and turanose, and the trisaccharide, melezitose, did not serve as substrates even after prolonged incubation (48 h) at both pH 4.0 and 6.0, it appears that the potato invertase behaves as a typical $\beta$-D-fructofuranosidase at both these pH values.

Experiments on the effect of varying substrate concentration yielded decidedly different $K_m$ and $V_{max}$ values at pH 4.0 and 6.0 (Figure 2).

When the enzyme was diluted five-fold to 0.07 mg protein nitrogen/ml, the phenomenon of the double pH optimum was barely in effect and disappeared completely when the enzyme was diluted ten-fold to 0.035 mg protein nitrogen (Table). Furthermore, the rate of hydrolysis of sucrose by the potato protein preparation was not proportional to the enzyme concentration at most of the pH's investigated, although the rate was zero order, and the enzyme activity was measured at a substrate concentration which effectively saturated the enzyme (Figure 2).

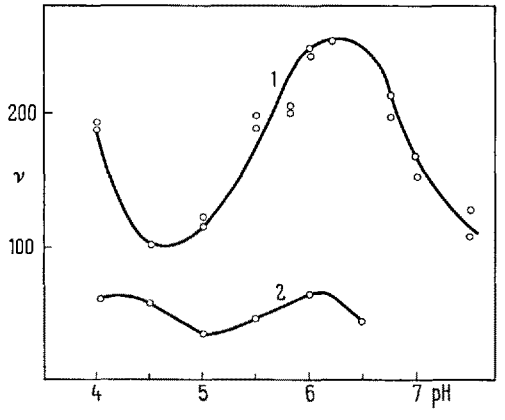


Fig. 1. Effect of pH on the rate of hydrolysis of sucrose and raffinose by protein preparation from cold stored Washington Russet potato tubers[1]. The rate ($v$) is expressed as μmoles of reducing sugar produced per h at 37°C per ml reaction mixture containing 40 μmoles of citrate buffer, protein preparation corresponding to 0.35 mg of protein nitrogen and either 138.8 μmoles of sucrose (curve 1) or 41.9 μmoles of raffinose (curve 2). The production of reducing sugar was linear with respect to time and was determined by adding aliquots after 1, 2, 4 and 7 h of incubation to 3,5-dinitro salicylic acid reagent[2]. Controls without enzyme or without substrate did not produce reducing sugars. Extended incubation of the enzyme at pH 4.0 did not result in loss of activity.

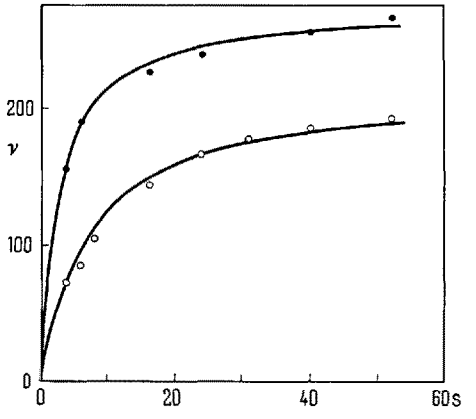


Fig. 2. Effect of substrate concentrations on the rate of hydrolysis ($v$) of sucrose by potato invertase at pH 4.0 (o) and at pH 6.0 (•). Rate $v$ is expressed as μg reducing sugar produced per h and substrate concentrations as μmoles per ml of enzyme reaction mixture under the same conditions as for Figure 1. The smooth curves are calculated from the Michaelis equation: For pH 4.0 (o), $K_m = 7.1$ $mM$, $V_{max} = 215$; for pH 6.0 (•), $K_m = 2.75$ $mM$, $V_{max} = 275$.

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The experimental results described are in accord with the hypothesis that potatoes contain a high molecular weight, nondialyzable ampholyte which can act as an inhibitor of potato invertase[3] and that the invertase exhibits double pH optima because only one ionic species of the inhibitor (whose pK values lie within the pH range of activity of the uninhibited enzyme) can combine with the enzyme to form an inactive enzyme-inhibitor complex inactive[4]. It appears that the inhibitor accompanies the enzyme in the preparation of the protein fraction. The different $K_m$ values which were observed would thus be expected in this case if a competitive inhibitor were present and active at pH 4.0 but not at pH 6.0.

Applying this theory to the data of the Table, one can calculate that the inhibition is greatest near the pH minimum, pH 4.7, but inhibition is negligible at pH 6, near one of the pH optima. It will also be noted in accordance with theory, that the calculated activity (as a function of pH) in absence of inhibitor, as well as that found at the lowest enzyme concentration investigated, shows no minimum. This suggests that the true optimum of the enzyme lies in the region of pH 3.5. The final proof of this hypothesis will be dependent upon the separation of the inhibitor from the preparation of potato protein.

It should be mentioned that protein preparations from potatoes stored at 2°C contained about four times more invertase activity than similar preparations from potatoes stored at room temperature, as measured at pH 6.0 by the method described for Figure 1.

Effect of enzyme concentration on rate of hydrolysis of sucrose by potato invertase

| Enzyme concentration, mg of protein nitrogen per ml of reaction mixture[a] | | | | | | |
|---|---|---|---|---|---|---|
| pH | 0.035 | 0.07 | 0.035 | 0.07 | 0.035 | 0.07 |
| | Observed rate[b] | | Rate in absence of inhibitor[c] | | % Inhibition[c] | |
| 4 | 41 | 54 | 87 | 174 | 53 | 69 |
| 4.5 | 30 | 38 | 72 | 143 | 58 | 74 |
| 5.0 | 24 | 34 | 42 | 83 | 42 | 59 |
| 6.2 | 20 | 40 | 20 | 40 | 0 | 0 |

[a] Conditions same as for Figure 1.
[b] µg reducing sugar produced per h per ml of reaction mixture.
[c] Calculated on assumption of the presence of an endogenous inhibitor in the enzyme preparation. The derivation of the equation for this calculation has been described earlier[3].

*Zusammenfassung.* Kartoffelinvertase scheint zwei pH-Optima zu haben; ein Optimum bei pH 6 und das andere bei oder unterhalb pH 3,5. Es scheint evident, dass das Phänomen nicht mit dem Vorhandensein von zwei Enzymen zusammenhängt. Untersuchungen über die Kinetik des Enzyms lassen daran denken, dass das doppelte pH-Optimum mit einer amphoteren Hemmungssubstanz der Invertase in Beziehung steht.

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## Gel Filtration of Protease Inhibitors from Potatoes

The inhibitor of proteases (IPP), isolated from potatoes[1], possesses pronounced anti-inflammatory activity[2,3]. By high-voltage electrophoresis, IPP was found to be composed of 5 protein fractions. By isolation procedures several preparations were obtained showing different degrees of purity and activity. It was of special interest to study whether some of the macromolecular fractions mixed with the anti-inflammatory fraction possess other characteristic activities and whether by their separation the anti-inflammatory activity remains unaltered. For this purpose, an isolation was carried out on an analytical scale such that the amount of fractions obtained sufficed for biological tests.

As the most suitable method, the column chromatography ('gel filtration') on Sephadex was suggested for preliminary information because of a large amount of inorganic material (mostly NaCl) present in the initial preparation and because of the lack of any information about the molecular weight of the individual fractions.

In experiments with Sephadex G-50 and G-25 (Pharmacia, Uppsala, Sweden) the presence of three maxima was found at 280 mµ. Figure 1 shows the chromatography of 1.5 g of lyophilized material dissolved in 30 ml of 0.01*M* acetic acid on Sephadex column G-25, water regain of 2.5 g/g of dry weight (3.2 × 200 cm) in equilibrium with 0.01*M* acetic acid. The elution was carried out by the same solution at room temperature. The localization of the maximum No. 3 (representing the anti-inflammatory activity) indicates a molecular weight of less than 3000. As the separation on Sephadex need not be complete, as was suggested in fact by the character of the maximum No. 3, a more detailed separation was realized before the lyophilization following the scheme in Figure 1 (fractions 1–3/3). Repeated chromatography of the main fractions (No. 2 and No. 3) gives identical pattern of separation, eliminating possible artefacts. The corresponding freeze-dried fractions obtained in the four individual chromatographic experiments were finally combined and evaluated biologically for irritant, haemagglutinating and anti-inflammatory activity.

As the preparation possess an inhibitory activity against a series of proteolytic enzymes, the inhibitions of trypsin, chymotrypsin, plasmin and plasmin-activator of the individual fractions were determined, and a possible correlation between the antiphlogistic and antiproteolytic activity was investigated. The results revealed (Figure 2 and Figure 3) that whereas fraction 2 is the main carrier of the antitrypsin, possibly antichymotrypsin activity and

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