Changes in Activity of Rat Epididymal Adipose Tissue *in vitro* Due to Time Elapsed since Last Feeding

The manometric method for insulin assay with epididymal fat pad[1], is mostly used for its sensitivity, but it is limited by not being entirely specific. Other substances like epinephrine[2,3], prolactin and growth hormone[4], showed an insulin like activity. In addition, results change according to the region from which fat is taken[5,6], and with animal weight[5]. Although some authors prefer to use fat from animals sacrificed after fasting[7,8], most authors recommend that animals should be fed[1,2,9–12]. The present paper deals with variations of activity of rat adipose tissue in relation to time elapsed since last feeding. In this way, we try to standardize the method in order to make it easier for future research work.

Material and Methods. Three groups of Wistar rats weighing approximately 150 g were used. The groups were sacrificed after 24, 5 or 2.30 h after last feeding. Animals were sacrificed by decapitation and epididymal fat was rapidly removed from both sides. In these animals the distal end of the epididymal fat is naturally divided, so a cut is made lengthwise in order to amplify the natural division. From each piece only the distal portion was taken. In this way, one animal gives four pieces, weighing approximately 50 mg. The incubation medium was Krebs-Henseleit bicarbonated solution with 200 mg% of glucose and 200 mg% of gelatin. On the side arm of the Warburg's flask insulin solution diluted in the same incubation medium is placed in order to obtain, after tipping, the final concentration of 20, 50, 125 and 10^4 μU of insulin (Insulin—Labor-terápica Bristol S.A.) per ml of medium. Insulin is added 1 h after starting incubation and this is carried on for 2 more h. The flasks are gassed with a gas mixture containing 95% of O_2 and 5% of CO_2. Bath temperature is 37°C at a frequency of 120 shakes/min. After incubation, fat is dried on filter paper and weighed in a torsion balance. Results are expressed in μl of CO_2 per g of tissue and per h.

To ascertain the influence of time elapsed after last feeding, we proceed as follows: animals are fasted overnight and fed next morning special food *ad libitum* during 1 h, after that changed to another cage and kept there for 24, 5 or $2^1/_2$ h, according to the group, and then sacrificed.

Results. In the Table, we can see that spontaneous activity (i.e. without adding insulin) is higher in the fat of rats sacrificed with 24 h of fasting than in those fed 5 h before. This difference is statistically significant ($P<0.01$). A similar difference ($P<0.02$) is found when comparing the results obtained with animals from the 1st group (24 h fasting) with those sacrificed $2^1/_2$ h after feeding. Results with animals fed $2^1/_2$ and 5 h before did not show statistically significant differences.

Production of CO_2 by adipose tissue to the same concentration of insulin was higher in rats 24 h of fasting than in fed animals. This difference, however, is not significant. We can observe in the Table that dispersion of results in the 1st group (24 h fasting) is very high. And comparing results corresponding to the answer to nearest insulin concentrations, such as 20 and 50 μU, 50 and 125, and 125 and 10^4, no statistically significant difference is found. The opposite happens in fat behaviour of animals sacrificed $2^1/_2$ and 5 h after feeding. In these we find statistically significant differences when we compare results between concentrations of 20, 50 and 125 μU/ml. There is no such difference between results obtained with concentrations of 125 and 10^4 μU/ml. In addition, it is interesting to note that in the results obtained with fat of animals fed 5 h before, dispersion is less than in the other two groups.

Discussion. Taking into consideration the fact that spontaneous activity and sensitivity of epididymal fat to insulin—when using the manometric method—change whether the animal is fasted or during digestion, it is convenient to establish the best moment to kill the animals.

From the results obtained we can verify that spontaneous activity shown by adipose tissue of animals during fasting is much higher than those in the digestive period.

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Epididymal adipose tissue activity in fasted and fed rats
μg of CO_2/g tissue/h

Insulin added μU/ml	24 h (9)	*P*	5 h (13)	*P*	2.30 h (12)	*P*
Without insulin	21 ± 5.0[a]		4 ± 0.9		6 ± 2.9	
		< 0.05		< 0.01		< 0.01
20	189 ± 67.5		76 ± 10.9		79 ± 10.2	
		> 0.05		< 0.01		< 0.01
50	271 ± 76.6		127 ± 8.6		160 ± 15.3	
		> 0.05		< 0.01		< 0.01
125	436 ± 124.0		268 ± 28.9		348 ± 50.0	
		> 0.05		> 0.05		> 0.05
10^4	531 ± 147.0		304 ± 22.7		354 ± 51.4	

[a] Mean ± standard error ( ) Number of animals

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 β-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).

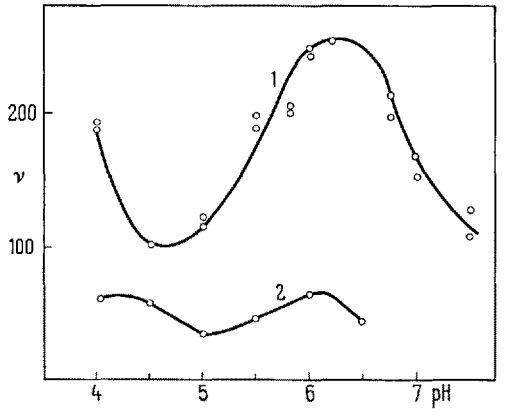

Fig. 1. Effect of pH on the rate of hydrolysis of sucrose and raffinose by protein preparation from cold stored Washington Russet potato tubers[1]. The rate (v) is expressed as μmoles of reducing sugar produced per h at 37°C per ml reaction mixture containing 40 μmoles of citrate buffer, protein preparation 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.

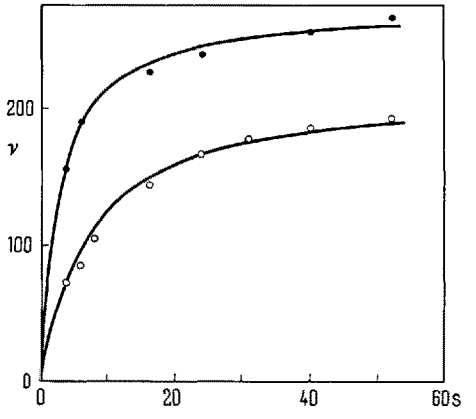

Fig. 2. Effect of substrate concentrations on the rate of hydrolysis (v) of sucrose by potato invertase at pH 4.0 (o) and at pH 6.0 (•). Rate v is expressed as μg reducing sugar produced per h and substrate concentrations as μmoles per ml of enzyme reaction mixture under the same conditions as for Figure 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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