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Commentary by

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on 'Insulin-mediated effect on the activity of UDPG-glycogen transglucosylase of muscle'
by C. Villar-Palasí and J. Larner
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In September 1957, the two of us arrived at the Department of Pharmacology of Western Reserve University. At that time, and for several years following, Earl Sutherland was head of the Department, and a great deal of the research being done was related to mechanisms of hormonal metabolic regulation. Our plans were to study several points on the metabolism of glycogen and its control by insulin that, although generally accepted, were far from clearly understood. The first one was the mechanism by which glycogen phosphorylase could switch from catalyzing the synthesis to catalyzing the degradation of glycogen. At the time, the 'in vitro' reversible reaction of phosphorylase led many biochemists to believe that it would also act 'in vivo' in a reversible manner. However, Sutherland's results had clearly demonstrated that when phosphorylase was activated 'in vivo', glycogen degradation always occurred, never synthesis. A series of studies on the tissue levels of hexose phosphates and inorganic phosphate [1] in control and insulin-stimulated rat dia-

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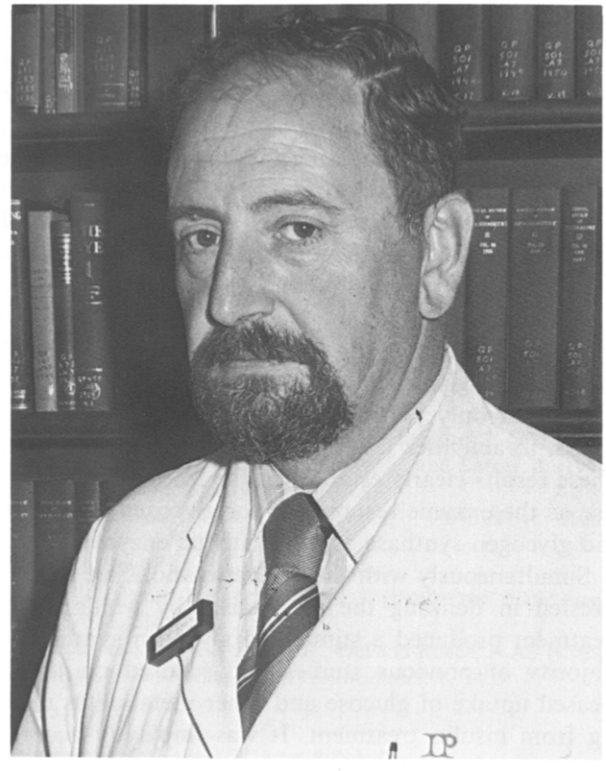

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phragms revealed that active glycogen synthesis occurred at a ratio of inorganic phosphate to glucose 1-phosphate of 300 to 1. This ratio is about 100-fold displaced towards glycogen degradation in the reaction catalyzed by phosphorylase.

In 1957, in view of these results, and partly inspired by a communication presented in Princeton by Leloir in which he hypothesized that UDPG could form a disaccharide (sucrose) which in this form could then transfer its glucose to form starch, or dextran, or transfer its fructose to form levan [2], Sutherland and both of us had begun speculating together about the possible existence of an alternative mechanism of glycogen synthesis. Uridine diphosphate glucose was discussed as a possible direct glucose donor. Shortly after this discussion, Leloir and Cardini published a report [3] on the direct formation of glycogen from uridine diphosphate glucose in a liver fraction. We were able to demonstrate the presence in skeletal muscle and rat diaphragm of two enzymic activities, uridine diphosphate glucose pyrophosphorylase, and Leloir's new enzyme, glycogen synthase. Together they constituted a reaction sequence for the conversion of glucose 1-phosphate to glycogen, bypassing phosphorylase and inorganic phosphate, thus favoring glycogen synthesis under physiological conditions in an essentially irreversible manner. The reason for the irreversibility became apparent when we tried out the new glycogen synthase reaction and found that it was a proton-forming reaction in contrast to phosphorylase.

Although it was clear by this time that the phosphorylase activity could not be responsible for both the synthesis and degradation of glycogen, but only for the latter, and that synthesis most likely occurred following the uridine diphosphate glucose pathway, there was great reluctancy in accepting these new ideas. Just then we were able to obtain a muscle biopsy from a patient with McArdle's disease kindly supplied by Drs. R. Schmid and P.W. Robbins. In patients with this disease, described as a myopathy due to a defect in muscle glycogen breakdown, the glycogen content may rise to 2.5–3%. After testing the various enzyme activities associated with glycogen synthesis and degradation, we found that only phosphorylase activity was greatly reduced, to activities less than 1% of normal individuals. These results clearly established the roles of phosphorylase as the enzyme responsible for glycogen breakdown and glycogen synthase as the synthetic enzyme.

Simultaneously with these investigations we were interested in defining the mechanism by which insulin treatment produced a stimulated glycogen synthesis. A majority of previous studies had focused on the increased uptake of glucose and other metabolites resulting from insulin treatment. It was implicitly accepted that an increased glucose uptake would cause a rise in the rate of glycogen formation simply by mass action. We decided to study the possibility of a metabolic effect of insulin independent of and present in addition to the increased transport effect of the hormone.

To this end, we used isolated rat diaphrams incubated with and without insulin at two concentrations of glucose, 140 and 280 mg/100 ml in the medium. After short incubation times (10 min) to approach initial reaction rates, glucose uptake from the medium, as well as glycogen and other metabolites present in the tissue, were measured. The controls incubated with 280 mg glucose/100 ml showed a higher glucose uptake than diaphragms incubated with 140 mg/100 ml treated with insulin; however, the glycogen content of the latter group was found to be higher. Furthermore, at both glucose concentrations, over 90% of the increased glucose uptake due to the addition of insulin could be accounted for as glycogen. These results suggested that insulin had a directing metabolic effect towards glycogen synthesis [4], independent of its effect on glucose transport.

A possible explanation for these results was suggested from another series of data obtained in these studies. In the insulin-treated diaphragms, the concentrations of glucose 6-phosphate appeared slightly but significantly higher than in the controls. In spite of this, the concentrations of glucose 1-phosphate were not elevated with insulin. This indicated that the stimulation by insulin occurred between glucose 1-phosphate and glycogen. We had previously shown that, in crude muscle extracts, glycogen synthase activity was greatly stimulated by concentrations of glucose 6-phosphate in the range of those present in diaphragms. Thus, the directive effect of insulin towards glycogen synthesis could still have been due to an increased glucose uptake followed by increased glucose phosphorylation by hexokinase. However, after considering the data obtained, this hypothesis appeared unlikely as an explanation of the results. The increases in glucose 6-phosphate concentrations due to insulin were too small to account for the large specific increase in glycogen synthesis.

An alternative hypothesis could be that insulin affected more directly the activity of one or more of the enzymes involved in glycogen metabolism, perhaps causing a covalent, stable conversion of the enzyme to a form with altered activity. To test this possibility, we measured in control and insulin-treated diaphragms the activities of phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, and phosphorylase. No difference between control and insulin-treated tissues was found. However, when the activities of glycogen synthase were assayed in the absence of glucose 6-phosphate in the test, the activity of the insulin-treated diaphragms was found to be increased over the controls. The differences disappeared when the activity was measured in the presence of excess glucose 6-phosphate in the test mixture. The increased activity in the insulin

extracts persisted after precipitation of the enzyme with ammonium sulfate or following dialysis. The explanation of these results that we suggested was the existence of two different forms of glycogen synthase, one active in the absence of added glucose 6-phosphate, the other requiring it for activity. The insulin effect could be interpreted as a conversion to the independent form from a dependent form [5].

The publication of these results was rapidly followed by the purification of glucose 6-phosphate dependent and independent forms of muscle and liver glycogen synthase. The mechanism of interconversion was shown to be phosphorylation-dephosphorylation reactions catalyzed by protein kinases and phosphatases [6]. Cyclic AMP, the second messenger responsible for increasing the activity of phosphorylase, was found to stimulate the phosphorylation and inactivation of glycogen synthase, thus controlling both glycogen synthesis and degradation in opposite directions. The effects of epinephrine in muscle could be explained then as a stimulation of the formation of cyclic AMP and the subsequent activation of cyclic AMP-dependent protein kinase. This enzyme activation would result in phosphorylation and inactivation of glycogen synthase, thus decreasing futile glycogen synthesis; simultaneously, cyclic AMP-dependent protein kinase would phosphorylate and activate phosphorylase *b* kinase and this last enzyme in turn would activate phosphorylase and glycogen breakdown to glucose 1-phosphate. This cascade of phosphorylations is now proven to occur in a variety of tissues.

The effect of insulin, on the other hand, could not be explained by a similar but opposite mechanism. It was clearly shown that, unless the tissue concentrations of cyclic AMP were previously elevated by epinephrine or a similar acting glycogenolytic agents, insulin treatment had no lowering effect on cyclic AMP intracellular concentrations [7]. Alternative mechanisms to explain the activation of glycogen synthase by insulin were proposed and discarded after testing, such as an elevation of the intracellular levels of cyclic GMP which would act, in general, opposite to cyclic AMP. Protein phosphatase activity was found decreased in diabetes, and restored to normal activity following insulin administration, but no clear effect of insulin was found on phosphatase activity in normal tissues. On the other hand, a decreased activity ratio and a decreased sensitivity to activation of cyclic AMP-dependent protein kinase by cyclic AMP was shown to occur following insulin treatment in diaphragm, skeletal muscle, and liver [8]. The molecular mechanism of this effect is still not worked out.

In subsequent years, it was found that a large variety of protein kinases present in the tissues could phosphorylate glycogen synthase in vitro, and in most cases this resulted in synthase inactivation. Most likely only some of these kinases have a regulatory role in vivo. Also, a variety of phosphatases, some with rather complex mechanisms of control, are now known to dephosphorylate and activate glycogen synthase. The picture that emerges is that glycogen synthase activity is controlled by the interplay of a multitude of protein kinases and phosphatases with activities often intercontrolled, and by a variety of allosteric activators and inhibitors. Glycogen synthase has possibly one of the most complex activity control mechanisms of any enzyme known so far [9].

The activation of glycogen synthase was the first metabolic effect of insulin found which could be directly related to a molecular change in the regulation of an enzyme activity and remains the best studied. It set the pattern for altered protein phosphorylation state with insulin action. It was the first case of decreased covalent phoshorylation and was followed by pyruvate dehydrogenase [10]. A list of enzymes usually biosynthetic can now be drawn up with similar decreased phosphorylation and activation with insulin action. Surprisingly, a list of enzymes with increased phosphorylation can now also be tabulated with insulin action, but the biological significance of these phosphorylations is as yet unclear. Of considerable interest is the fact that, as a direct result of the action of insulin in decreasing the activity of the cyclic AMP-dependent protein kinase, an assay for insulin mediator has been devised and used to detect a cluster of novel molecules which have the proper credentials to act as insulin mediators [11]. This has now opened a new and exciting chapter in the ongoing story of the mechanism of action of insulin.

References

1 Larner, J., Villar-Palasí, C. and Richman, D.J. (1959) Ann. N.Y. Acad. Sci. 82, 345–353.
2 Springer, G.F. (ed.) (1957) Polysaccharides in Biology, Josiah Macy, Jr. Foundation.
3 Leloir, L.F. and Cardini, C.E. (1957) J. Am. Chem. Soc. 79, 6340.
4 Larner, J., Villar-Palasí, C. and Richman, D.J. (1960) Arch. Biochem. Biophys. 86, 56–60.
5 Villar-Palasí, C. and Larner, J. (1960) Biochim. Biophys. Acta 39, 171–173.
6 Friedman, D.L. and Larner, J. (1963) Biochemistry 2, 669.
7 Goldberg, N.B., Villar-Palasí, C., Sasko, H. and Larner, J. (1967) Biochim. Biophys. Acta 148, 665–672.
8 Shen, L.C., Villar, Palasí, C. and Larner, J. (1970) Phys. Chem. Phys. 2, 536.
9 Larner, J. (1989) Adv. Enzymol., in press.
10 Lynn, T.C., Pettit, F.H. and Reed, L.J. (1969) Proc. Natl. Acad. Sci. USA 62, 234.
11 Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A.A., Huang, L., Daggy, P. and Kellogg, J. (1979) Science 206, 1408.

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Insulin-mediated effect on the activity of UDPG-glycogen transglucosylase of muscle

The increased accumulation of glycogen found in the presence of insulin in previous experiments appeared to be difficult to explain solely on the basis of increased glucose uptake[1], and suggested the possibility of an insulin-mediated effect on the enzymes of the glycogen cycle. We have therefore measured the activities of phosphoglucomutase, UDPG pyrophosphorylase, UDPG-glycogen transglucosylase, and phosphory-

Abbreviations: UDPG, uridine diphosphate glucose; G-6-P, glucose 6-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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lase in parallel extracts of control and insulin-treated diaphragms. An increased activity of UDPG-glycogen transglucosylase has been found in extracts of diaphragms incubated with insulin as compared to controls. As this enzymic activity appears to be rate-limiting in glycogen synthesis[2], an increased efficiency in this step could explain the accumulation of glycogen observed.

Isolated cut rat diaphragms were used, employing the techniques of incubation, freezing, and extraction of the tissues previously described[1]. The powdered frozen diaphragms were homogenized either in 0.1 *M* NaF – 0.002 *M* EDTA[3], for the measurement of phosphorylase activity, or in 0.05 *M* Tris-maleate – 0.0025 *M* EDTA (pH 8.2), for phosphoglucomutase, UDPG pyrophosphorylase, and UDPG-glycogen transglucosylase activities. Extracts were prepared by centrifugation of the homogenates at 20,000 × *g* for 30 min*.

Phosphorylase activity was measured at pH 6.1 by the method of ILLINGWORTH AND CORI[4], with the results expressed in μmoles/g/h. Phosphoglucomutase and UDPG pyrophosphorylase activities were determined spectrophotometrically[5]. Table I shows that no significant difference was observed in any of these three activities.

TABLE I

PHOSPHOGLUCOMUTASE, UDPG PYROPHOSPHORYLASE AND PHOSPHORYLASE ACTIVITIES OF EXTRACTS OF CONTROL AND INSULIN-TREATED DIAPHRAGMS

Mean values (μmoles/g tissue/h) of 6 experiments ± standard error of mean.

	Control	*Insulin*	*Mean difference*	*P*
Phosphoglucomutase	7484	7671	187 ± 100	> 0.15
UDPG pyrophosphorylase	440	437	3 ± 15.6	> 0.5
Phosphorylase, total §	2262	2364	102 ± 148	> 0.5

§ The ratios of a/total phosphorylase activities found in these experiments (control, 87; insulin 85, $P > 0.3$) appear to be much higher than previously reported values. This discrepancy may be due to the increase in the proportion of phosphorylase *a*[6] that occurs when freezing is used as a method of fixation.

TABLE II

UDPG-GLYCOGEN TRANSGLUCOSYLASE ACTIVITY

Mean results (μmoles/g tissue/h) of 13 experiments.

	Control	*Insulin*	*Mean difference*	*P*
No G-6-P in test	13.2	18.0	4.8 ± 0.5	< 0.001
G-6-P added	59.8	58.4	1.4 ± 3.4	> 0.50

For the determination of UDPG-glycogen transglucosylase activity, [^{14}C-*glucose*]-UDPG and glycogen were incubated at pH 8.2 with the tissue extracts, with or without added G-6-P. After 10–20 min at 30°, the incubation mixtures were deproteinized by addition of 5 % trichloroacetic acid, glycogen was isolated from aliquots of the supernatant, and its radioactivity measured. In Table II are presented the results of these determinations.

* No loss of UDPG-glycogen transglucosylase activity was found in the extracts, as compared with homogenates.

It has been found previously[1] that the G-6-P content is increased in insulin-treated as compared to control diaphragms. To test the possibility that the observed difference in activity of UDPG-glycogen transglucosylase could be accounted for by an increase in a free or loosely bound co-factor, the enzyme was precipitated by $(NH_4)_2SO_4$ (60 % satn.), the precipitates washed with 60 % satd. $(NH_4)_2SO_4$, and dialyzed (1–3 h). After this treatment, the increased activity of insulin extracts persisted when tested in the absence of added G-6-P. Recovery of enzyme activity was virtually complete. Furthermore, when extracts were incubated at 0° with a large excess of added G-6-P prior to the $(NH_4)_2SO_4$ treatment, the activity recovered (measured in the absence of added G-6-P in the test system) accounted for only that initially present, which appears to indicate that added G-6-P is not tightly bound to the enzyme.

It is known[1] that high glucose concentrations in the incubation medium may increase the G-6-P content of diaphragms even over that found in diaphragms incubated with insulin at lower glucose concentrations; however, the glycogen content is not correspondingly increased. Accordingly, diaphragms were incubated at medium glucose concentrations between 70 and 360 mg/100 ml without added insulin. No differences were observed at different concentrations in the medium (6 experiments) in the UDPG-glycogen transglucosylase activity of diaphragms incubated without added insulin in the face of a consistently higher activity of extracts prepared from insulin-treated diaphragms.

From these results it appears that observed differences in UDPG-glycogen transglucosylase activity cannot be explained on the basis of an increased G-6-P content of diaphragms incubated with insulin. The activity of extracts measured in the presence of G-6-P seems to remain constant whatever the activity determined in the absence of added G-6-P may be: a reasonable explanation would be the existence in muscle of two species of UDPG-glycogen transglucosylase: one active in the absence of added G-6-P, the other requiring it for activity. The insulin effect here reported could then be interpreted as an interconversion between the two species.

It has been demonstrated that in the presence of insulin increased glycogen synthesis occurs associated with an increased activity of UDPG-glycogen transglucosylase. This finding represents additional evidence for assigning the synthetic role in the glycogen cycle to the uridine-linked enzymes.

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[1] J. LARNER, C. VILLAR-PALASÍ AND D. J. RICHMAN, *Ann. N.Y. Acad. Sci.*, 82 (1959) 345.
[2] C. VILLAR-PALASÍ AND J. LARNER, *Arch. Biochem. Biophys.*, 86 (1960) 270.
[3] G. T. CORI AND B. ILLINGWORTH, *Biochim. Biophys. Acta*, 21 (1956) 105.
[4] B. ILLINGWORTH AND G. T. CORI, *Biochem. Preparations*, 3 (1953) 1.
[5] J. LARNER AND C. VILLAR-PALASÍ, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1234.
[6] C. F. CORI, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, Inc., New York, 1956, p. 573.

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