Effects of Insulin, Epinephrine, and Cyclic Adenosine Monophosphate on Pyruvate Dehydrogenase of Adipose Tissue†

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ABSTRACT: Homogenates of fat pads (from fed or starved-refed rats) incubated with physiological concentrations (10–100 μunits/ml) of insulin demonstrate increased activity of mitochondrial pyruvate dehydrogenase. The increase in activity caused by insulin after prolonged incubation does not result simply from conversion of the inactive (phospho) form to the active (dephospho) form of the enzyme since *in vitro* conversion of the enzyme by incubation with high concentrations of Mg^{2+} demonstrates that compared to the control the total enzyme activity is increased by insulin. The activity of pyruvate dehydrogenase of fat pad homogenates falls rapidly when the intact tissue is incubated for 40–60 min in the absence of hormone. Insulin causes a more rapid fall in enzyme activity in the initial period (5 min) of incubation, but this is followed quickly (10–15 min) by a rise in enzyme activity which usually equals but sometimes exceeds the activity observed in the unincubated fat pads. The specific effect of hormones on enzyme activity is thus highly dependent on the spontaneous changes occurring in the control enzyme and on the exact time during which the tissues are examined. Kinetic studies can distinguish two major activities of pyruvate dehydrogenase phosphatase in homogenates of fat pads. These two activities differ markedly in their affinity for the substrate, the inactivated (phospho) form of pyruvate dehydrogenase. Fat pads incubated for 60 min with insulin exhibit a substantial increase in the activity of the high-affinity phosphatase activity. Homogenates of fat pads incubated for 60 min with dibutyryl-cAMP (1 mM) or with epinephrine (0.1–1 μg/ml) display, as in the case of insulin, elevated activities of pyruvate dehydrogenase. Epinephrine does not diminish the effects of insulin on the basal or the Mg^{2+}-stimulated activities of the enzyme. The effect of epinephrine differs from that of insulin, however, since after incubation with epinephrine all of the enzyme appears to be in the active (dephospho) form while with insulin the most prominent effect is an increase in the phospho form which results in an increase in the total amount of enzyme. Neither dibutyryl-cAMP nor epinephrine alters the activity of the phosphatase and neither compound appears to appreciably decrease the activity of the specific pyruvate dehydrogenase kinase. Insulin does not alter the activity of the kinase. Insulin, epinephrine, dibutyryl-cAMP, or cAMP have no effects on the activity of pyruvate dehydrogenase, the phosphatase, or the kinase when they are added directly to fat pad homogenates. Actinomycin D markedly depresses the activity of pyruvate dehydrogenase of fat pads incubated in the absence of hormone but in the presence of insulin it has little effect on this enzyme. Cycloheximide and puromycin on the other hand selectively block the effects of insulin on pyruvate dehydrogenase. Since these two compounds do not alter the effect of insulin on phosphatase activity it appears that the most evident effects of insulin on pyruvate dehydrogenase activity may be mediated by changes in protein synthesis. The effects of epinephrine and of dibutyryl-cAMP on pyruvate dehydrogenase are not blocked by inhibitors of protein synthesis. Pyruvate dehydrogenase is a complex enzyme whose activity in adipose tissue is subject to rapid spontaneous changes and to complicated hormonal and metabolic regulation through such factors as protein synthesis and the activities of related enzymes (phosphatases, kinases) which can alter the degree of phosphorylation of the enzyme.

The increased synthesis of fatty acids in adipose tissue incubated in the presence of glucose and insulin (Winegrad and Renold, 1958) has been explained on the basis of the enhancement of glucose transport by insulin (Crofford and Renold, 1965). Recently considerable evidence has accumulated which suggests that insulin activates one of the steps controlling the conversion of pyruvate to extramitochondrial acetyl-CoA (Denton *et al.*, 1968; Denton and Halperin, 1968; Saggerson and Greenbaum, 1970; Jungas, 1970a,b; Halperin, 1970). In adipose tissue the enzyme which is involved in this activation by insulin is pyruvate dehydrogenase (Jungas, 1970a,b; Denton *et al.*, 1971; Coore *et al.*, 1971; Jungas and Tayor, 1972; Taylor and Jungas, 1972). Activation of pyruvate dehydrogenase by insulin has also been observed in rat liver (Wieland *et al.*, 1972).

Pyruvate dehydrogenase is a mitochondrial multienzymic complex which catalyzes the following reaction

$$CH_3COCOO^- + CoASH + NAD^+ \longrightarrow CoASCOCH_3 + CO_2 + NADH$$

(Reed and Cox, 1966). In mitochondria from beef kidney (Linn *et al.*, 1969a,b), pork liver (Linn *et al.*, 1969b), and rat epididymal fat pads (Coore *et al.*, 1971) the activity of the pyruvate dehydrogenase complex is regulated by a phosphorylation–dephosphorylation reaction of one of the components of the complex, pyruvate decarboxylase. The dephosphorylated form of this protein is enzymically active while the phosphorylated form appears to be inactive. Phosphorylation is mediated by the activities of a specific ATP-dependent kinase and of a specific phosphatase. The kinase requires low concentrations of Mg^{2+} for activity whereas the phosphatase re-

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quires Mg^{2+} at a concentration of about 10 mM for optimal activity. In pig heart mitochondria the kinase does not appear to be cAMP-dependent (Wieland and Siess, 1970). Although early studies suggested the possible existence of a regulatory role of cAMP in the activity of pig heart phosphatase (Wieland and Siess, 1970), more recent studies (Siess and Wieland, 1972; Hucho *et al.*, 1972) indicate that the activity of this enzyme is not regulated by cAMP. This report presents the results of studies designed to elucidate the specific mechanisms by which physiological concentrations of insulin, epinephrine, and cAMP modulate the activity of pyruvate dehydrogenase of adipose tissue.

Materials and Methods

Chemicals. Crystalline pork zinc insulin (24 units/mg) was purchased from Eli Lilly; [1-^{14}C]pyruvate (6.4 Ci/mol) from New England Nuclear; ATP, epinephrine, sodium pyruvate, N^6,O^2-dibutyryl-cAMP, protamine sulfate, actinomycin D, and cycloheximide from Sigma; dithiothreitol, thiamine pyrophosphate, and CoASH from Calbiochem; β,γ-methyleneadenosine 5′-triphosphate from P-L Biochemicals; hydroxide of Hyamine 10-X from Packard; bovine plasma albumin (fraction V) from Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex. Puromycin was a gift from Dr. D. Nathans. All other reagents were of analytical grade.

Incubation of Fat Pads. Epididymal fat pads were obtained from Sprague-Dawley rats (100–150 g) which were either fed *ad libitum* or fasted 2 to 3 days and refed for 15 hr. Fat pads were cut in small pieces, weighed, and randomly distributed in 2-oz. plastic Nalgene narrow-mouth bottles. Unless indicated otherwise, the fat pads were preincubated at 37° for 30 min in a volume equal to ten times the weight of the tissue of freshly prepared Krebs–Ringer bicarbonate buffer (Krebs and Henseleit, 1932) containing half the recommended calcium concentration (0.5 mM), no magnesium, and 0.2% (w/v) albumin; before use the buffer was oxygenated by bubbling an O_2–CO_2 (95:5) gas mixture for 30 min, and the pH of the buffer was adjusted to 7.4 with 1 N NaOH. After the 30-min period of preincubation the fat pads were incubated at 37° in fresh buffer (as described above) in the presence or absence of insulin, dibutyryl-cAMP, epinephrine, actinomycin D, puromycin, or cycloheximide. At the end of this incubation the fat pads were rinsed thoroughly with ice-cold 30 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM dithiothreitol and 50 mM NaCl. The fat pads were suspended in a volume of buffer equal to five times the weight of the tissue and homogenized for 60 sec in an ice bath using a Polytron PT-10 (Brinkmann) at a setting of 3.5. The homogenate was centrifuged for 10 min at 700*g*. The infranatant layer present between pellet and floating fat was collected (crude homogenate) and used for enzymic and protein assays. Protein concentration was measured by the method of Lowry *et al.* (1951) using serum bovine albumin as the standard.

Pyruvate Dehydrogenase Assay. Pyruvate dehydrogenase activity was determined by measuring the rate of formation of $^{14}CO_2$ from [1-^{14}C]pyruvate (Jungas, 1970b). Each assay was performed in duplicate or triplicate. Protein (100–300 μg) was added to 2-oz. plastic bottles containing an incubation mixture (kept at 4°) which consisted of 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, 50 mM NaCl, 1 mM thiamine pyrophosphate, 0.5 mM NAD, 0.1 mM CoASH, and 0.25 μmol of [1-^{14}C]pyruvate (specific activity 2.56 Ci/mol) in a total volume of 0.5 ml. The bottles were immediately stoppered with rubber serum stoppers equipped with plastic center wells (Kontes Glass) and incubated in a Dubnoff shaker for 6 min at 37°. Control samples were not incubated at 37° but kept instead at 4°. At the end of the incubation the reaction was stopped by placing the samples in an ice bath and injecting through the rubber stopper 0.6 ml of 2.5 M H_2SO_4. Hydroxide of Hyamine 10-X (0.2 ml) was injected into the center wells. The bottles were shaken gently at room temperature for 30 min to facilitate CO_2 absorption. The center wells were transferred to counting vials containing 3 ml of ethanol and 12 ml of 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene, and radioactivity was determined (94% efficiency) with a liquid scintillation spectrometer.

A unit of pyruvate dehydrogenase activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of $^{14}CO_2$/min. Specific activity is the units of pyruvate dehydrogenase activity per milligram of protein. With this assay the enzyme activity is directly proportional to the amount of protein up to a concentration of 280 μg/ml. The amount of CO_2 produced is linear with time of incubation up to 8 min so that initial velocities are obtained with the 6-min incubation period used in these studies. No activity is observed if NAD, thiamine pyrophosphate, or CoASH is omitted from the incubation mixture.

Partial Purification of Inactivated Pyruvate Dehydrogenase. Inactivated (phosphorylated) pyruvate dehydrogenase was prepared for use as a substrate for pyruvate dehydrogenase phosphatase. The inactive enzyme from adipose tissue was purified by a modification of the method used by Linn *et al.* (1969b) for purification of pyruvate dehydrogenase of beef kidney, beef heart and pork liver. Fat pads from 15 Sprague–Dawley rats weighing 200–250 g were homogenized for 20 sec at 24° with a Polytron PT-10 (Brinkmann) in a volume of 0.4 M sucrose equal to three times the weight of the tissue. All subsequent steps were performed at 4°. The homogenate was centrifuged at 700*g* for 15 min and the infranatant was collected and incubated at 30° for 30 min in the presence of 8 mM $MgCl_2$. The suspension was centrifuged at 35,000 rpm for 30 min (Spinco Model L2-65B, Rotor 42). The supernatant was discarded and the pellet was suspended in 7 ml of 30 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM dithiothreitol and 4 mM $MgCl_2$; it was dispersed by homogenization for 10 sec with a Polytron PT-10.

The suspension was sonicated for 1 min and frozen and thawed twice. The sample was centrifuged for 20 min at 20,000 rpm and the pellet was suspended by sonication using 2 ml of 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, and 4 mM $MgCl_2$. After centrifugation for 20 min at 20,000 rpm the supernatant was combined with the supernatant of the prior centrifugation, adjusted to pH 6.2 with 0.5% (v/v) acetic acid, and precipitated with 0.002 volume of 2% protamine sulfate. After 5 min at room temperature the precipitate was removed by centrifugation for 15 min at 10,000 rpm. The supernatant was precipitated with 70% $(NH_4)_2SO_4$. The pellet obtained by centrifugation for 30 min at 10,000 rpm was dissolved in 2.5 ml of 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, and 50 mM NaCl, and dialyzed overnight at 4° with 1 l. of the same buffer. After dialysis the solution was incubated for 30 min at 30° with 0.5 mM ATP and 0.5 mM $MgCl_2$ and dialyzed again for 12 hr with four changes of the same buffer to remove ATP and $MgCl_2$.

Table I summarizes the results of a typical purification of pyruvate dehydrogenase by the procedures described above. It has been shown that incubation of purified pork liver pyruvate dehydrogenase complex (Linn *et al.*, 1969b) or of

TABLE I: Purification of Adipose Tissue Pyruvate Dehydrogenase.[a]

Step	Vol (ml)	Protein (mg/ml)	Total Protein (mg)	Sp Act.[b]	Total Act.	Yield (%)[c]
Crude homogenate	43	2.1	89.4	1.6	143.1	31.0
Crude homogenate, incubated with Mg^{2+}	46.5	1.9	89.4	6.1	545.6	100.0
Pellet, 35,000 rpm	7	1.4	10.2	25.0	253.8	46.5
Freezing and thawing	7	1.4	10.2	24.8	251.7	46.2
Supernatant, 20,000 rpm	8.8	0.8	7.0	26.2	184.4	33.9
Supernatant after protamine precipitation	8.8	0.4	3.8	38.4	145.2	26.8
Pellet of 70% $(NH_4)_2SO_4$ precipitation	2.5	1.2	3.2	43.7	139.8	25.8

[a] The fat pads of 30 rats were processed by the procedures described in the text. [b] Expressed as units of enzyme activity per milligram of protein. [c] Calculated on the basis of the activity of the crude homogenate incubated with Mg^{2+}.

crude or purified mitochondria of rat fat pads (Coore *et al.*, 1971) with ATP in the presence of Mg^{2+} at a concentration as low as 0.5–1 mM produces a rapid loss of pyruvate dehydrogenase activity. This results from the action of a specific dehydrogenase kinase which is present in these preparations. Dilution of the inhibited (phosphorylated) pyruvate dehydrogenase complex followed by incubation in the presence of a high concentration (10 mM) of magnesium restores the enzymic activity, presumably as the result of the action of pyruvate dehydrogenase phosphatase. The pyruvate dehydrogenase activity of the crude fat pad homogenate used in the present experiments demonstrates similar reversible effects upon incubation with ATP and Mg^{2+} (Table II).

TABLE II: Effect of $MgCl_2$ and ATP on the Activity of Pyruvate Dehydrogenase of Crude Homogenates of Rat Fat Pads.[a]

Addition	Pyruvate Dehydrogenase Act. (nmol of $^{14}CO_2$/min per mg of Protein)
None	1.8
8 mM Mg^{2+}	4.8
1 mM Mg^{2+}, 0.5 mM ATP,	0.9
8 mM Mg^{2+}, 0.5 mM ATP, diluted ten times with 8 mM Mg^{2+}	4.0

[a] Fat pads from fed rats were homogenized and centrifuged at 700*g* as described in the text. The crude homogenate was adjusted with $MgCl_2$ to 8 mM and incubated for 15 min at 30°. At the end of this incubation the reaction was stopped by adding 0.2 ml to an ice-cold solution containing 0.4 ml of 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, 50 mM NaCl, and 0.2 ml of 8 mM NaEDTA. The remaining portion of the crude homogenate was adjusted with NaEDTA (7 mM) and ATP (0.5 mM) and the incubation was continued for 5 min at 30°. Samples (0.2 ml) were added to 0.6 ml of the ice-cold EDTA-containing solution described above. Another portion (0.5 ml) of the remaining crude homogenate incubated with ATP was diluted ten times with potassium phosphate buffer containing 8 mM $MgCl_2$ and incubated for 15 min at 30°; this reaction was stopped by adding NaEDTA (8 mM) and cooling to 4°. All the samples were assayed in triplicate for pyruvate dehydrogenase activity by the procedures described in the text.

The activity of the purified adipose tissue pyruvate dehydrogenase falls rapidly when the enzyme is incubated in the presence of 0.5 mM ATP and 0.5 mM $MgCl_2$. At 30° the activity falls by nearly 80% in 10 min and 95% in 26 min. The partially purified pyruvate dehydrogenase was nearly free of pyruvate dehydrogenase phosphatase since tenfold dilution followed by incubation with 8 mM $MgCl_2$ does not restore pyruvate dehydrogenase activity. This inactivated preparation of pyruvate dehydrogenase, which contained 1.1 units of activity/20 μl in its final form, was used as substrate for measurements of phosphatase activity.

Assay of Pyruvate Dehydrogenase Phosphatase Activity. The activity of this enzyme in the fat pad homogenates was determined by measuring the rate of activation of the partially purified, inactivated (phosphorylated), and phosphatase-free pyruvate dehydrogenase in the presence of high concentrations of $MgCl_2$. For all assays, samples of the crude fat pad homogenate (0.1 ml), prepared as described earlier, were incubated at 30° for 20 min in a total volume of 250 μl of (a) 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, and 50 mM NaCl, (b) the same buffer but containing varying amounts of purified, inactivated pyruvate dehydrogenase and 8 mM $MgCl_2$, (c) the same buffer containing 8 mM $MgCl_2$ but no added pyruvate dehydrogenase; and (d) samples identical with b but without crude fat pad homogenate were also examined. At the end of the incubation period aliquots (0.1 ml) of each of these incubation mixtures were assayed for pyruvate dehydrogenase activity. The activity of pyruvate dehydrogenase phosphatase was calculated by subtracting the activities obtained in incubations c and d from the activity measured in b. The values of d in different preparations are between 0 and 2% of the b values. The c values, which are constant over the entire range of concentration of purified pyruvate dehydrogenase, are equal to about 70–30% of the total phosphatase activity respectively for the low and high range of substrate concentration. The blank value (c, above) was not assumed to remain constant at different substrate concentrations; it was determined independently for every assay performed. The variability in the phosphatase assay in different experiments performed during a period of several weeks was about 15–20%. The differences between triplicate samples, after correction for the blank values, were seldom greater than 5–10%. Despite the large blank corrections required for low substrate concentrations and the rather

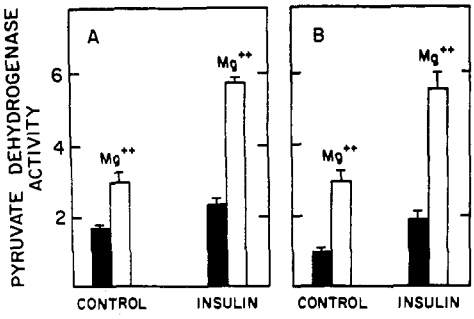

FIGURE 1: Pyruvate dehydrogenase activity of homogenates from fat pads incubated with insulin. Fat pads from fed (A) and fasted (3 days) refed (15 hr) (B) rats were preincubated for 30 min at 37° in Krebs–Ringer bicarbonate buffer containing 0.2% (w/v) albumin which was gassed under an atmosphere of O_2–CO_2 (95–5%) and adjusted to pH 7.4. The fat pads were then incubated for 1 hr at 37° in the same buffer in the absence (control) or in the presence of 120 μunit of insulin/ml. The fat pads were homogenized and centrifuged as described in the text; 2 ml of each homogenate was adjusted to 8 mM with $MgCl_2$ (□) and incubated for 30 min at 30° in a Dubnoff bath, shaking at 80 cycles/min. Other samples of the homogenate were similarly incubated in the absence of added $MgCl_2$ (■). The Mg^{2+} activation was stopped by diluting a portion (0.2 ml) of the homogenate into 0.4 ml of ice-cold 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, 50 mM NaCl, and 2.4 mM EDTA. Samples were assayed in triplicate for pyruvate dehydrogenase activity as described in the text. The data shown are the mean of four experiments. Pyruvate dehydrogenase activity is expressed as nanomoles of $^{14}CO_2$ produced per minute per milligram of protein.

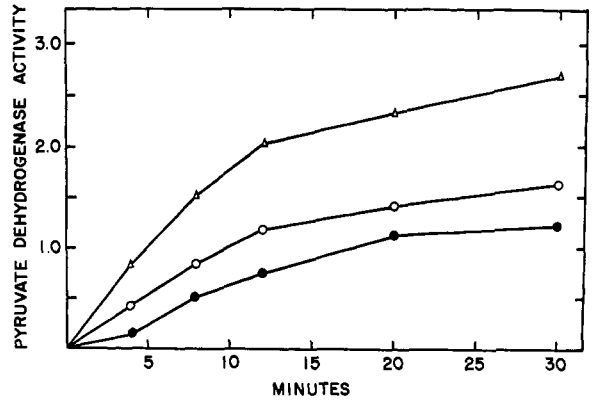

FIGURE 2: Time course of Mg^{2+} activation of pyruvate dehydrogenase activity of homogenates of fat pads incubated with insulin. The fat pads from eight fasted (48 hr) and refed (10 hr) rats were preincubated in Krebs–Ringer bicarbonate buffer for 30 min at 37° as described in the text. The fat pads were incubated for 60 min at 37° in the absence (●) and presence of 24 μunits (○) and 120 μunits (△) of insulin per ml. At the end of the incubation the tissue was homogenized and centrifuged at 700*g*. The homogenate was adjusted with $MgCl_2$ to 8 mM and incubated for different time periods at 30°. At indicated time intervals, samples (0.2 ml) of the incubation mixture were diluted in ice-cold buffer, as described in Figures 4 and 5, and assayed for pyruvate dehydrogenase activity. The zero time values were subtracted from the values obtained at the different time periods. The time-dependent plateau of activity is not apparently explicable on the basis of inactivation of the phosphatase since the activity of this enzyme is unimpaired after an incubation period of 20 min under similar conditions.

complex nature of the assay procedure, the shape of the curves as well as the effects of insulin were highly reproducible. One unit of phosphatase activity is defined as the amount of enzyme that catalyses the activation of one unit of inactivated pyruvate dehydrogenase in 20 min at 30°. The activity of the phosphatase under the conditions used here is directly proportional to the amount of enzyme added at least up to a concentration of 500 μg/ml. Furthermore, the activity of the phosphatase is linear with time of incubation for at least 20 min when the concentration of substrate (inactivated pyruvate dehydrogenase) corresponds to 6.4 units/ml.

Results

Effects of Insulin on Pyruvate Dehydrogenase. Incubation of fat pads obtained from fed or fasted-fed rats for 60 min with physiological concentrations of insulin results in a substantial increase in the pyruvate dehydrogenase activity of the fat pad homogenates (Figure 1). Insulin increases the basal activity of the enzyme as well as the activity which is observed after incubating the homogenate with high concentrations of $MgCl_2$. The data indicate that insulin has a greater effect on the Mg^{2+}-stimulated than on the basal pyruvate dehydrogenase activity. The base-line enzyme activity is increased by 50% in the fat pad homogenates of fed and by 76% in those of starved-fed rats, while Mg^{2+}-stimulated activity is enhanced 97 and 88%, respectively. The concentration of $MgCl_2$ used in these experiments was 8 mM, which is the optimal concentration under these conditions. Incubation of the homogenates with $MgCl_2$ for longer time periods does not lead to a further increase in enzyme activity (Figure 2), indicating that the Mg^{2+} effects were equally complete in both the absence and presence of insulin. Since the Mg^{2+} effect almost certainly results from the conversion of inactive (phospho) pyruvate dehydrogenase to the active (dephospho) form of the enzyme by the action of a specific phosphatase, the enzyme activity measured after complete Mg^{2+} activation is presumably a measure of the total quantity of both the dephosphorylated and phosphorylated forms of the enzyme. These results suggest that insulin causes an increase in the total amount of enzyme in the tissue, and that the effects of the hormone do not result simply from a shift in the relative proportions of phospho and dephospho forms of the enzyme.

The maximal effects of insulin on pyruvate dehydrogenase activity are observed with concentrations of the hormone near 120 μunits/ml (Figure 3). The dependence on insulin concentration is the same for base-line enzyme activity and for Mg^{2+}-stimulated enzyme activity. It is notable that at all concentrations of insulin the Mg^{2+}-stimulated enzyme activity is increased substantially more by the hormone than is the base-line activity.

Fat pads which are incubated at 37° in the absence of insulin demonstrate a rapid fall in the activity of pyruvate dehydrogenase (Figure 4). In the presence of insulin this fall in enzyme activity is prevented. Although the activity of pyruvate dehydrogenase of homogenates of fat pads incubated with insulin for longer than 20 min is considerably higher than that of tissue incubated in the absence of the hormone, the activity is not substantially greater than that observed in the homogenates of the unincubated fat pads. This suggests that insulin may act primarily by preventing the normal fall in enzyme activity which occurs during incubation. Although considerable variability in the absolute effect of insulin on pyruvate dehydrogenase activity is observed in different experiments, the magnitude of the insulin effect is nearly always inversely related to magnitude of the enzyme activity present in the control homogenate of the tissue incubated in the absence of

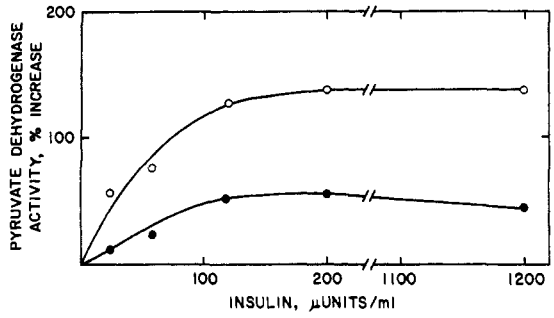

FIGURE 3: Dependence on insulin concentration of activation of adipose tissue pyruvate dehydrogenase activity. Fat pads from fasted (52 hr) and refed (17 hr) rats were cut into small pieces and distributed in randomized form into 2-oz plastic bottles. In this experiment the fat pads were not preincubated but were instead incubated directly for 1 hr at 37° in freshly prepared Krebs–Ringer bicarbonate buffer containing half the recommended calcium concentration (0.5 mM) and no Mg^{2+}, with 0.2% (w/v) albumin as described in the text. At the end of the incubation the tissue was extensively rinsed in ice-cold 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, and 50 mM NaCl, and homogenized in the same buffer. After centrifugation at 700*g* for 10 min samples of the homogenate supernatant were preincubated for 30 min at 30° in the absence (●) and presence (○) of 8 mM $MgCl_2$ (as described in Figure 4 before assaying for pyruvate dehydrogenase activity). Pyruvate dehydrogenase activity is expressed as the per cent increase over the activity measured in the absence of insulin.

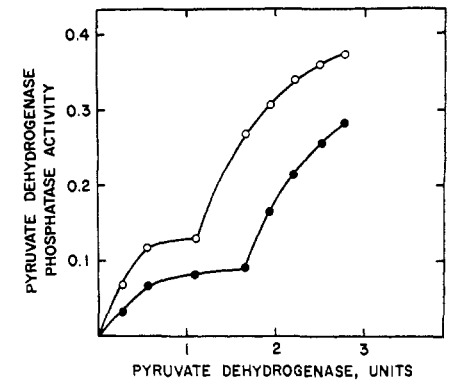

FIGURE 5: Effect of increasing concentrations of the phospho form of purified pyruvate dehydrogenase on the activity of phosphatase of homogenates from fat pads incubated in the presence of insulin. Fat pads were incubated for 60 min at 37° with (○) or without (●) 120 μunits of insulin/ml. Homogenate samples (0.2 ml) were incubated for 20 min at 30° under various conditions (see Methods) to determine phosphatase activity. The phosphatase activity is expressed as units of pyruvate dehydrogenase activated in 20 min.

insulin. When the activity of the latter is high, the apparent increase in enzyme activity induced by insulin is small.

It is evident, however, that the effect of insulin is more complicated than simply to prevent a fall in enzyme activity with time of incubation since in some experiments (for example, see Figure 9) the enzyme activity after exposure to insulin for 60 min is twice that observed in the unincubated tissue. Furthermore, during the first few minutes of exposure of the fat pads to insulin there is a fall in enzyme activity which is nearly always more pronounced than that observed in the tissue incubated without insulin (Figure 4). This very early effect of insulin is quite reproducible and probably represents more than a latency phase required for the diffusion of insulin through the tissue since the activity at this early time is nearly always significantly lower than that of the control tissue. The data suggest that there may be biphasic effects of insulin on pyruvate dehydrogenase which may be mediated by different mechanisms.

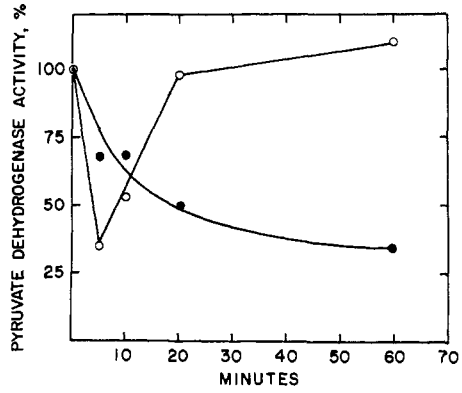

FIGURE 4: Pyruvate dehydrogenase activity as a function of duration of incubation with insulin. Fat pads from fed rats were cut in small pieces and incubated at 37° in the presence (○) or in the absence (●) of 120 μunits of insulin/ml for different time periods. At indicated time intervals, tissue aliquots were homogenized and assayed for pyruvate dehydrogenase activity as described in the text.

Kinetic studies in which the concentration of pyruvate is varied from 6×10^{-5} to 3×10^{-4} M insulin (24 μunits/ml) alters the V_{max} but not the apparent K_M of pyruvate dehydrogenase. These results, which are similar to those reported by Coore *et al.* (1971), are also consistent with the data presented earlier in this report which suggest that the effect of insulin on the total quantity of the enzyme may be more important than effects which the hormone may have in the overall state of phosphorylation of the enzyme.

Various attempts to demonstrate effects of insulin on pyruvate dehydrogenase activity by adding the hormone directly to homogenates of fat pads were not successful. These results, in agreement with the studies of Coore *et al.* (1971) and Jungas and Taylor (1972), indicate that insulin does not affect mitochondria directly but acts instead indirectly through signals generated by primary interactions with the cell membrane (Cautrecasas, 1969, 1971).

Effects of Insulin on Pyruvate Dehydrogenase Phosphatase Activity. The increased activity of pyruvate dehydrogenase observed after incubating fat pads with insulin could at least in part reflect changes in the relative proportions of the phospho and dephospho forms of the enzyme resulting from changes in the activities of the specific phosphatase or kinase. The inactivated (phospho) form of pyruvate dehydrogenase was purified (see Methods) for use as a substrate in assays of phosphatase activity.

Studies of fat pad homogenates suggest the presence of at least two kinetically distinct forms of phosphatase in this tissue (Figure 5). These two enzymic activities, which are equally apparent in homogenates of fat pads incubated in the absence or presence of insulin, appear to differ markedly in their affinity for the phosphorylated form of pyruvate dehydrogenase.

Although the exact nature of these two phosphatase activities is not yet resolved, insulin clearly causes an increase in the overall phosphatase activity (Figure 5). The effect of insulin appears to be most pronounced on the high-affinity phosphatase activity. Insulin increases the V_{max}, or the

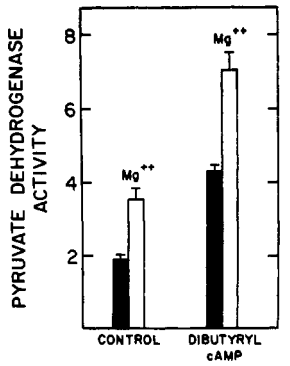

FIGURE 6: Effect of dibutyryl-cAMP on the activity of pyruvate dehydrogenase of adipose tissue. Fat pads from fed rats were incubated in the presence or absence of 1 mM dibutyryl-cAMP for 60 min at 37°. The fat pads were homogenized and assayed for basal (■) and Mg^{2+}-stimulated (□) pyruvate dehydrogenase activity by the same procedures described in Figure 1. The data represent the mean of three experiments.

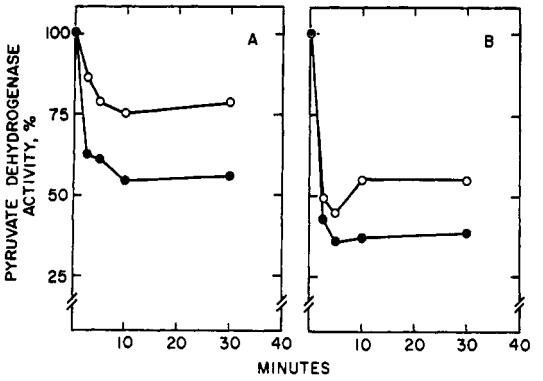

FIGURE 7: Time course of ATP inhibition of pyruvate dehydrogenase activity in homogenates of fat pads incubated with dibutyryl-cAMP. Fat pads were incubated in Krebs–Ringer bicarbonate buffer for 60 min at 37° in the presence (○) and absence (●) of 3 mM dibutyryl-cAMP (A), and in the presence (○) and absence (●) of 120 μunits of insulin/ml (B) as described in Figures 5 and 6. Samples (1 ml) of each crude homogenate were incubated for 15 min at 30° with 1.5 ml of potassium phosphate buffer (pH 7.4), containing 8.0 mM $MgCl_2$. Aliquots (0.2 ml) were then removed and adjusted with NaEDTA to a concentration of 7.5 mM. The reaction was started by the addition of ATP (0.5 mM). After varying times of incubation at 30°, samples (0.2 ml) were added to tubes containing 50 μl of 3 mM β,γ-methylene-ATP which was kept in an ice bath. The phosphonic acid analog of ATP was used to stop the reaction since it is a potent inhibitor of ATP-requiring kinases (Krug *et al.*, 1973). The samples were then assayed for pyruvate dehydrogenase activity as described in the text. The results are expressed in comparison (%) to the activities obtained in the samples incubated for 15 min with $MgCl_2$.

apparent total quantity of this enzyme, but it does not alter the apparent K_M for the phospho enzyme. It is also possible that insulin alters the activity of the low-affinity phosphatase, but such changes are not clearly discernible from the present data.

Effect of Dibutyryl-cAMP on Pyruvate Dehydrogenase and Phosphatase Activities. Incubation of fat pads with 3 mM dibutyryl-cAMP results, like insulin (Figure 1), in an increase of the basal (133%) as well as of the Mg^{2+}-stimulated (97%) pyruvate dehydrogenase activity (Figure 6). Similar effects of this nucleotide on adipose tissue have recently been reported (Schimmel and Goodman, 1972). The enhancement of enzyme activity differs from that observed with insulin since the basal activity is stimulated to a greater extent than is the Mg^{2+}-stimulated activity. These results differ from the report of Coore *et al.* (1971) who described no change in the activity of pyruvate dehydrogenase with this cyclic nucleotide; in the presence of insulin they reported that dibutyryl-cAMP caused a fall in enzyme activity.

Unlike insulin, dibutyryl-cAMP (1 mM) does not increase the activity of pyruvate dehydrogenase phosphatase. The nucleotide may cause a slight fall in phosphatase activity.

Effect of Insulin and Dibutyryl-cAMP on Pyruvate Dehydrogenase Kinase Activity. The initial rate of inactivation of pyruvate dehydrogenase activity upon incubation with 50 μM ATP and 0.5 mM $MgCl_2$ was used to assess the endogenous activity of the kinase in homogenates of fat pads incubated under various conditions (Figure 7). The initial rate of fall of enzyme activity in the homogenates from fat pads incubated with dibutyryl-cAMP is approximately the same as that of the control tissue. The observed increase in pyruvate dehydrogenase activity of fat pads incubated with dibutyryl-cAMP is therefore not obviously explained by a lowered activity of the kinase. The initial rate of inhibition of enzyme activity by ATP in homogenates from insulin-treated fat pads shows no difference from the control homogenates (Figure 7). Thus, insulin does not appear to alter in a very significant way the activity of the pyruvate dehydrogenase kinase.

Various attempts were made to detect effects of dibutyryl-cAMP and cAMP on the activities of pyruvate dehydrogenase phosphatase or kinase by adding the nucleotides directly to homogenates or to mitochondrial fractions of fat pads. In agreement with the results of others (Coore *et al.*, 1971; Jungas and Taylor, 1972), no such effects could be demonstrated in the present studies. Effects on enzyme activities occurred only when the intact tissue was incubated with the nucleotide.

Effect of Epinephrine on Pyruvate Dehydrogenase, Phosphatase, and Kinase Activities. Coore *et al.* (1971) reported that exposure of fat pads to epinephrine for 30 min almost completely abolished the stimulating effects of insulin on pyruvate dehydrogenase activity but it failed to significantly alter the activity of the enzyme in fat pads not incubated with insulin. In the present studies the pyruvate dehydrogenase activity of fat pads incubated for 60 min with 1 μg/ml of epinephrine was substantially increased (Figure 8). Furthermore, epinephrine did not depress the increase in enzyme

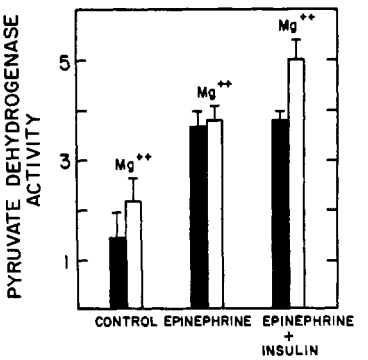

FIGURE 8: Effect of epinephrine on pyruvate dehydrogenase activity of adipose tissue. Fat pads from fed rats were incubated in the presence of epinephrine (1 μg/ml) or epinephrine (1 μg/ml) plus insulin (120 μunits/ml) for 60 min at 37°. The details of the experimental procedures are the same as described in Figures 1 and 6.

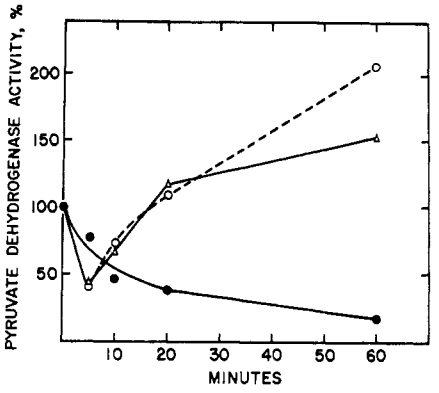

FIGURE 9: Effect of length of incubation of fat pads with epinephrine or insulin on the activity of pyruvate dehydrogenase of homogenates. Fat pads from fed rats were incubated at 37° for varying times without additions (●), or in the presence of 1 μg/ml of epinephrine (△) and 1 μg/ml of epinephrine plus 120 μunits/ml of insulin (○). The experiments were performed as described in Figure 4.

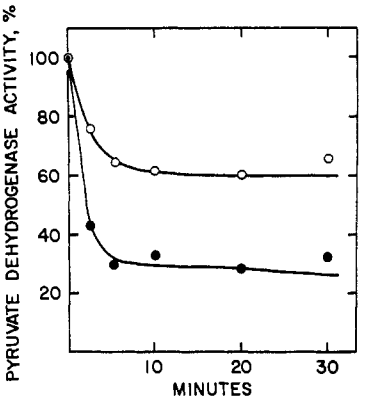

FIGURE 10: Time course of ATP inhibition of pyruvate dehydrogenase activity of homogenates of fat pads incubated in the presence of epinephrine. Fat pads were incubated at 37° for 60 min in the presence (○) or in the absence (●) of epinephrine. The homogenates of the fat pads were assayed for pyruvate dehydrogenase activity after addition of 50 μM ATP and 0.5 mM $MgCl_2$. The experimental details are the same as those described in Figure 7.

activity observed in fat pads incubated with insulin (120 μunits/ml).

Definite differences exist, however, between the nature of the activation of pyruvate dehydrogenase observed with the two hormones. Whereas epinephrine causes principally an increase in the basal activity of the enzyme and an abolition of the Mg^{2+} effect (Figure 8), the effect of insulin is principally on the total enzyme activity and on the Mg^{2+}-stimulatable activity (Figure 1). These results suggest that epinephrine may be acting at least in part by depressing the activity of the pyruvate dehydrogenase kinase, thus causing conversion of the inactive phospho form of the enzyme to the active dephospho form. It is notable, however, that epinephrine like insulin causes a substantial increase in the total quantity of pyruvate dehydrogenase activity.

The time course of the epinephrine effect on pyruvate dehydrogenase activity (Figure 9) indicates that during the first 5-min incubation there is a fall in enzyme activity similar to that observed with insulin. The effects of both hormones become increasingly pronounced with longer periods of incubation, and this effect becomes quite exaggerated if the activity of the enzyme is expressed in relation to the basal enzyme activity since this falls progressively with time. Jungas and Taylor (1972) have recently described similar changes in the activity of the enzyme during incubation of fat pads with epinephrine for varying time periods. If the effects of insulin and epinephrine on total enzyme activity occurs by different mechanisms, it is not clear why additivity between the effects of both hormones is not observed.

The effects on pyruvate dehydrogenase activity of fat pads incubated with lower concentrations (0.1 μg/ml) of epinephrine are qualitatively identical to those described in the experiments depicted in Figures 8 and 9. Furthermore, similar studies performed with isolated adipose tissue cells instead of intact fat pads show identical results. In this case the enhancement of pyruvate dehydrogenase activity by epinephrine and insulin are even more pronounced than those described in fat pads, and epinephrine does not diminish the effects of insulin.

When fat pads are incubated (37°, 60 min) in the presence of epinephrine (1 μg per ml), there is no alteration in the apparent activity of pyruvate dehydrogenase phosphatase of adipose tissue. Epinephrine, like dibutyryl-cAMP and insulin (Figure 7), does not appear to significantly enhance the activity of pyruvate dehydrogenase kinase (Figure 10).

Effect of Inhibitors of RNA and Protein Synthesis. Since the total activity of pyruvate dehydrogenase (revealed by incubation with Mg^{2+}) of homogenates of fat pads incubated with insulin (Figure 1) appears to be increased, it was of interest to examine the possibility that insulin might in part modulate this enzyme by changing its rate of synthesis or degradation. Incubation of fat pads with actinomycin D causes a fall in the basal as well as in the Mg^{2+}-stimulated activities of pyruvate dehydrogenase (Figure 11). Actinomycin D, however, is only partially effective in decreasing the effects of insulin on this enzyme. In the presence of this inhibitor the relative increase in enzyme activity is considerably greater than it is in its absence. Although these results suggest that insulin does not act by increasing specific RNA synthesis, they do not exclude effects of this hormone on translational processes. Furthermore, these results suggest that in the time intervals utilized in the present experiments significant changes in the synthesis or degradation of pyruvate dehydrogenase can occur.

Incubation of fat pads with cycloheximide completely prevents the effects of insulin on the activity of pyruvate dehydrogenase while no significant effect is observed on the activity of this enzyme in the absence of insulin (Figure 11). Puromycin also diminishes the insulin effect without altering the activity of the enzyme in fat pads incubated in the absence of hormone (Figure 11). These results suggest that insulin alters pyruvate dehydrogenase at least in part by increasing the net synthesis of the enzyme. It is significant, furthermore, that puromycin (30 μg/ml) and cycloheximide (30 μg/ml) do not block the effect of insulin on phosphatase activity despite their profound effect on the insulin-induced change in pyruvate dehydrogenase activity. Thus, the increase in total activity of pyruvate dehydrogenase may result predominantly if not exclusively from effects of insulin on protein synthesis at the translational level.

It is of interest that actinomycin D causes a fall in the activity of the high-affinity pyruvate dehydrogenase phosphatase but has no effect on the second, low-affinity enzyme activity (Figure 12). These results are consistent with the earlier evidence suggesting that the high-affinity phosphatase

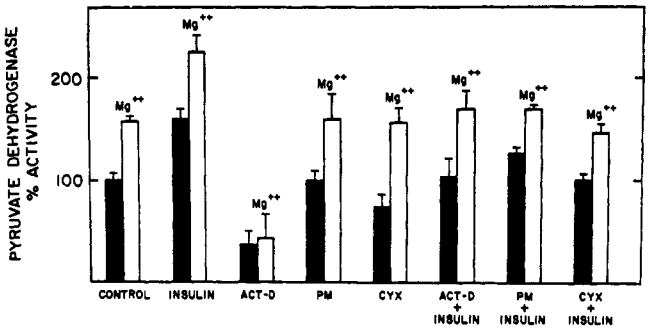

FIGURE 11: Effect of actinomycin D (ACT-D; 25 μg/ml), puromycin (PM; 30 μg/ml), and cycloheximide (CYX; 30 μg/ml) on the activity of pyruvate dehydrogenase of fat pads incubated with insulin (INS; 120 μunits/ml). Fat pads from fed rats were incubated with these compounds for 60 min at 37° as described in Figures 1 and 6. The fat pads were thoroughly rinsed in 30 mM potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, and 50 mM NaCl before homogenization.

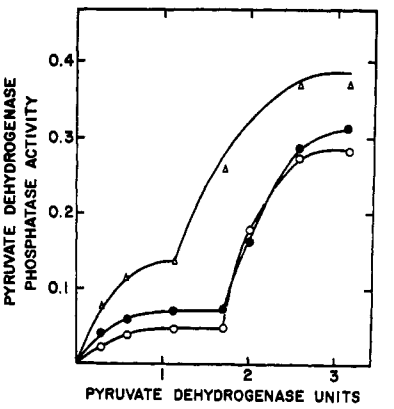

FIGURE 12: Effect of actinomycin D on the activity of pyruvate dehydrogenase phosphatase of adipose tissue. Fat pads were incubated for 60 min at 37° without additions (●) or in the presence of 120 μunits/ml of insulin (△) or of 25 μg/ml of actinomycin D (○). The experimental details are the same as those described in Figure 5.

activity is physiologically more relevant with respect to the overall effects on the activity of pyruvate dehydrogenase.

In contrast to the studies described for insulin, puromycin and cycloheximide do not significantly impede the rise in pyruvate dehydrogenase activity which is observed after incubating fat pads with epinephrine (Figure 13). Actinomycin D markedly decreases the effects of epinephrine (Figure 13). However, the activity of pyruvate dehydrogenase is not as severely depressed by actinomycin D (Figure 11) if this hormone is also present.

Discussion

Although several reports (Jungas, 1970a,b; Denton *et al.*, 1971; Coore *et al.*, 1971; Jungas and Taylor, 1972; Taylor and Jungas, 1972) have described increased activity of pyruvate dehydrogenase in homogenates of rat fat pads previously incubated with insulin, the mechanisms by which this occurs have not been elucidated. Coore *et al.* (1971) felt that insulin caused an activation of the enzyme by increasing the proportion of active (dephospho) pyruvate dehydrogenase. It was not established, however, whether this effect resulted from an increased activity of phosphatase or from a decreased activity of the specific kinase.

The present studies confirm the observations that insulin treatment of adipose tissue causes an increase in the active form of pyruvate dehydrogenase. In addition, however, there is an increase in the inactive (phospho) form of the enzyme which is quantitatively more pronounced than is the increase in the active form of the enzyme. There is, therefore, an increase in the *total* amount of pyruvate dehydrogenase in insulin-treated fat pads.

The activity of pyruvate dehydrogenase phosphatase is clearly increased in homogenates prepared from fat pads incubated with insulin. However, the physiological significance of this elevated activity in relation to the elevated activity of pyruvate dehydrogenase is not clear. It is tempting to speculate that the increased activity of the phosphatase may be the basis of the increased amount of the active (dephospho) form of the enzyme in the insulin-treated fat pads. This does not, however, explain the concomitant increase in the inactive form of pyruvate dehydrogenase, which is the predominant effect of insulin. It is also surprising that the active form of the enzyme is only modestly increased despite the apparent increase in the phosphatase activity as well as the substrate for the phosphatase.

The results of the studies with inhibitors of RNA and protein synthesis indicate that insulin may regulate at a translational level the synthesis of pyruvate dehydrogenase. Furthermore, cycloheximide and puromycin do not prevent the insulin-induced increase in phosphatase activity but do block the effect of insulin on pyruvate dehydrogenase. This suggests that the effect of insulin on phosphatase activity may be of minor importance compared to the effect of the hormone on protein synthesis under the conditions of these studies. The ability of insulin to modify protein synthesis in a manner similar to that suggested in the present studies has been well established in several tissues (Wool *et al.*, 1968).

Although insulin, epinephrine, and dibutyryl-cAMP all increase the total activity of pyruvate dehydrogenase in epididymal fat pads, there is evidence that these compounds may be acting at least in part by different mechanisms. Insulin appears to increase the activity of the phosphatase and it enhances the synthesis of pyruvate dehydrogenase but it does not alter the activity of the kinase. Epinephrine and dibutyryl-cAMP on the other hand do not appear to appreciably affect the activity

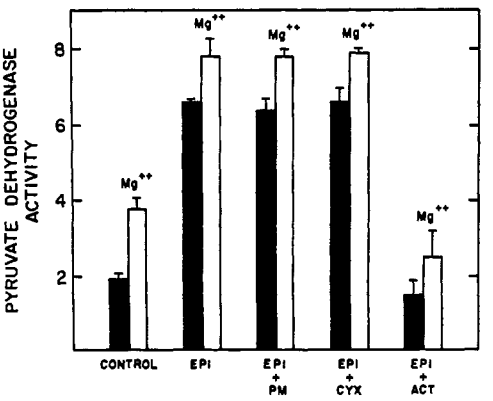

FIGURE 13: Effect of actinomycin D (ACT-D, 25 μg/ml), puromycin (PM; 30 μg/ml), and cycloheximide (CYX; 30 μg/ml) on the activity of pyruvate dehydrogenase of homogenates of fat pads incubated with epinephrine (EPI; 0.1 μg/ml). The experiments were performed as described in Figure 11.

of the kinase or of the phosphatase. In tissues treated with epinephrine, in contrast to those treated with insulin, virtually no inactive (phospho) pyruvate dehydrogenase is detectable. Furthermore, the inhibitors of protein synthesis do not alter the effect of epinephrine on pyruvate dehydrogenase but they nearly abolish the effects of insulin. The apparent increase in the total quantity of pyruvate dehydrogenase after epinephrine administration therefore appears to occur by different mechanisms than in the insulin treated tissue. The effects of actinomycin D (Figure 13) suggest that perhaps a step involving RNA synthesis may be involved. Possible effects of epinephrine on degradative processes must also be considered. It should also be noted that it is curious that the effects of insulin and epinephrine are not additive, especially if the effects are mediated by different mechanisms.

The apparent lack of antagonism between insulin and epinephrine is of special interest since the actions of these hormones in many physiological processes are mutually antagonistic. It is generally accepted (Robison *et al.*, 1971) that in most if not all tissues the fundamental basis of epinephrine action is stimulation of adenylate cyclase activity with a resultant increase in intracellular levels of cAMP. In contrast, many biological effects of insulin are explicable on the basis of a presumed fall in intracellular levels of cAMP, and there is some evidence which suggests that a fundamental effect of insulin may be to inhibit the activity of adenylate cyclase. Insulin decreases the intracellular levels of cAMP of fat cells or fat pads which have been stimulated by epinephrine (Butcher *et al.*, 1966, 1968; Jungas, 1966; Manganiello *et al.*, 1971), and it also inhibits the glucagon-stimulated increase of this nucleotide in liver (Exton *et al.*, 1971). Furthermore, insulin can directly inhibit the activity of adenylate cyclase in membrane fractions obtained from homogenates of fat or liver cells (Illiano and Cuatrecasas, 1972), and in fat cell ghosts (Hepp and Renner, 1971). Furthermore, there is some evidence that insulin can increase the activity of adipose tissue cAMP phosphodiesterase (Loten and Sneyd, 1970). These studies suggest that at least some of the biological effects of insulin may be mediated by direct or primary modulation of the concentration of cAMP.

Although the present studies do not provide support for the thesis that the primary effect of insulin on cells is to inhibit the activity of adenylate cyclase or to lower intracellular levels of cAMP, they also do not constitute direct proof against these points of view. Even though the apparent end effects of exogenous cAMP, epinephrine and insulin are presumably the same in terms of an elevation in the activity of pyruvate dehydrogenase, the mechanisms by which these hormones do this is clearly different. As described in this report and emphasized by others (Jungas and Taylor, 1972), pyruvate dehydrogenase is a complex enzyme which is subject to multiple regulatory controls. It has not been possible to demonstrate here or in other studies (Coore *et al.*, 1971; Jungas and Taylor, 1972) effects of any of these compounds on the activity of any of the components of the pyruvate dehydrogenase complex in broken-cell preparations. For this reason it is necessary to consider the possibility that the effects of insulin on pyruvate dehydrogenase synthesis or on phosphatase activity, or of epinephrine on total enzyme activity, result indirectly by compensatory metabolic processes perhaps quite remote from the original biochemical process initiated by the hormone–receptor interaction. This may be especially important in studies like the present which involve mitochondrial functions which are apparently quite distant from the cytoplasmic membrane, which is the initial site of action of insulin (Cuatrecasas, 1969, 1972). The changing patterns of the hormone effects on fat pads which occur with increasing time of incubation (Figures 4 and 9, and Jungas and Taylor, 1972) suggest strongly that secondary metabolic events are important in the observed changes in pyruvate dehydrogenase activity.

Since it has not been possible to demonstrate direct effects of hormones on the activity of enzymes in homogenates or on purified enzymes, the present studies like all those previously reported must by necessity utilize homogenates of intact tissues which have been incubated under varying conditions. Under these circumstances differences in the composition of metabolites (*e.g.*, pyruvate, nucleotides, Ca^{2+}, etc.) might be important in modifying the activity of various enzymes (Jungas and Taylor, 1972; Martin *et al.*, 1972; Hucho *et al.*, 1972). Because of such inherent difficulties, results obtained on such complicated systems must be considered provisional and subject to modification in interpretation as more information becomes available.

The possibility that all of the metabolic effects of insulin are related basically to changes in the activity of adenylate cyclase or intracellular levels of cAMP cannot be rejected on the basis that certain effects of the hormone, such as glucose transport and pyruvate dehydrogenase activity, are not immediately explicable by such a hypothesis. As described above, pyruvate dehydrogenase is subject to complex regulatory controls. In examining the possible relationship between insulin, cAMP, and events such as transport processes it is unreasonable to expect that because a given metabolic function is stimulated (or depressed) by cAMP that it necessarily follows that a fall in the basal concentration of the nucleotide will result in a metabolically opposite effect. The many and varied biochemical reactions which are modulated by cAMP can be expected to differ in their specific sensitivity to the concentration of the nucleotide. Some processes, for example, may be normally in an "inhibited" state at the basal concentrations of cAMP which prevail in the cell. In this case a selected depression of the nucleotide concentration may lead to an event which cannot be experimentally "antagonized" simply by elevating in a normal cell the already "high" concentration of the nucleotide. Such considerations may for example be applicable to certain insulin-sensitive processes, such as glucose and amino acid transport, which to date have defied explanation on the basis of changes in the activity of adenylate cyclase. These questions will probably remain unresolved until more information is available concerning the specific molecular events involved in the processes which are being studied.

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Kinetic Studies on Catechol *O*-Methyltransferase. Product Inhibition and the Nature of the Catechol Binding Site†

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ABSTRACT: The potent inhibition of catechol *O*-methyltransferase by one of the products, *S*-adenosylhomocysteine, has necessitated the development of two new assay techniques for monitoring this enzyme-catalyzed reaction. Using these techniques, initial velocity and product inhibition studies have been carried out. The results of these studies are in accord with a mechanism involving random binding of substrates and products to the enzyme. These data, together with data from the literature, suggest a mechanism of enzyme catalysis which involves two modes of catechol binding and participation of the adjacent hydroxyl group in enzyme-catalyzed methylation of catechols.

The metabolic fate of (nor)epinephrine has been extensively studied (Axelrod, 1971) and the important role of the enzyme catechol *O*-methyltransferase (EC 2.1.1.6) in the inactivation of catecholamines is now well established. The enzyme has been isolated and purified by several groups of workers (Nikodejevic *et al.*, 1970; Assicot and Bohuon, 1970; Flohe and Schwabe, 1970; Ball *et al.*, 1971) and some of its physical and chemical properties have been described. In order to get some insight into the mechanism of biological transmethylation processes, we have carried out kinetic studies on several nonenzymic (Coward and Sweet, 1971) and enzyme-catalyzed methyl transfer reactions. Initial kinetic studies on the reaction catalyzed by the catechol methylase revealed a strong inhibition by one of the products, AdoHcy[1] (Coward *et al.*, 1972). This type of inhibition has been observed in the methylation of tRNA (Hurwitz *et al.*, 1964; Kjellin-Straby, 1969; Pegg, 1971), homocysteine (Shapiro *et al.*, 1965), histamine and *N*-acetylserotonin (Zappia *et al.*, 1969), fatty acids (Akamatsu and Law, 1970), and biogenic amines (Deguchi and Barchas, 1971). This is of considerable interest in regulation of methylation (Deguchi and Barchas, 1971) and in the design of methylase inhibitors (Coward and Sweet, 1972; Coward and Slisz, 1973). However, our initial kinetic results showed that because of the strong product inhibition, reliable kinetic data would be difficult to obtain

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[1] Abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; TTO, toluene–Triton X-100–Omnifluor scintillation fluid; *p*-Cl-HgBzO, *p*-chloromercuribenzoate.