Methylation of Newly Synthesized and Older Deoxyribonucleic Acid

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Billen (1968) demonstrated that in *Escherichia coli* methylation of DNA normally occurs only on nascent DNA. In mouse L929 cells synchronized by treatment with aminopterin there is a burst of DNA methylation that peaks about 1 h after the time of maximum DNA synthesis (Burdon & Adams, 1969). However, the rate of methylation does not fall to zero in cells in phase G1, and moreover (in the presence of 2 mM-hydroxyurea) methylation still occurs at 35% the control rate although DNA synthesis is inhibited by 95%. These results suggested that in animal cells DNA could be methylated at a time other than immediately after synthesis, a suggestion that is substantiated by the following results.

Mouse L929 cells were grown in Roux flasks in minimum essential medium (Eagle's) containing 10% (v/v) of calf serum. After incubation for 1–3 h with [32 P]orthophosphate (2 μ Ci/ml) and L-[Me- 3 H]-methionine (2 μ Ci/ml) in the presence of 5-bromodeoxyuridine (20 μ M), sodium formate (20 mM) and aminopterin (0.2 μ M), DNA was extracted by the method of Kirby (1964) and subjected to equilibrium centrifugation in alkaline caesium chloride to separate the heavy, newly synthesized, 32 P-labelled DNA from light DNA. Tritium radioactivity is associated with both peaks and was shown by chromatography to be present almost exclusively in 5-methylcytosine.

When the cells were prelabelled with [32 P]orthophosphate in the presence of 5-bromodeoxyuridine and then incubated with L-[Me- 3 H]methionine in the presence of hydroxyurea (2 mM) tritium is again found in both peaks, suggesting the occurrence of methylation of recently synthesized DNA as well as of older DNA.

On release from stationary phase L929 cells do not make DNA for at least 12 h (Adams, 1969). In such cells, and also in phytohaemagglutinin-stimulated horse lymphocytes, there is no methylation of DNA before the onset of DNA synthesis. Moreover, as L929 cells re-enter a stationary phase of growth and the rate of synthesis of DNA slows there is an almost parallel fall-off in the rate of methylation.

Thus although methylation of old DNA does occur it seems unlikely that the time-lag between synthesis and methylation can be more than a generation.

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Effects of Insulin and Adrenaline on Rat Epididymal-Fat-Pad Pyruvate Dehydrogenase

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When fat-pads were incubated in Krebs bicarbonate buffer insulin stimulated fatty acid synthesis from glucose or fructose to a greater extent than explicable by its action on sugar transport. For example, with fructose (2 mg/ml) insulin (0.01 unit/ml) increased the rate of fatty acid synthesis to $180 \pm 13\%$ (15) of the control rate whereas the outputs of pyruvate and lactate into the incubation medium fell to 70 ± 4 and $57 \pm 4\%$ respectively of the control. An additional action of insulin on pyruvate metabolism to fatty acids is therefore indicated, as also concluded by Halperin (1970) and Jungas (1970).

We measured maximal activities of enzymes involved in the pathway of fatty acid synthesis from pyruvate after 30 min incubation of fat-pads. No effect of insulin was observed on pyruvate carboxylase, citrate synthase, ATP citrate-lyase or acetyl-CoA carboxylase, but pyruvate dehydrogenase was stimulated by $87 \pm 12\%$ (16). The mitochondrial marker enzyme glutamate dehydrogenase was unaffected by insulin, and the effect on pyruvate dehydrogenase was observed with or without added glucose or fructose.

Adrenaline (1 μ g/ml) or dibutyryl cyclic AMP greatly diminished the effect of insulin on fat-pad pyruvate dehydrogenase when present in the incubation mixture. On collagenase-released fat-cells incubated in Krebs bicarbonate buffer with 2% bovine serum albumin adrenaline decreased pyruvate dehydrogenase activity even below the value observed in the absence of insulin. This adrenaline effect was antagonized by the β -adrenergic inhibitor propanolol (10 μ g/ml).

The total activity of pyruvate dehydrogenase and the magnitude of these effects of insulin and adrenaline may be sufficient to account for the effects of these hormones on rates of fatty acid synthesis in epididymal adipose tissue. They are substantially greater than those given by Jungas (1970). The present findings provide an explanation for earlier observations on the interactions of insulin and adrenaline on fatty acid synthesis in epididymal fat-pad (Denton & Halperin, 1968) and are in accord with the suggestion that these hormones

may regulate the metabolism of pyruvate to fatty acids (Denton, Halperin & Randle, 1969).

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Properties of Pyruvate Dehydrogenase from Fat-Cell Mitochondria and its Hormonal Control

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The increased pyruvate dehydrogenase activity in fat-pads exposed to insulin (Coore, Denton, Martin & Randle, 1971) persists in mitochondria prepared from isolated fat-cells by the method of Martin & Denton (1970). Exposure of fat-pads and subsequently cells to insulin increased the pyruvate dehydrogenase/glutamate dehydrogenase activity ratio in extracts of mitochondria prepared from them by $80 \pm 15\%$ (12). Exposure to insulin also increased the rate of conversion of pyruvate into citrate by intact mitochondria incubated with pyruvate + bicarbonate or pyruvate + malate.

Linn, Pettit & Reed (1969) find that pyruvate dehydrogenase from kidney, liver or heart can occur in phosphorylated (inactive) and non-phosphorylated (active) forms, which may be interconverted by an ATP-requiring kinase and an Mg^{2+} -activated phosphatase. Similar transformations of fat-pad pyruvate dehydrogenase can be assumed from the following. In an extract of fat-cell mitochondria prepared in 30 mM-phosphate buffer, pH 7.0, containing 0.5 mM-magnesium chloride, 5 mM-2-mercaptoethanol and 1 μ g of oligomycin/ml the pyruvate dehydrogenase/glutamate dehydrogenase activity ratio was 0.28 throughout incubation for 30 min at 30°C. With addition of 10 mM-magnesium chloride the ratio rose to 0.53 and with addition of 0.1 mM-ATP the ratio fell to 0.04 (within 30 min). The effect of 10 mM-magnesium chloride could be reversed by subsequent dilution and addition of 0.1 mM-ATP, and that of 0.1 mM-ATP by subsequent addition of 10 mM-magnesium chloride. Addition of 5 mM-ATP to intact mitochondria incubated with pyruvate (5 mM), malate (5 mM), carbonyl cyanide *p*-chlorophenylhydrazide (0.5 μ M) and

oligomycin (0.5 μ g/ml) for 5 min was found to decrease the conversion of pyruvate into citrate by 35% and the activity of extracted pyruvate dehydrogenase was halved.

When extracts of mitochondria prepared from adipose tissue with or without insulin were incubated with 10 mM-magnesium chloride before assay the effects of insulin on pyruvate dehydrogenase activity were largely eliminated. This finding, together with the observation that insulin activation of pyruvate dehydrogenase did not alter the K_m for pyruvate (67 μ M), suggests that insulin action may increase the proportion of active (dephospho) pyruvate dehydrogenase. The mechanism of activation by insulin and of reversal by adrenaline are not known. An obvious mechanism is one involving cyclic AMP (adenosine cyclic 3':5'-monophosphate), but no clear inhibitory effects of cyclic AMP on pyruvate dehydrogenase activity in intact mitochondria or mitochondrial extracts have been found.

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An Immuno-electrophoretic Assay of β -Lipoprotein and Albumin in Human Aortic Intima by Direct Electrophoresis from the Tissue Sample into an Antibody-Containing Gel

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In human atherosclerosis much of the lipid accumulated in the artery wall is probably derived from plasma β -lipoprotein. Previously (Smith & Slater, 1970) we measured the immunologically intact lipoprotein by extraction, ultracentrifugation and immuno-plate assay (Fahey & McKelvey, 1965), which required 30–150 mg of tissue. On electrophoresis of minced intima in agarose lipoprotein migrated as a broad and straggling band, but re-mincing and re-running indicated that its removal was mainly complete. This suggested that intimal β -lipoprotein might be measured immunologically by migration into an antibody-containing gel, as suggested by Ressler (1960) for isolated proteins.

Plates (10 cm $\times$ 10 cm) are prepared with 28 ml of