SC 2217

Stimulation by insulin of incorporation of [^{32}P]phosphate and ^{14}C from acetate into lipid and protein of isolated rat diaphragm

Insulin *in vitro* stimulates sugar uptake[1–3], glycogen synthesis[4], amino acid incorporation into protein[5,6] and nucleotide incorporation into nucleic acid[7] by isolated rat diaphragm. It is therefore to be expected that insulin will also increase incorporation in this tissue of various precursors into fat and phospholipid. BODEL *et al.*[8] have recently observed a stimulation of incorporation of [^{14}C]palmitate into total lipid, though such an effect was not found in a previous study by FRITZ AND KAPLAN[9]. This communication demonstrates promotion by insulin of incorporation of ^{14}C from [^{14}C]acetate into fat and phospholipid and of [^{32}P]phosphate into phospholipid and protein. An effect of insulin to enhance incorporation of ^{14}C from acetate into protein has been previously reported[10].

Hemidiaphragms, from non-fasting female albino Wistar rats, 100–120 g, were incubated for 2 h in Krebs bicarbonate buffer, gassed with O_2–CO_2 (95:5), pH 7.4, at 37°. The buffer contained no glucose or other additions unless otherwise stated. Radioisotopes were obtained from the Radiochemical Centre, Amersham, England. Sodium [1-^{14}C]acetate, specific activity about 4.5 μC/μmole, was added at a concentration of 0.8 μC/ml (0.2 mM). [^{32}P]Phosphate was used at the rate of about 4 μC/ml, which in view of the concentration of phosphate in the buffer (1.2 mM) gave a specific activity of 3.3 μC/μmole. Measurement of incorporation of radioactivity from acetate and phosphate into neutral fat and phospholipid of diaphragm was based on techniques described by FOLCH-PI *et al.*[11] and NEPTUNE *et al.*[12]. After incubation the hemidiaphragms were washed in 1% NaCl, blotted, and ground in a mortar with 1–2 ml of a mixture of chloroform and methanol (2:1, v/v). The suspension was transferred to a filter funnel and the filtrate collected in a 25-ml volumetric flask. The residue in the filter was washed with successive portions of chloroform–methanol until the filtrate reached 25 ml. The filtrate was then evaporated to dryness on a rotary evaporator. The residue, contained in a round-bottomed flask, was extracted with 1 ml of *n*-hexane. To the hexane solution, still in the flask, was then added 10 ml of acetone and the residue now extracted with the mixture of hexane and acetone. The soluble fraction, which on addition of the acetone had turned cloudy due to partial precipitation of phospholipid, was transferred away from the residue and the further precipitation of phospholipid facilitated by addition of 0.1 ml of a saturated solution of anhyd. $MgCl_2$ in ethanol. The mixture was placed at −10° for at least 2 h, after which the precipitate was collected by centrifugation, washed with acetone and dissolved in 1.5 ml of ethanol. Samples (1 ml) of this solution and of the supernatant from the precipitation of phospholipid, being phospholipid and neutral fat fractions, respectively, were pipetted on to aluminium discs 2, cm in diameter and the liquid allowed to evaporate. The last traces of liquid were removed by gentle warming under an infrared lamp. The content of radioactivity of the discs was assessed in an end-window counter (Panex-model D-554). No corrections for self-absorption were made. Extraction of the hexane–acetone solution, after evaporation to dryness and redissolving in chloroform or hexane, with an equal volume of 4% Na_2CO_3 or of 0.4 N NaOH in 50% ethanol, led to removal of less than 10% of the ^{14}C in the organic phase and only minute quantities of radioactivity could be recovered

TABLE I

EFFECT OF INSULIN AND GLUCOSE ON THE INCORPORATION OF ^{14}C FROM ACETATE AND OF [^{32}P]PHOSPHATE INTO NEUTRAL FAT, PHOSPHOLIPID AND PROTEIN OF ISOLATED RAT DIAPHRAGM

Each figure is the mean ± S.E. of 12 observations.

Fractions in which radioactivity is contained after incubation with	*Additions to the medium*				*Significance of differences (P)*
	No additions (a)	*Insulin (b)*	*Glucose (c)*	*Glucose and insulin (d)*	
[1-^{14}C]*Acetate*					
Neutral fat (counts/min/g wet wt. of diaphragm)	16500 ± 1500	29200 ± 3000	32400 ± 4600	43700 ± 2800	$(b-a) = 0.001$ $(c-a) < 0.001$ $(d-b) < 0.01$ $(d-c) < 0.05$
Phospholipid (counts/min/g wet wt. of diaphragm)	2820 ± 190	4170 ± 260	3390 ± 210	4040 ± 310	$(b-a) < 0.001$
[^{32}P]*Phosphate*					
Phospholipid (counts/min/g wet wt. of diaphragm)	10200 ± 420	13000 ± 1130	10500 ± 360	12100 ± 460	$(b-a) < 0.05$ $(d-c) = 0.02$
Protein (counts/min/mg of protein)	266 ± 18	363 ± 21	295 ± 14	376 ± 25	$(b-a) < 0.001$ $(d-c) = 0.01$

from the aqueous phase on extraction with hexane after acidification. The free fatty acid content of muscle has been found by other workers to be small by comparison with that of neutral fat[13]. Virtually no ^{32}P was found in the neutral fat fraction. Measurement of incorporation of radioactivity into protein was as previously described[14]. Acetate incorporated into fat and phospholipid is presumably first activated to acetyl-CoA and used in the synthesis of longer-chain fatty acids. The form in which ^{32}P is incorporated into protein is at present unknown.

The results are contained in Table I. Addition of insulin (0.1 unit/ml) in the absence of added glucose stimulated the incorporation of ^{14}C from acetate into fat and phospholipid and of ^{32}P into phospholipid and protein. The effect of insulin on incorporation of ^{32}P was equally apparent whether glucose was present or not, glucose alone being without significant effect. Glucose did enhance incorporation of ^{14}C from acetate into fat and possibly into phospholipid, but a stimulation by insulin of incorporation into fat was still seen in the presence of glucose.

The effect of insulin in the absence of added glucose is reminiscent of its action on amino acid incorporation[15,16] and is similar to the observation of FIELD AND ADAMS[17] of the stimulation by insulin of incorporation of acetate into fat of sciatic nerve. The results again provide evidence of an action of insulin on anabolic processes in diaphragm not dependent on a stimulation of glucose transport. In view of the stimulation by insulin of uptake of sugars[1-3] the possibility that insulin raises amino acid incorporation by influencing the entry into the cell of amino acids also was put forward[18], but the balance of the available evidence[19] suggests that this is not the mechanism involved. Similarly it is unlikely that the influence of insulin on incorporation of ^{14}C and ^{32}P into fat and phospholipid results from a stimulation of their

uptake. VOLFIN *et al.*[20] looked for but did not find an effect of insulin on the entry of inorganic phosphate into diaphragm and MANCHESTER AND KRAHL[10] found no effect on the accumulation of acetate. In order to account for the action of insulin in stimulating amino acid incorporation into protein[15,16] and of nucleotides into nucleic acid[7] in the absence of added glucose, it has been suggested[19,21,22] on the basis of an idea put forward by RANDLE AND SMITH[23] that insulin affects the translocation of energy-rich phosphate within the cell in such a way as to increase the availability of such materials at the sites of synthetic processes whose rates are limited by their requirement for energy-rich compounds. Since fat and phospholipid synthesis is an energy-consuming process an action of insulin in the manner indicated would equally explain the observed stimulation of incorporation of radioactivity from acetate and phosphate.

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