Re-evaluation of the Effect of Insulin on Nucleic Acid Synthesis in Muscle

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Several groups have recently found that, though growth hormone *in vitro* stimulates incorporation of amino acids into protein of isolated rat diaphragm, it does not enhance nucleic acid synthesis (Martin & Young, 1965; Kostyo, 1966; Dawson, Patey, Rubenstein & Beck, 1966). Though not remarked upon by the authors, this is an observation of some interest, for hitherto there has been a good parallel between the action of insulin and growth hormone *in vitro*, e.g. on glucose uptake, on incorporation of amino acids into protein and on accumulation of amino acids, whereas with nucleic acid synthesis a divergence appears. That insulin promotes nucleic acid synthesis in muscle was reported by Wool (1960, 1963), and on the face of it his results show a clear stimulation. However, my experience has been that an effect of insulin on nucleic acid synthesis in muscle is not readily seen, and, in view of the findings with growth hormone, it seemed desirable to re-examine the effect of insulin.

Past methods that have been used for isolation of RNA from tissues include extraction with hot 10% NaCl. In my experience this method is unsatisfactory for, as can be seen from the results of Wool (1963), it extracts only 5–10% of the RNA that is supposedly present in muscle (Leslie, 1955; Devi, Mukundan, Srivastava & Sarkar, 1963; Margreth & Novello, 1964), as well as variable amounts of protein. In the present investigation two other procedures have been used: (a) extraction with alkali by the method of Fleck & Munro (1962) and (b) extraction with phenol. In the latter, diaphragm muscle, after incubation with labelled precursor, was homogenized at 4° in 0.3M-sodium trichloroacetate, pH 7.5, in the presence of an equal volume of phenol and was heated to 65° for 20 min. in an attempt to extract as much RNA as possible (Georgiev, Samarina, Lerman, Smirnov & Severtzov, 1963). The mixture was cooled and the two layers were separated by centrifugation. The aqueous layer was made 2M with respect to NaCl (or sodium acetate) and after addition of 2 vol. of ethanol the precipitate forming on standing overnight at –10° collected. This material, after being washed in ethanol and dissolved in water, had an E_{260}/E_{280} ratio about 1.7. The yield of RNA based on E_{260} 34.2 mg. $^{-1}$ cm.² varied considerably between experiments. It was improved by the presence of

1% (w/v) sodium lauryl sulphate during homogenization and averaged about 1 mg. of RNA/g. of tissue, which is about 50% of the total content of the tissue. Addition of 0.3M-KCl, as suggested by Heywood, Dowben & Rich (1967) for isolation of muscle polysomes, improved the yield to between 1.5–1.8 mg./g., but it did not significantly change the specific activity of the material. The re-dissolved material on 5–20% (w/v) sucrose gradients separated into three peaks as described by Wool & Munro (1963) and Kostyo (1966). At the interface between the phenol and water layers a variable amount of material collected, possibly a nuclear RNA fraction (Georgiev *et al.* 1963). This material was not analysed. However, on standing a precipitate gradually collected in the phenol phase and this material after washing with ethanol and cold 10% (w/v) trichloroacetic acid was found to possess considerable radioactivity that could be extracted by trichloroacetic acid at 90°. It is possible that this material is a portion of the nuclear or chromosomal RNA (Greenman, Wicks & Kenney, 1965). Although the above indicates that a fully satisfactory fractionation of diaphragm-muscle RNA has not been obtained, the relative incorporation of radioactivity from the different precursors adenine, uracil and orotic acid was in proportions similar to those observed when total RNA was extracted with alkali (Table 1). In numerous experiments no hint of any influence of insulin to increase their incorporation was observed.

The results obtained with alkali are shown in Table 1. In four experiments the quantity of RNA phosphorus extracted by alkali was fairly consistent at about 150 μ g./g. of tissue, and negligible radioactivity remained in the residues. The quantity present after incubation was very slightly increased by insulin. The presence of insulin clearly did not stimulate incorporation of adenine, but some indication of an enhanced incorporation could be seen with orotic acid and uracil. The reason for this difference I do not know. It is possible that the greater incorporation of adenine results from terminal labelling of transfer RNA (Franklin, 1963), a process that might not be expected to be responsive to hormones, but incorporation of adenine is almost completely prevented by the presence of actinomycin.

Table 1. *Effect of insulin on the synthesis of RNA in isolated rat diaphragm muscle*

Diaphragms were incubated as described previously (Manchester, 1966) for 2 hr. with the appropriate precursor. The tissue was then homogenized in N-HClO_4 at 0° and the precipitate washed three times with N-HClO_4 . The precipitate was then suspended in 2 ml. of 0.5N-NaOH for 1 hr. at 37° and reprecipitated by addition of 2 ml. of N-HClO_4 . The quantity of RNA P in solution was estimated from E_{260} and E_{275} according to the formula of Fleck & Munro (1962):

$$\text{RNA P } (\mu\text{g./ml.}) = 11.87E_{260} - 10.40E_{275}$$

Each precursor was present at a concentration of about $0.4 \mu\text{Ci/ml.}$, which was for adenine $11.4 \mu\text{M}$, for orotic acid $9.0 \mu\text{M}$ and for uracil $6.5 \mu\text{M}$. Insulin, where present, was at a final concentration of 0.1 unit/ml. Each result is the mean $\pm$ s.e.m. of six observations. Combining the values for the RNA extracted the means of 24 observations are: without insulin, $152.4 \pm 2.75 \mu\text{g}$ of RNA P/g.; with insulin, $158.5 \pm 3.6 \mu\text{g}$ of RNA P/g.; difference, $6.1 \pm 2.46 \mu\text{g}$ of RNA P/g. ($P=0.02$).

Precursor	RNA extracted (μg of RNA P/g. of tissue)			Specific activity of RNA extracted (disintegrations/min. μg of P)			RNA synthesis (disintegrations/min. incorporated/mg. of tissue)		
	Without insulin	With insulin	Percentage increase	Without insulin	With insulin	Difference	Without insulin	With insulin	Difference
[8- ^{14}C]Adenine	174 $\pm$ 10	183 $\pm$ 11	— 1.2	2170 $\pm$ 164	2143 $\pm$ 146	27 $\pm$ 67	369 $\pm$ 29	386 $\pm$ 25	18 $\pm$ 18
[8- ^{14}C]Adenine	147 $\pm$ 3.2	151 $\pm$ 7.0	— 0.7	2080 $\pm$ 90	2065 $\pm$ 63	15 $\pm$ 47	311 $\pm$ 20	316 $\pm$ 18	5 $\pm$ 12
[6- ^{14}C]Orotic acid	134 $\pm$ 3.2	143 $\pm$ 2.7	5.4	313 $\pm$ 14	344 $\pm$ 17	17 $\pm$ 9.2	41.5 $\pm$ 0.97	48.3 $\pm$ 1.86	6.9 $\pm$ 1.8
[2- ^{14}C]Uracil	156 $\pm$ 5.8	159 $\pm$ 7.7	16	118 $\pm$ 5.4	137 $\pm$ 12	19 $\pm$ 9.9	24.3 $\pm$ 0.94	27.2 $\pm$ 2.0	2.9 $\pm$ 1.8

The significance of the results, unfortunately, is equivocal. Although they do not refute the possibility of a stimulation of nucleic acid synthesis in muscle by insulin, they do suggest that the influence is not very marked and not nearly as great as on other parameters, e.g. glucose and glycogen metabolism or accumulation and incorporation into protein of amino acids. If an effect of insulin is irregular and dependent on conditions and choice of precursor, it would be possible for it to have been missed in the studies with growth hormone. The lack of any observable effect of growth hormone on nucleic acid synthesis therefore remains consistent with its possessing activities *in vitro* paralleling those of insulin. It is noteworthy that Bransome & Reddy (1963) found with adrenal slices that insulin increased amino acid incorporation but not nucleic acid synthesis.

Since so many anabolic parameters in diaphragm appear to respond to insulin *in vitro* (Manchester 1965), it becomes almost more difficult to explain why nucleic acid synthesis should be excluded from this list rather than included. However, if nucleic acid synthesis takes place in the nucleus and if the primary action of insulin that leads to the observed consequences is to bring about some form of conformational change in the tissue (Krahl, 1957) (a vague concept, but as yet as good as any), it is conceivable that nuclear processes are but little affected. If in the experiments of Wool (1963) incomplete extraction of nucleic acid was taking place, as seems very likely, it is possible that the effect of insulin observed on precursor incorporation resulted from a change in the degree of extractability of the nucleic acid, which indeed he indirectly confirmed in suggesting that the presence of insulin led to net synthesis of RNA during incubation. The exact significance of any such change in extractability is difficult to evaluate. However, if for example the extent of binding of polysomes to membranes is an important aspect in the control of their activity, as is suggested in other systems (Tata, 1967), then change in degree of binding would provide a mechanism by which insulin could rapidly alter in cellular preparations the rate of their protein-synthetic activity independently of any requirement for nucleic acid synthesis. Thus for insulin and growth hormone we probably have to look elsewhere than to enhancement of nucleic acid synthesis or messenger RNA production for the basis of the mechanism of their influence on protein synthesis in muscle.

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