Biochem. J. (1975) **150**, 441–451
Printed in Great Britain

Studies on the Role of Insulin in the Regulation of Glyceride Synthesis in Rat Epididymal Adipose Tissue

By SUREN R. SOORANNA and E. DAVID SAGGERSON
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

(*Received* 7 *March* 1975)

1. When rat isolated fat-cells were incubated with fructose and palmitate, insulin significantly stimulated glyceride synthesis as measured by either [^{14}C]fructose incorporation into the glycerol moiety or of [^{3}H]palmitate incorporation into the acyl moiety of tissue glycerides. Under certain conditions the effect of insulin on glyceride synthesis was greater than the effect of insulin on fructose uptake. 2. In the presence of palmitate, insulin slightly stimulated (*a*) [^{14}C]pyruvate incorporation into glyceride glycerol of fat-cells and (*b*) $^{3}H_2O$ incorporation into glyceride glycerol of incubated fat-pads. 3. At low extracellular total concentrations of fatty acids (in the presence of albumin), insulin stimulated [^{14}C]fructose, [^{14}C]pyruvate and $^{3}H_2O$ incorporation into fat-cell fatty acids. Increasing the extracellular fatty acid concentration greatly inhibited fatty acid synthesis from these precursors and also greatly decreased the extent of apparent stimulation of fatty acid synthesis by insulin. 4. These results are discussed in relation to the suggestion [A. P. Halestrap & R. M Denton (1974) *Biochem. J.* **142**, 365–377] that the tissue may contain a specific acyl-binding protein which is subject to regulation. It is suggested that an insulin-sensitive enzyme component of the glyceride-synthesis process may play such a role.

White adipose tissue makes an important contribution to the overall metabolism of many animals through its ability to store fatty acids as triglyceride and then to make these metabolites available again when required. Since the opposing processes of fatty acid esterification and triglyceride lipolysis in adipose tissue have extremely high potential activities, it is important that they should be maintained under tight metabolic control. A considerable body of knowledge exists on the direct activation of adipose-tissue lipolysis by several hormones and the ability of insulin to antagonize these effects (Gordon & Cherkes, 1958; Jungas & Ball, 1963; Fain *et al.*, 1966; Blecher *et al.*, 1968). On the other hand, current ideas about the control of triglyceride synthesis are concerned essentially with regulation of the supply of precursors to fat-cells. First, the activity of lipoprotein lipase which may influence the supply of exogenous fatty acyl precursors appears to be subject to hormonal regulation (Salaman & Robinson, 1966; Wing & Robinson, 1968; Patten, 1970; Davies *et al.*, 1974). Secondly, stimulation by insulin of glucose entry demonstrated with various adipose-tissue preparations (Crofford & Renold, 1965; Martin & Carter, 1970; Illiano & Cuatrecasas, 1971) has been shown to increase the rate of glyceride synthesis (Bally *et al.*, 1960) and to increase the tissue concentration of glycerol phosphate *in vitro* (Denton *et al.*, 1966; Saggerson & Greenbaum, 1970). An analogy may be made with the metabolism of glycogen, the synthesis and degradation of which appears to be under synchronized, but opposite, hormonal control (see review by Nuttall, 1972). It might be argued that direct hormonal regulation should exist at the level of the intracellular synthesis of triglyceride as well as on the lipolysis of triglyceride. There have been reports of direct or indirect effects of insulin and adrenaline on two complex enzymes in adipose tissue, namely acetyl-CoA carboxylase (EC 6.4.1.2) (Halestrap & Denton, 1973, 1974) and pyruvate dehydrogenase (EC 1.2.4.1) (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971; Martin *et al.*, 1972; Sica & Cuatrecasas, 1973). This sets a precedent for the possibility of there being direct or indirect hormonal effects on some enzyme(s) in the intracellular pathway of triglyceride synthesis. The present paper presents the results of preliminary experiments suggesting that this may in fact be the case in that insulin is shown to stimulate glyceride synthesis. Further, the previously reported effects

of this hormone on acetyl-CoA carboxylase and pyruvate dehydrogenase may be reasonably explained as being secondary to an effect on glyceride synthesis.

Materials and Methods

Chemicals

All enzymes, nucleotides and sodium pyruvate were obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.). Fructose and calf thymus DNA were obtained from the Sigma (London) Chemical Co. (London S.W.6, U.K.). Bovine serum albumin (fraction V) from the Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) was treated to remove fatty acids as described by Saggerson (1972). Sodium palmitate was obtained from Nu Chek Prep, Elysian, Minn., U.S.A., and was associated with fatty acid-poor albumin as described by Evans & Mueller (1963). All radiochemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Radioactive palmitic acid supplied either as a benzene or as a benzene–hexane solution was subjected to a procedure which appreciably decreased the counting backgrounds in the experiments performed. The labelled palmitate was extracted into 50mM-$NaHCO_3$ in ethanol–water (1:1, v/v). The pH was then adjusted to 2.5–3.0 by addition of 2.5M-HCl. The palmitic acid was extracted into hexane which was then evaporated under N_2 at 50–60°C. The palmitic acid was finally converted into the sodium salt and associated with albumin as described above. 2,5-Bis-(5-t-butylbenzoxazol-2-yl)-thiophen was obtained from CIBA (A.R.L.) Ltd. (Duxford, Cambs., U.K.), 2-methoxyethanol from Reeve Angel Scientific (London E.C.4, U.K.) and Hyamine hydroxide from Nuclear Enterprises (Edinburgh, U.K.). Bovine insulin (6×recrystallized) was obtained from Boots Pure Drug Co. (Nottingham, Notts., U.K.), anti-insulin serum from the Wellcome Research Laboratories (Beckenham, Kent, U.K.) and prostaglandin E_1 from Cambrian Chemicals (Croydon, Surrey, U.K.). Insulin was freshly dissolved at a concentration of 20i.u./ml in 3.3mM-HCl and diluted appropriately in incubation media. Prostaglandin E_1 was dissolved to give a concentration of 770μg/ml in a solution consisting of 1.5mM-KH_2PO_4 adjusted to pH7.8 with KOH to which ethanol was then added (23%, v/v) and diluted appropriately in incubation media. Similarly diluted samples of KH_2PO_4 containing 23% (v/v) ethanol were added to appropriate control incubations. All other chemicals were of A.R. grade and were used without further purification.

Animals

Male Sprague–Dawley rats weighing 140–190g were purchased from Bantin and Kingman, Hull, Humberside, U.K. or were bred in the animal colony at University College London from animals originally obtained from Bantin and Kingman. The animals were maintained on cube diet 41B (Bruce & Parkes, 1949) with water supplied *ad libitum*. Animals designated as starved–re-fed were starved for 48h and then re-fed for 48h on a high-carbohydrate diet of which 70% (w/w) was sucrose (Saggerson & Tomassi, 1971). These animals were within the weight range quoted above at death. All animals were killed by cervical dislocation.

Techniques with fat-cells

Preparation. Isolated fat-cells were prepared by the method of Rodbell (1964) as described by Saggerson & Tomassi (1971).

Incubation of fat-cells in shaken flasks. Portions of fat-cells equivalent to 50–70mg (dry wt.) were incubated in 4ml of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) previously gassed with O_2+CO_2 (95:5) and containing albumin as described by Saggerson & Tomassi (1971). Normally the incubations were performed in 25ml silicone-treated Erlenmeyer flasks. However, when $^{14}CO_2$ was to be measured, 50ml silicone-treated flasks fitted with glass centre wells were used. Where appropriate ^3H_2O was present at 1.25mCi/ml and [9,10(n)-^{3}H]palmitate at 1μCi/ml. [2-^{14}C]Pyruvate, [U-^{14}C]pyruvate, [U-^{14}C]fructose and [U-^{14}C]glucose were used at 0.25μCi/ml.

Incubation of fat-cells with stirring. In one series of experiments in which fat-cells were withdrawn at frequent times, it was not convenient to incubate the cells in sealed shaken flasks. In this case therefore starting volumes of 12ml of fat-cells plus incubation medium were stirred by a magnetic follower in a cylindrical (2.5cm diameter), flat-bottomed glass tube of 8cm height which was surrounded by a 37°C water jacket. The glass of the vessel and of the coating of the follower were silicone-treated. O_2+CO_2 (95:5) was continuously passed into the air space above the stirred liquid through a lid which could be easily removed for sampling. The stirring procedure did not appear to result in any more damage to the cells than is encountered on incubation with shaking in the conventional manner, since rates of incorporation of radioactive substrates into metabolic products were similar in both systems.

Measurement of incorporation of radioactivity into metabolic products. After incubation, lipids from fat-cells were extracted into hexane (Saggerson & Tomassi, 1971). In experiments where labelled palmitate was present, the hexane extracts were washed first with 3vol. and then with 1vol. of freshly prepared 50mM-$NaHCO_3$ in ethanol–water (1:1, v/v) previously cooled to 4°C. This procedure is a

modification of that used by Borgström (1952). A further washing with 1 vol. of ethanolic bicarbonate was used when ^3H_2O was present in incubations. The washed hexane extracts were then analysed for incorporation of ^{14}C and of ^3H into glyceride fatty acids and glyceride glycerol as described by Saggerson & Greenbaum (1970) and Saggerson & Tomassi (1971) except that for the measurement of ^3H incorporation from ^3H_2O into glyceride glycerol the scintillation fluid used, which was a modification of that described by Bray (1960), consisted of 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen and 80g of naphthalene per litre of 2-methoxyethanol–toluene (2:3, v/v). Collection and determination of $^{14}CO_2$ was as described by Saggerson & Greenbaum (1970). Controls were performed in parallel with each experiment to correct for the trapping in Hyamine of ^{14}C-labelled substances made volatile by acidification.

Techniques with fat-pads

Incubation of epididymal fat-pieces was performed as described by Saggerson & Greenbaum (1970), and the conditions of individual experiments are given in the headings to individual Tables. All analytical techniques were identical with those described for fat-cells except that fat-pads were subjected to homogenization in a motor-driven ground-glass homogenizer during extraction of lipids into hexane as described above.

Measurement of unesterified fatty acids in incubation media

Incubation media containing fat-cells were centrifuged for 20s at approx. $400g_{av.}$. The resulting infranatant was removed by aspiration and from this a 2ml portion was taken and the fatty acids were extracted into hexane (Saggerson & Tomassi, 1971). Duplicate portions of the hexane extracts were then evaporated to dryness at 60–70°C, 6ml of chloroform was added and determination of fatty acids was performed as described by Itaya & Ui (1965) with palmitic acid as a standard.

Measurement of fructose, lactate and pyruvate in incubation media

Samples of media were deproteinized and lactate and pyruvate assayed enzymically as described by Saggerson & Greenbaum (1970). Fructose was assayed in deproteinized media by the method of Klotzsch & Bergmeyer (1963).

Measurement of fat-cell DNA

This was as described by Saggerson (1972). A mean value of 178μg of DNA/g dry wt. of cells was obtained for cells prepared from normally fed rats.

Expression of results

Units for incorporation of substrates are indicated in the individual legends to Figures and Tables. For labelled palmitate incorporation, the values presented are calculated from the specific radioactivity of this precursor at the start of incubation unless otherwise stated. In all fat-cell experiments different cell preparations were used for each determination. Where presented, statistical significance of results was determined by Student's *t* test.

Results and Discussion

The purpose of this study was to investigate whether, in addition to the various effects of insulin on adipose tissue that have already been documented (see review by Avruch *et al.*, 1972), the hormone may exert a direct stimulatory effect on glyceride synthesis. For such a demonstration it is necessary to supply the tissue with both a glycerol phosphate precursor and a sufficiently large pool of unesterified fatty acid to sustain glyceride synthesis. Glucose cannot satisfactorily be used as the glycerol phosphate precursor since insulin stimulated its plasma-membrane transport. For this reason Coore *et al.* (1971), in a study that implicated pyruvate dehydrogenase as a site of insulin stimulation of fatty acid synthesis in adipose tissue, used fructose as a carbohydrate substrate. These workers concluded that insulin had a very small effect on the uptake of this sugar and that in percentage terms insulin stimulation of fatty acid synthesis was greater than the observed increase in fructose utilization. It is particularly noteworthy that although it has been postulated that insulin stimulates plasma-membrane transport of D-fructose in rat adipose tissue (Froesch & Ginsberg, 1962; Fain, 1964; Gutman *et al.*, 1966), this conclusion is based entirely on the observation that insulin stimulates fructose utilization and appearance of [^{14}C]fructose in metabolic products such as fatty acids and CO_2. Unlike D-glucose, for which insulin stimulation of plasma-membrane transport has been demonstrated indirectly in adipose-tissue pieces or in fat-cells (Crofford & Renold, 1965; Crofford *et al.*, 1966) and directly in fat-cell membrane preparations or 'ghosts' (Martin & Carter, 1970; Illiano & Cuatrecasas, 1971), insulin stimulation of fructose transport into adipose tissue does not appear to have been demonstrated.

Table 1 shows that when fat-cells were incubated with [^{14}C]fructose alone, insulin stimulated the incorporation of ^{14}C into fatty acids by 117% whereas the calculated fructose utilization ($^{14}CO_2$ + ^{14}C-labelled lipids + lactate and pyruvate production) was only increased by 51% in accord with the observations of Coore *et al.* (1971).

Table 1. *Effect of insulin on fructose utilization by fat-cells in the presence and absence of palmitate*

Fat-cells were incubated for 1h in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, 2mM-[U-^{14}C]fructose and 0.2mM-sodium palmitate, anti-insulin serum (10m-i.u./ml) or insulin (20m-i.u./ml) where indicated. The results are the means±S.E.M. of four determinations and are expressed as μg-atoms of C/h per 100μg of DNA. *, **, indicate $P<0.05$ or <0.001 respectively for comparison of insulin-treated incubations versus anti-insulin serum-treated controls. The mean fat-cell DNA was 2.8μg/ml of incubation medium.

	Fructose incorporation			
Additions to incubation medium ...	Anti-insulin serum	Insulin	Anti-insulin serum +palmitate	Insulin+palmitate
Incorporation of fructose into:				
CO_2	1.50±0.21	2.62±0.11**	1.21±0.10	2.03±0.15**
Total lipids	1.01±0.20	2.15±0.27**	1.41±0.16	2.89±0.24**
Fatty acids	0.46±0.04	1.00±0.13**	0.21±0.04	0.32±0.08
Glyceride glycerol	0.61±0.11	0.83±0.10	0.92±0.13	1.89±0.20
Lactate+pyruvate production	2.92±0.51	3.56±0.47	2.87±0.07	3.21±0.22**
Lactate/pyruvate ratio	4.89	3.55	2.71	1.81
Calculated fructose utilization	5.52±0.80	8.33±0.64*	5.48±0.26	8.13±0.46**

Table 2. *Effect of insulin on fructose utilization by epididymal fat-pads in the presence of palmitate*

Tissue from three halved fat-pads (0.79–1.15g) was incubated in each flask for 2h in 4ml of Krebs–Ringer bicarbonate containing 15mg of albumin/ml, 1mM-[U-^{14}C]fructose, 0.5mM-sodium palmitate and either anti-insulin serum (10m-i.u./ml) or insulin (20m-i.u./ml). From each animal the tissue from one pad was used for determination of $^{14}CO_2$ and the other pad was used for determination of all other measured parameters. The data for pads incubated with and without insulin were obtained with tissues from different animals. The results are means±S.E.M. of the numbers of determinations (indicated in parentheses) and are expressed as μg-atoms of C/2h per g wet wt. of tissue. *, ** indicate $P<0.05$ and <0.001 respectively for comparison of insulin-treated tissues versus anti-insulin serum-treated controls.

	With anti-insulin serum	With insulin
[^{14}C]Fructose into:		
CO_2	2.56±0.08 (7)	4.59±0.13** (7)
Total lipids	2.74±0.09 (8)	3.64±0.08** (8)
Fatty acid	0.69±0.07 (5)	0.86±0.08 (5)
Glyceride glycerol	1.66±0.13 (5)	2.51±0.09** (5)
Lactate+pyruvate production	0.89±0.04 (7)	0.38±0.03** (7)
Lactate/pyruvate ratio	2.92 (7)	1.75 (7)
Measured fructose utilization (A)	7.22±1.01 (7)	10.23±0.78* (7)
Calculated fructose utilization (B)	6.17±0.15 (7)	8.68±0.11** (7)
Mean ratio A/B	1.16	1.17

In this experiment it was not possible to determine accurately and directly the utilization of fructose by the fat-cells which was usually less than 3% even over a 90min period of incubation. The experiment summarized in Table 2 was therefore performed with fat-pads and a low concentration of fructose to ascertain whether summation of the metabolic products ($^{14}CO_2$+^{14}C-labelled total lipids+lactate and pyruvate) gave an adequate measurement of fructose utilization in adipose tissues. The calculated utilization of fructose agreed quite well with the directly measured utilization which was 30–40%. When fat-cells were incubated with [^{14}C]fructose in the presence of palmitate (Table 1) the incorporation of fructose carbon into glyceride glycerol was then the most insulin-sensitive parameter measured (insulin stimulation of 105%). Insulin stimulated fructose utilization by only 48% under these conditions. Although not conclusive, these data suggested that insulin may change the disposition of pathways inside the fat-cell to promote glyceride synthesis in a manner that cannot fully be explained by the promotion of fructose entry into the cells. In fact, as is more fully discussed below, the 48% insulin-stimulation of fructose utilization is most likely to be a considerable overestimate of the size of any transport stimulation by this hormone since much of the fructose uptake may result from processes whose enhancement is secondary to stimulation of synthesis.

In the experiment summarized in Fig. 1 glyceride synthesis was linear with either glucose or fructose in the presence and absence of insulin. The experiment was performed in the stirred chamber to permit rapid sampling so that the time-dependence of the insulin stimulation of glyceride synthesis could be investigated. Insulin stimulated glyceride synthesis in the presence of glucose by 258%. As far as could be ascertained this effect was essentially immediate and no lag could be detected. Insulin stimulated

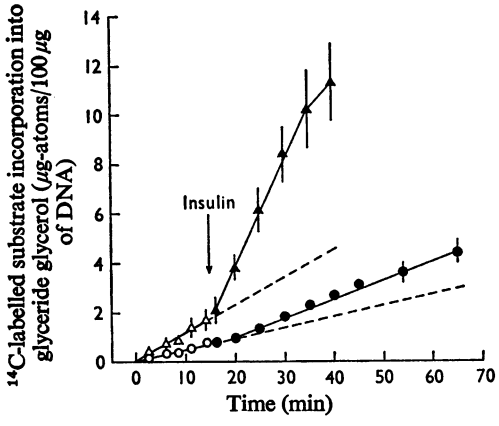

Fig. 1. *Effect of insulin on glyceride glycerol synthesis from glucose and fructose*

Fat-cells were incubated in the stirred chamber in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, 2mM-[U-^{14}C]glucose or 2mM-[U-^{14}C]fructose and 0.5mM-sodium palmitate. Time = 0 indicates addition of cells; 20m-i.u. of insulin/ml was added at time = 15min. The results are the means of three (for glucose) and five (for fructose) determinations. The bars indicate S.E.M. The mean fat-cell DNA was 4.0 and 3.5μg/ml of incubation media for glucose and fructose respectively. △, Glucose; ▲, glucose with insulin; ○, fructose; ●, fructose with insulin.

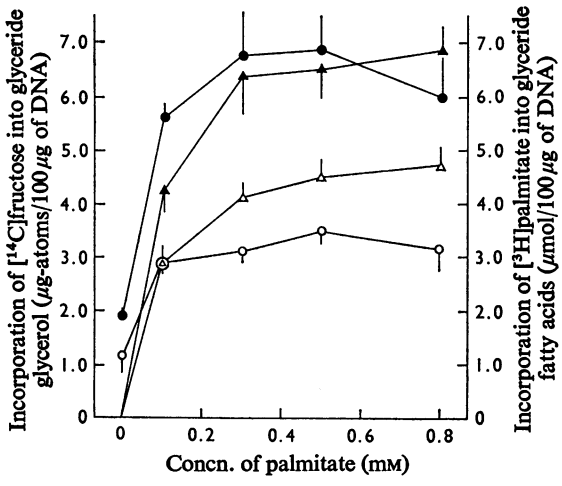

Fig. 2. *Effect of insulin and palmitate concentration on the incorporation of [^{3}H]palmitate into glyceride fatty acids and of [^{14}C]fructose into glyceride glycerol*

Fat-cells were incubated for 1h in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, 2mM-[U-^{14}C]-fructose, the indicated concentration of sodium [9,10(n)-^{3}H]palmitate and either 20m-i.u. of insulin/ml or 10m-i.u. of anti-insulin serum/ml. The results are the means of four determinations. The bars indicate S.E.M. The mean fat-cell DNA was 2.0μg/ml of incubation medium. ○, [^{14}C]Glyceride glycerol with anti-insulin serum; ●, [^{14}C]glyceride glycerol with insulin; △, [^{3}H]glyceride-labelled fatty acid with anti-insulin serum; ▲, [^{3}H]-glyceride-labelled fatty acid with insulin.

glyceride synthesis in the presence of fructose by 74%. There was some indication that this effect was not immediate and that the stimulated rate was not maximum until approx. 5min after insulin administration. The data do not, however, allow this fact to be established unequivocally by statistical analysis. Earlier experiments in which fat-cells were incubated in shaken flasks under similar conditions showed that a negligible proportion of the [^{14}C]fructose carbon incorporated into glyceride glycerol could be attributed to formation of glycerol esterified to fatty acids synthesized *de novo* derived from fructose carbon. However, when glucose was used, in the presence of insulin, a significant amount (approx. 40%) of the net ^{14}C in glyceride glycerol could be accounted for on this basis rather than for esterification of palmitate or endogenous unlabelled fatty acids. This calculation was made on the basis of 1μg-atom of glyceride glycerol carbon being involved in the esterification of 1μmol of newly synthesized fatty acid of average carbon chain length 17 (Kather *et al.*, 1972). These results indicate that a significant proportion of the insulin stimulation of glyceride synthesis in the presence of glucose could be driven by postulated effects of the hormone on fatty acid synthesis (e.g. on pyruvate dehydrogenase or acetyl-CoA carboxylase). This, however, cannot be the case when low concentrations of fructose are used since fatty acid synthesis in the presence of palmitate represents an extremely small proportion of the utilization of fructose carbon.

A further series of experiments was performed in which the general experimental design adopted was to incubate fat-cells with a ^{14}C-labelled carbohydrate precursor and [^{3}H]palmitate. The incorporation of ^{14}C into glyceride glycerol and of ^{3}H into glyceride fatty acids provided two simultaneous estimates of glyceride synthesis. The incorporation of ^{14}C was considered to be the more reliable method since there is less likelihood of dilution of the label during incubation.

At all concentrations of palmitate tested insulin caused a significant stimulation ($P < 0.05$ in all cases) of both [^{3}H]palmitate (46–56%) and [^{14}C]fructose (89–118%) incorporation into glycerides (Fig. 2). Tables 3 shows that insulin stimulated glyceride synthesis by cells incubated with 0.2mM-palmitate in a manner that was essentially independent of the concentration of fructose. The proportion of

Table 3. *Effect of insulin and of fructose concentrations on the rate of incorporation of [^{3}H]palmitate into glyceride fatty acids and of [^{14}C]fructose into glyceride glycerol*

Fat-cells were incubated for 1 h in Krebs–Ringer bicarbonate containing 16 mg of albumin/ml, 0.2 mM-sodium [9,10(n)-^{3}H]-palmitate, the indicated concentration of [U-^{14}C]fructose and either anti-insulin serum (10 m-i.u./ml) or insulin (20 m-i.u./ml) The results are the means ± S.E.M. of four determinations and are expressed either as μmol/h per 100 μg of DNA for palmitate incorporation or as μg-atoms of C/h per 100 μg of DNA for fructose incorporation. The values in parentheses refer to incorporations in the presence of insulin expressed as percentages of the respective controls in the presence of anti-insulin serum. *, **, *** indicate $P<0.05$, <0.02, <0.01 respectively. The mean fat-cell DNA was 2.0 μg/ml of incubation medium.

Additions to incubation medium	Concn. of fructose (mM)	[^{3}H]Palmitate incorporation into glyceride fatty acids	[^{14}C]Fructose incorporation into glyceride glycerol
Anti-insulin serum	0	2.40 ± 0.81	—
	1	3.11 ± 0.79	0.84 ± 0.12
	3	5.29 ± 0.78	3.85 ± 0.70
	5	5.83 ± 1.08	5.19 ± 0.55
	10	7.72 ± 0.96	8.27 ± 0.79
	15	7.91 ± 0.92	10.93 ± 0.34
Insulin	0	2.55 ± 0.84 (106)	—
	1	4.45 ± 1.22 (143)	2.43 ± 0.39*** (289)
	3	7.03 ± 1.14 (133)	7.75 ± 0.98** (201)
	5	8.78 ± 1.26 (151)	10.94 ± 1.55** (211)
	10	10.23 ± 1.48 (133)	17.22 ± 2.12*** (208)
	15	11.47 ± 1.06* (145)	18.14 ± 1.53*** (166)

glyceride glycerol synthesis which could be attributed to esterification of fatty acids synthesized *de novo* derived from fructose carbon was again insignificant except for the incubations with 10 mM-fructose (6%) and 15 mM-fructose (9%). Complete utilization of [^{3}H]palmitate occurred within the 1 h period of incubation when 15 mM-fructose was used and may therefore account for the lower insulin stimulation of glyceride glycerol synthesis under this particular experimental condition. In further experiments (results not shown) using 2.0 mM-[U-^{14}C]-fructose and 0.2 mM-sodium[^{3}H]palmitate incubated with fat-cells for 1 h, it was established that the concentration of insulin used in the above experiments (20 m-i.u./ml) gave maximum stimulation of glyceride synthesis. The effect of a range of prostaglandin E_1 concentrations on glyceride synthesis was also tested in the same experiment (0.05–1.0 μg of prostaglandin E_1/ml). In some cases a slight (approx. 20%) stimulation of glyceride synthesis was found in the presence of this agent. This effect was, however, not consistently observed. A combination of insulin (25 m-i.u./ml) and prostaglandin E_1 (0.125 μg/ml) tested in the same experiment gave no greater stimulation of glyceride synthesis than was found with insulin alone. In this respect it is pertinent that Martin *et al.* (1972) reported that prostaglandin E_1 (1 μg/ml) had no significant effect on adipose-tissue pyruvate dehydrogenase activity *in vitro*.

The experiment summarized in Fig. 3 comprises a more detailed time-course of glyceride synthesis by fat-cells incubated with fructose and palmitate. In this experiment the specific radioactivities of

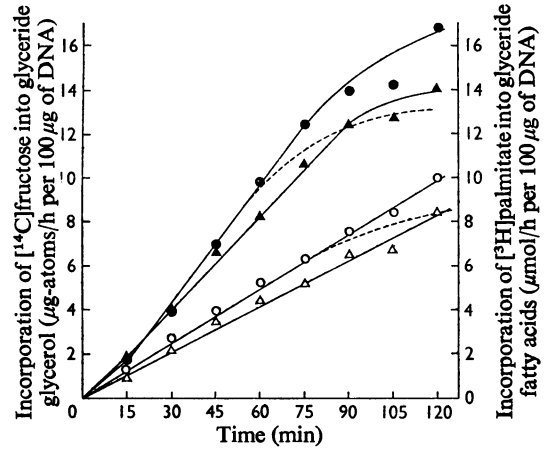

Fig. 3. *Effect of insulin on the time-course of glyceride synthesis in the presence of fructose*

Fat-cells were incubated for the indicated time in Krebs–Ringer bicarbonate containing 16 mg of albumin/ml, 0.03 mM-sodium [9,10(n)-^{3}H]palmitate and 5 mM-[U-^{14}C]fructose. Insulin (20 m-i.u./ml) or anti-insulin serum (10 m-i.u./ml) was added as indicated. The results are the means of four determinations. The mean fat-cell DNA was 2.0 μg/ml of incubation medium. The fatty acid-incorporation data were corrected for changes in the specific radioactivity of medium fatty acids over each 15 min time-interval. The brocken lines indicate these uncorrected data. ●, [^{3}H]Glyceride-labelled fatty acid with insulin; ○, [^{3}H]glyceride-labelled fatty acid with anti-insulin serum; ▲, [^{14}C]glyceride glycerol with insulin; △, [^{14}C]glyceride glycerol with anti-insulin serum.

tritiated fatty acids in the incubation media were determined at each time-interval. Assuming that this medium pool represented the precursor pool for the observed glyceride synthesis, the values obtained for [^{3}H]palmitate incorporation were corrected accordingly to give absolute values for fatty acid incorporation into glycerides. This was done by calculating the mean medium fatty acid specific radioactivity during each 15 min time-period and then correcting the corresponding increment in ^{3}H-labelled fatty acid incorporation. In both the presence and absence of insulin glyceride synthesis, as represented by fatty acid or carbohydrate precursor incorporation, was linear from 15 to 75 min. Considering the best-fit straight lines through the values over this time-period, the insulin stimulation of fatty acid esterification was 123% and of glyceride formation was 112%. The ratio

$$\frac{\text{fatty acid-esterification rate } (\mu\text{mol/unit time})}{\text{glyceride glycerol-formation rate } (\mu\text{g-atoms of C/unit time})}$$

was 1.26 in the presence of insulin and 1.18 in the absence of the hormone, suggesting that triglyceride is the major glyceride product formed. The proportion of glyceride glycerol formation that could be attributed to esterification of fatty acids synthesized *de novo* was negligible at all time-intervals in both the presence and absence of insulin. The possibility that appreciable label may be lost from [^{3}H]palmitate before incorporation into glycerides through the action of the desaturase system was discounted since the ratio

$$\frac{^3\text{H disappearance from the incubation medium}}{^3\text{H appearance in glycerides}}$$

at each 15 min time-interval was 0.98 ± 0.04 in the absence of insulin (32 measurements) and 0.94 ± 0.04 in the presence of insulin (28 measurements).

Table 4 shows that when fat-cells were incubated with palmitate in the presence of pyruvate, which is a poor precursor for glyceride glycerol synthesis (Saggerson & Tomassi, 1971), the esterification of [^{3}H]palmitate was, as expected, less than that observed in the presence of glucose or fructose (Table 3, Figs. 2 and 3). At 0.2 mM-palmitate there was a slight, but significant, stimulation by insulin of [^{14}C]pyruvate incorporation into glyceride glycerol.

In a study by Jungas (1970) using fat-pads from starved–re-fed rats incubated with ^3H_2O in the absence of any other substrate, insulin stimulation of ^{3}H incorporation into fatty acids was taken to indicate an effect of the hormone on some step in the lipogenic pathway which could obviously not be explained by promotion of the entry of a precursor into the cells of the tissue. It was expected that

Table 4. *Effect of insulin and of palmitate concentrations on the rate of incorporation of [^{3}H]palmitate into glyceride fatty acids and of [^{14}C]pyruvate into glyceride glycerol*

Fat-cells were incubated for 1 h in Krebs–Ringer bicarbonate containing 16 mg of albumin/ml, 5 mM-[U-^{14}C]-pyruvate, the indicated concentration of sodium [9,10(n)-^{3}H]palmitate and either anti-insulin serum (10 m-i.u./ml) or insulin (20 m-i.u./ml). The results are the means ±S.E.M. of three determinations and are expressed either as μmol/h per 100 μg of DNA for palmitate incorporation or as μg-atoms/h per 100 μg of DNA for pyruvate incorporation. *, ** indicate $P < 0.05$, < 0.01 respectively for comparison of insulin-treated incubations versus anti-insulin serum-treated controls. The mean fat-cell DNA was 3.8 μg/ml of incubation medium.

Additions to incubation medium	Concn. of palmitate (mM)	[^{3}H]Palmitate incorporation into glyceride fatty acids	[^{14}C]Pyruvate incorporation into glyceride glycerol
Anti-insulin serum	0	—	1.55±0.02
	0.2	2.24±0.40	2.00±0.09
	0.4	2.57±0.34	2.34±0.56
	0.6	2.71±0.32	2.27±0.31
	1.0	2.97±0.32	2.30±0.16
Insulin	0	—	1.42±0.01**
	0.2	2.03±0.33	2.30±0.05*
	0.4	2.46±0.30	2.58±0.07
	0.6	2.65±0.20	2.35±0.20
	1.0	2.81±0.25	2.25±0.43

insulin stimulation of glyceride synthesis in fat-cells incubated with ^3H_2O and palmitate alone and therefore relying on endogenous precursors to supply the glycerol moiety should be reflected in insulin stimulation of ^{3}H incorporation into glyceride glycerol. However, in incubated fat-cells such incorporation of ^{3}H is too low to differentiate from the experimental background. It was therefore decided to investigate whether such an effect could be observed instead with incubated fat-pieces (Table 5). When the adipose tissues were incubated with ^3H_2O alone, insulin stimulated the incorporation of ^{3}H into fatty acids and decreased the incorporation into glyceride glycerol in accord with the observation of Jungas (1970). The decreased incorporation into glyceride glycerol presumably reflects the anti-lipolytic effect of insulin which would lead to a decrease in the quantity of fatty acid precursor for glyceride synthesis. When the tissues were incubated with palmitate and ^3H_2O, insulin again stimulated ^3H_2O incorporation into fatty acids and the incorporation into glyceride glycerol was now also slightly increased. In 10 of the 12 determinations recorded the incorporation of ^{3}H into glyceride glycerol was increased; however, the magnitude of the effect was insufficient to be statistically significant. It appears to be technically

Table 5. *Effect of insulin on ^3H_2O incorporation into glycerides in incubated fat-pieces from starved–re-fed rats*

Single paired fat-pads were each cut into two pieces (108–315mg) and preincubated in Krebs–Ringer bicarbonate containing 15mg of albumin/ml. After 30min the paired tissues were briefly rinsed in warm Krebs–Ringer bicarbonate and incubated for a further 90min with either anti-insulin serum (10m-i.u./ml) or insulin (20m-i.u./ml) in a medium consisting of Krebs–Ringer bicarbonate containing 15mg of albumin/ml, ^3H_2O and 0.5mM-sodium palmitate where appropriate. The experiments with palmitate present used tissues obtained from different animals from those used in the absence of this substrate. The results, which are means±S.E.M. of the numbers of determinations (indicated in parentheses), are expressed as μg-atoms of H/90min per g wet wt.

	^3H_2O incorporation			
Additions to incubation medium ...	Anti-insulin serum	Insulin	Anti-insulin serum +palmitate	Insulin+ palmitate
^3H_2O incorporation into:				
Fatty acids	3.03±0.88	4.85±1.12	1.91±0.40	2.93±0.65
Glyceride glycerol	7.82±0.91	4.87±0.58	4.00±0.37	4.43±0.27
	(6)	(6)	(12)	(12)
Insulin effect on fatty acid synthesis	+1.82±0.34		+0.91±0.34	
Insulin effect on glyceride glycerol synthesis	−2.95±0.70		+0.43±0.26	

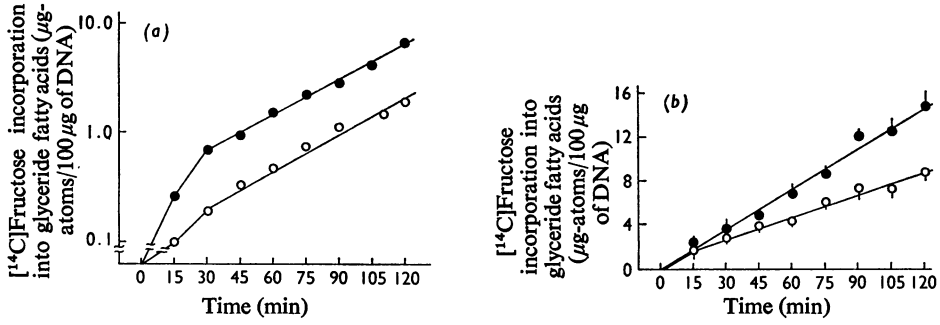

Fig. 4. *Effect of insulin on time-courses of fructose incorporation into fatty acids*

Fat-cells were incubated for the indicated times in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, 5mM-[U-^{14}C]-fructose and either insulin (20m-i.u./ml) or anti-insulin serum (10m-i.u./ml). (*a*) An initial concentration of 0.3mM-sodium palmitate was present. The data were obtained in the same experiment as the data shown in Fig. 3. ●, With insulin; ○, with anti-serum. (*b*) Free fatty acid was absent from the incubation medium at the start of incubations. The results are the means of three determinations, the bars indicating S.E.M. The mean fat-cell DNA was 1.7μg/ml of incubation medium. ●, With insulin; ○, with anti-insulin serum.

difficult to demonstrate an effect of insulin on ^3H_2O incorporation into glyceride glycerol because, particularly for isolated cells, the amount of suitable endogenous glycerol precursor appears to be very low, and, for fat-pieces, uniform penetration of the albumin-bound precursor palmitate to the surfaces of the constituent cells cannot be ensured.

The findings presented and discussed above are not incompatible with an effect of insulin on glyceride synthesis in adipose tissue. The observations of Bagul *et al.* (1972) are noteworthy and offer support to this hypothesis. These workers found that homogenates of rat fat-cells show increased rates of [^{14}C]glycerol phosphate acylation after prior exposure of the cells to insulin, suggesting an insulin-promoted enzyme activation that persists through tissue homogenization and partial fractionation.

In the same experiment from which the data of Fig. 3 were obtained, the incorporation of [^{14}C]-fructose into glyceride fatty acids was also determined. The time-courses of this parameter in the presence and absence of insulin are shown in Fig. 4(*a*). It may be seen that after an initial transient period both time-courses are exponential and that the two plots of the logarithm of the incorporation versus time are parallel. This suggested that the profile of the time-course in the absence of insulin was the same as that in the presence of the hormone, but lagged behind by approx. 45min under the conditions used. Fig. 3 shows that glyceride synthesis proceeds at

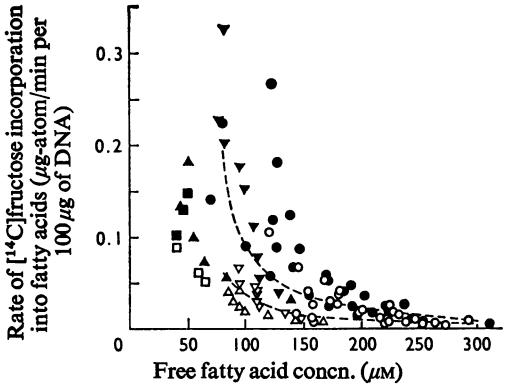

Fig. 5. *Effect of insulin and of the extracellular unesterified fatty acid concentration on the rate of fatty acid synthesis from fructose*

Fat-cells were incubated in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, 5mM-[U-^{14}C]fructose and various initial concentrations of sodium palmitate. Rates of fatty acid synthesis at various times were obtained by differentiation of time-courses of [^{14}C]fructose incorporation as described in the text in the Results and Discussion section. Closed symbols indicate incubation in the presence of insulin (20m-i.u./ml); open symbols indicate incubations in the presence of anti-insulin serum (10m-i.u./ml). ○, ●, 0.3mM-Sodium palmitate present initially (data obtained in the same experiments as are summarized in Figs. 3 and 4*a*). △, ▲, 0.2mM-Sodium palmitate present initially. ▽, ▼, 0.15mM-Sodium palmitate present initially. □, ■, Zero sodium palmitate present initially (means of the data obtained in the three experiments summarized in Fig. 4*b*). The upper and lower broken lines refer to the insulin and anti-insulin serum data respectively and are obtained from non-weighted regression lines relating the reciprocal of the rates of fatty acid synthesis to the concentration of free fatty acid. No statistical importance is assigned to these lines; they are solely to give guidance to the reader.

approximately twice the rate in the presence of insulin as in the absence and this is reflected in a higher rate of disappearance of fatty acid from the incubation medium (results not shown). It was concluded that the exponential profiles of fructose incorporation into fatty acid (Fig. 4*a*) could be explained by progressive removal of inhibitory fatty acid from the incubation medium. Insulin stimulation of this process would then account for the exponential time-course of fatty acid synthesis in the presence of insulin preceding a similar exponential in the absence of the hormone. The conclusion that may then be reached is that at least part of the effects of insulin on rates of fructose incorporation into fatty acids are not primary effects of the hormone on the lipogenic pathway but are secondary effects due to promotion of glyceride synthesis. Other processes may be expected to increase in rate under these conditions. First, hexose monophosphate-pathway CO_2 production will passively follow the increased fatty acid synthesis (Katz *et al.*, 1966; Kather *et al.*, 1972). Secondly, enhancement of glyceride synthesis in incubated fat-pads results in considerable increases in tricarboxylic acid-cycle CO_2 production (Saggerson & Greenbaum, 1970) and in elevated tissue concentrations of AMP which may be expected to enhance phosphofructokinase activity and hence glycolysis (Saggerson & Greenbaum, 1970). This may account for the slight increases in lactate and pyruvate production observed in the presence of insulin (Table 1). It should be concluded therefore, as referred to above, that any effects of insulin that may be attributed to plasma-membrane transport of fructose must be significantly less than the 48% stimulation of fructose utilization shown in Table 1. In two other similar experiments (single determinations, original results not shown), using 5mM-[U-^{14}C]fructose and either 0.2M- or 0.15mM-palmitate as substrate, the relationship between the logarithm of ^{14}C incorporation into fatty acids and time was also linear in the presence and absence of insulin. On the other hand, if cells were incubated with 5mM-[U-^{14}C]fructose in the absence of any added palmitate, time-courses of [^{14}C]fructose incorporation into fatty acids were linear after 15min and the rate of ^{14}C incorporation was greater in the presence of insulin (Fig. 4*b*).

Fig. 5 represents a treatment of the data obtained in the experiments summarized in Figs. 4(*a*) and 4(*b*) in which rates of fatty acid synthesis df/dt at individual times are plotted against the measured fatty acid concentrations in the medium at those times. For non-linear time-courses (i.e. when palmitate was present initially) the rates were obtained as follows.

For each individual experiment in which incorporation of [^{14}C]fructose into fatty acids (f) was determined at various times (t), best-fit straight lines (r values of 0.956–0.996, at least seven values of each variable in each case) of gradient m and ordinate intercept C were obtained by plotting $\ln f$ versus t (30–120min). At any time t therefore (at which the medium free fatty acid concentration was also determined), the rate of fatty acid synthesis was determined from:

$$\frac{df}{dt} = me^{(mt + C)}$$

For linear time-courses (i.e. when palmitate was not present initially), df/dt was obtained from the best-fit straight lines through the points of the time-courses shown in Fig. 4(*b*). In these experiments there was an initial rapid release of fatty acids by the cells to give a steady-state concentration in the medium. Between 30 and 120min these medium free fatty acid concentrations were $47 \pm 2\mu$M and $55 \pm 3\mu$M for cells

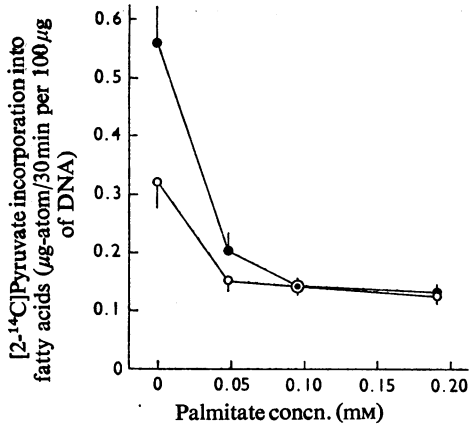

Fig. 6. *Effect of palmitate on the rate of pyruvate incorporation into fatty acids in the presence and absence of insulin*

Fat-cells were incubated for 30min in Krebs–Ringer bicarbonate containing 14mg of albumin/ml and 0.5mM-sodium [2-^{14}C]pyruvate together with the indicated concentration of sodium palmitate and either 20m-i.u. of insulin/ml or 10m-i.u. of anti-insulin serum/ml. The results are the means of six determinations. The bars indicate S.E.M. The mean fat-cell DNA was 1.2μg/ml of incubation medium. ●, With insulin; ○, with anti-insulin serum.

Table 6. *Effect of palmitate and insulin on the rate of ^3H_2O incorporation into fatty acids by fat-cells from starved–re-fed rats*

Fat-cells from rats which were starved for 48h and re-fed on a high-carbohydrate diet for 48h were incubated for 1h in Krebs–Ringer bicarbonate containing 16mg of albumin/ml, ^3H_2O, the required concentration of sodium palmitate and either anti-insulin serum (10m-i.u./ml) or insulin (20m-i.u./ml). The results are the means±S.E.M. of five determinations. The values in parentheses refer to incorporations in the presence of insulin expressed as percentages±S.E.M. of their respective controls in the presence of anti-insulin serum. The mean fat-cell DNA was 3.0μg/ml of incubation medium.

Additions to incubation medium	Concn. of palmitate (mM)	^3H_2O incorporation into glyceride fatty acids (μg-atoms of H/h per 100μg of DNA)	
Anti-insulin serum	0	2.33±0.71	
	0.25	1.54±0.50	
	0.50	1.52±0.59	
	0.75	1.82±0.77	
	1.00	1.36±0.48	
Insulin	0	4.01±1.81	(182±40)
	0.25	2.36±1.18	(137±27)
	0.50	2.23±0.92	(122±9)
	0.75	1.83±0.72	(103±5)
	1.00	1.56±0.61	(108±7)

incubated with and without insulin respectively (21 determinations).

Fig. 5 shows that as the fatty acid concentration external to the cells is increased, the rate of fatty acid synthesis from the fructose carbohydrate precursor is very greatly inhibited. At low fatty acid concentrations the rates of fatty acid synthesis appeared to be higher in the presence of insulin. At higher concentrations of fatty acid insulin stimulation of the rate of fatty acid synthesis was barely discernible. The results imply that insulin may stimulate fatty acid synthesis from fructose, but that long-chain fatty acids may over-ride the effects of the hormone. A similar interplay between the effects of insulin and external fatty acids on the rate of fatty acid synthesis is evident in two other experimental systems. First, when fat-cells were incubated with 0.5mM-[^{14}C]pyruvate in the absence of external fatty acid, insulin significantly ($P<0.01$) stimulated ^{14}C incorporation into fatty acids (Fig. 6). Palmitate inhibited the incorporation of pyruvate and over-rode the stimulatory effects of the hormone. It is noteworthy that, in accord with the observations of Halperin (1971), insulin did not stimulate the incorporation of higher concentrations of pyruvate (5mM; E. D. Saggerson, unpublished work), although inhibition of pyruvate incorporation by palmitate is still observed with 5mM-pyruvate (Saggerson & Tomassi, 1971; Saggerson, 1972). Secondly, when fat-cells from starved–re-fed rats were incubated only with ^3H_2O and ^3H incorporation was determined as a measure of fatty acid synthesis from endogenous substrates (Table 6), there was an appreciable insulin stimulation of ^3H incorporation in accord with the observations of Jungas (1970). Increasing the external concentration of palmitate both inhibited ^3H incorporation and again over-rode the stimulatory effects of insulin. At present it is not possible to locate clearly the site(s) that responds to changes in extracellular fatty acid concentration to produce changes in fatty acid-synthesis rate. However, both pyruvate dehydrogenase and acetyl-CoA carboxylase appear to be capable of regulation by long-chain fatty acyl derivatives and have also been implicated in insulin regulation of fatty acid synthesis in adipose tissue (Coore *et al.*, 1971; Halestrap & Denton, 1974). First, long-chain fatty acyl-CoA inhibition of acetyl-CoA carboxylase has been demonstrated by Goodridge (1972, 1973) for the chicken liver enzyme and by Halestrap & Denton (1973, 1974) for the rat adipose-tissue enzyme. Secondly, fatty acyl-carnitine displacement of mitochondrial pyruvate and inhibition of a postulated mitochondrial monocarboxylate transporter could ultimately result in phosphorylation and inactivation of pyruvate dehydrogenase as discussed by Mowbray (1975). Although the proposals of Mowbray relate to rat liver and heart muscle, palmitoyl-carnitine

greatly decreases the activity of pyruvate dehydrogenase in isolated fat-cell mitochondria (S. J. Smith & E. D. Saggerson, unpublished work), and long-chain fatty acyl-carnitine concentrations have been shown to increase considerably in rat adipose tissue under conditions of increased fat mobilization (Bøhmer, 1967). At the present time the relationships between extracellular fatty acid concentration and intracellular concentrations of long-chain fatty acyl-CoA and carnitine derivatives in adipose tissue remain to be established.

General conclusions

In the present study two effects of insulin are apparent, stimulation of glyceride synthesis which is apparent at all fatty acid concentrations, and stimulation of fatty acid synthesis which is mainly apparent at low fatty acid concentrations. We suggest that these two effects of the hormone may be very closely related. Halestrap & Denton (1974) have proposed the possible existence of an adipose-tissue specific binding protein for fatty acyl-CoA thioester which may be subject to regulation. An enzyme of glyceride synthesis whose activity is regulated by insulin could possibly play such a role and combine insulin stimulation of activity with increased fatty acyl-CoA thioester binding. The attendant decrease in the free concentrations of fatty acyl-CoA (and possibly fatty acyl-carnitine) would permit an increase in the rate of fatty acid synthesis as discussed above. On this basis insulin stimulation of fatty acid synthesis would, as is in fact observed, be expected to be more pronounced at lower fatty acid concentrations and to be over-ridden partially or totally at higher fatty acid concentrations. The extent of such effects must depend on the relationships between concentrations of fatty acid and of intracellular fatty acyl derivatives.

S. R. S. holds a Medical Research Council Studentship.

References

Avruch, J., Carter, J. R. & Martin, D. B. (1972) *Handb. Physiol. Section 7*, **1**, 545–562

Bagul, C. D., Boshell, B. R. & Reddy, W. J. (1972) *Diabetes* **21**, 351–352

Bally, P. R., Cahill, G. F., LeBoeuf, B. & Renold, A. E. (1960) *J. Biol. Chem.* **235**, 333–336

Blecher, M., Merlino, N. S. & Ro'Ane, J. T. (1968) *J. Biol. Chem.* **243**, 3973–3977

Bøhmer, T. (1967) *Biochim. Biophys. Acta* **144**, 259–279

Borgström, B. (1952) *Acta Physiol. Scand.* **25**, 111–119

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Bruce, H. M. & Parkes, A. S. (1949) *J. Hyg.* **47**, 202–206

Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–127

Crofford, O. B. & Renold, A. E. (1965) *J. Biol. Chem.* **240**, 14–21

Crofford, O. B., Stauffacher, W., Jeanrenaud, B. & Renold, A. E. (1966) *Helv. Physiol. Acta* **24**, 45–57

Davies, P., Cryer, A. & Robinson, D. S. (1974) *FEBS Lett.* **45**, 271–275

Denton, R. M., Yorke, R. E. & Randle, P. J. (1966) *Biochem. J.* **100**, 407–419

Evans, H. H. & Mueller, P. S. (1963) *J. Lipid Res.* **4**, 39–45

Fain, J. N. (1964) *J. Biol. Chem.* **239**, 958–962

Fain, J. N., Kovacev, V. P. & Scow, R. O. (1966) *Endocrinology* **78**, 773–778

Froesch, E. R. & Ginsberg, J. L. (1962) *J. Biol. Chem.* **237**, 3317–3324

Goodridge, A. G. (1972) *J. Biol. Chem.* **247**, 6946–6952

Goodridge, A. G. (1973) *J. Biol. Chem.* **248**, 4318–4326

Gordon, R. S. & Cherkes, A. (1958) *Proc. Soc. Exp. Biol. Med.* **97**, 150–154

Gutman, A., Autor, A. P. & Lynn, W. S. (1966) *Biochim. Biophys. Acta* **130**, 19–27

Halestrap, A. P. & Denton, R. M. (1973) *Biochem. J.* **132**, 509–517

Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **142**, 365–377

Halperin, M. L. (1971) *Biochem. J.* **124**, 615–621

Illiano, G. & Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 2472–2479

Itaya, K. & Ui, M. (1965) *J. Lipid Res.* **6**, 16–20

Jungas, R. L. (1970) *Endocrinology* **86**, 1368–1375

Jungas, R. L. & Ball, E. G. (1963) *Biochemistry* **2**, 383–388

Kather, H., Rivera, M. & Brand, K. (1972) *Biochem. J.* **128**, 1089–1096

Katz, J., Landau, B. R. & Bartsch, G. E. (1966) *J. Biol. Chem.* **241**, 727–740

Klotzsch, H. & Bergmeyer, H. U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 156–159, Verlag Chemie, Weinheim

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Martin, D. B. & Carter, J. R. (1970) *Science* **167**, 873–874

Martin, B. R., Denton, R. M., Pask, H. T. & Randle, P. J. (1972) *Biochem. J.* **129**, 763–773

Mowbray, J. (1975) *Biochem. J.* **148**, 41–47

Nuttall, F. Q. (1972) *Handb. Physiol. Section 7*, **1**, 395–413

Patten, R. L. (1970) *J. Biol. Chem.* **245**, 5577–5584

Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380

Saggerson, E. D. (1972) *Biochem. J.* **128**, 1057–1067

Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 193–219

Saggerson, E. D. & Tomassi, G. (1971) *Eur. J. Biochem.* **23**, 109–117

Salaman, M. R. & Robinson, D. S. (1966) *Biochem. J.* **99**, 640–647

Sica, V. & Cuatrecasas, P. (1973) *Biochemistry* **12**, 2282–2291

Weiss, L., Löffler, G., Schirmann, A. & Wieland, O. H. (1971) *FEBS Lett.* **15**, 229–231

Wing, D. R. & Robinson, D. S. (1968) *Biochem. J.* **106**, 667–676