Metabolism of Isolated Rat Retina

THE ROLE OF NON-ESTERIFIED FATTY ACID

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1. Retina *in vitro* showed progressive uptake of non-esterified fatty acid, given in the form of complexes with bovine serum albumin. The rate of influx was related linearly to the concentration of non-esterified fatty acid in the incubation medium and was enhanced by glucose. 2. There was some exchange between medium and tissue non-esterified fatty acid, particularly in the early stages of incubation, and this probably corresponded to the rapid labelling of one component of the tissue non-esterified fatty acid pool. A much slower exchange with tissue non-esterified fatty acid was also evident, not reaching equilibrium even after 7½ hr. incubation. Within the tissue, non-esterified fatty acid also found its way into the fat esters, and about 10% of the uptake was oxidized to carbon dioxide. 3. At high concentrations of non-esterified fatty acid in the medium, disproportionately more of the ingoing non-esterified fatty acid was found in the fat esters of the tissue, whereas the proportion in the tissue non-esterified fatty acid pool or oxidized to carbon dioxide did not change. 4. The role of non-esterified fatty acid as an energy-producing oxidizable substrate is discussed and the regulating influence of glucose on the metabolism of non-esterified fatty acid considered.

The importance of long-chain NEFA* as an energy source for living tissue attracts increasing attention. In the intact animal, fatty acids are taken up by a number of tissues, and an important fraction is oxidized to carbon dioxide (Gordon, 1957; Fredrickson & Gordon, 1957; McCalla, Gates & Gordon, 1957; Fredrickson, McCollister, Havel & Ono, 1958). Studies of isolated adipose and muscle tissue have elucidated the role of NEFA in fat mobilization and turnover, and have shown the metabolic interdependence of NEFA and glucose (Reshef, Shafrir & Shapiro, 1958; Gordon & Cherkes, 1958; Neptune, Sudduth, Foreman & Fosh, 1960; Fritz & Kaplan, 1961). The present work is concerned with the study of NEFA metabolism in isolated surviving retina of normal rats, and was undertaken as part of an enquiry into biochemical factors in the aetiology of human diabetic retinopathy.

EXPERIMENTAL

Material. Male Wistar albino rats weighing 100–150 g. were fed on a standard diet (rat cake from North Eastern Agricultural Co-operative Society Ltd.) but starved for 24 hr. before the experiment. After decapitation, the eyes were removed into chilled buffer and the lens and vitreous body discharged by gentle pressure through a

small incision just behind the corneo-scleral junction. The eye was then completely hemisected in the sagittal plane and the retina gently lifted off with a blunt spatula. Tissue damaged by this procedure was rejected. After being drained by sliding them across a clean glass surface, retinas were weighed to the nearest 0.1 mg. on a torsion balance, immersed in medium within a micro-incubation unit (Keen, Field & Pastan, 1963), gassed with CO₂ + O₂ (5:95), and then sealed and incubated in a shaking metabolic incubator (Gallenkamp) at 60 cyc./min. Tissues were macroscopically undamaged at the end of incubation. Depending on the nature of the experiment, one to four retinas were used per incubation unit and, apart from the long-term incubation experiments, 0.25 ml. of medium was added for each retina.

The standard incubation medium consisted of Krebs-Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1957) to which NEFA-crystalline bovine albumin complex was added to give a final concentration of 3 g./100 ml. The complexes with albumin were formed with a constant trace amount of [1-¹⁴C]palmitic acid and various quantities of non-radioactive palmitic acid by the following method. [1-¹⁴C]-Palmitic acid in benzene (0.5 mc/ml.) (purchased from The Radiochemical Centre, Amersham, Bucks.) was used. The solvent was evaporated and an ethanolic solution containing the required amount of unlabelled palmitic acid was added to the residue. An excess of N-NaOH in water was added and the solution well stirred. Crystalline bovine albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) was made up to a concentration of approx. 5 g./100 ml. in distilled water, and N-NaOH solution was added, drop by

* Abbreviation: NEFA, non-esterified fatty acid.

drop, until the pH was 9-9.5; the solution was kept at 40° in an incubator and vigorously stirred as the alkaline-ethanolic palmitate solution was poured in. The clear mixture was transferred to Visking dialysis membrane (18/32 in.) previously prepared by boiling in three changes of distilled water, and dialysed first against two changes of distilled water adjusted to pH 9-9.5 with NaOH and then against three changes of distilled water. After dialysis, the solution was freeze-dried and stored at 4°. This method of preparation obviated the problem of uncombined palmitic acid or insoluble palmitate arising from other procedures. Only those preparations that, when dissolved in buffer, were crystal-clear were used. Solutions of the freeze-dried complex were freshly made up in buffer before each experiment. Glucose concentrations were adjusted by the addition of suitable volumes of a solution of glucose (10%, w/v) in buffer.

Methods. The NEFA in the medium was estimated by Dole's (1956) method with a modified titration method (Chlouverakis, 1963). In several experiments, the heptane phase after simple extraction was washed with an equal volume of distilled water or with blank lower phase, as described by Dole & Meinertz (1960), to remove lower acids, such as lactic acid, which appeared in the heptane phase of the extracting mixture. The radioactivity of the NEFA extracts was measured in 1 ml. of the heptane phase added to 8 ml. of scintillation liquid, consisting of (by vol.) 29 parts of dioxan, 23 parts of ethanol and 39 parts of xylene in which diphenyloxazole was dissolved at a concentration of 0.5 g./100 ml. Samples were counted in a Nuclear Enterprise liquid-scintillation counter; quenching was measured and allowed for when necessary. Samples of the incubation medium, appropriately diluted with 1% (w/v) KF, were estimated for glucose in a Technicon Autoanalyser by the ferricyanide reduction method (Hoffman, 1937).

Changes in [^{14}C]palmitate, total NEFA and glucose in tissue-containing media were estimated from the differences in the concentrations between these media after incubation and media incubated without tissue; when appropriate, they were expressed as uptake/g. wet wt. of retina/incubation period.

The $^{14}\text{CO}_2$ was measured as follows. At the end of incubation 0.7 ml. of a solution of phenethylamine (Woeller, 1961) was injected through the rubber cap into the centre well of the incubation unit, and 0.2 ml. of 5 N- H_2SO_4 into the medium. After 2 hr. agitation at room temperature, the phenethylamine solution was transferred to counting vials along with 8 ml. of scintillation liquid. Quenching with phenylethylamine varied between 30 and 50%, and was allowed for in each experiment by including standards of [^{14}C]hexadecane (The Radiochemical Centre) with and without the amine. The production of $^{14}\text{CO}_2$ was expressed in terms of counts/min./g. wet wt. of retina/incubation period.

Tissue lipids were extracted after rinsing retinas in three successive 100 ml. volumes of chilled 0.9% NaCl; retinas were homogenized in 3 ml. of propan-2-ol-heptane-N- H_2SO_4 (40:10:1, by vol.) extraction mixture (Dole, 1956) in Potter homogenizing tubes with a motor-driven pestle. The tubes were centrifuged and 2.5 ml. samples of supernatant transferred to glass-stoppered tubes, to each of which were added 1.5 ml. of heptane and 1 ml. of water. Separation of fat esters from NEFA in the heptane phase was effected by Borgström's (1952) procedure, as modified

by Kerpel, Shafrir & Shapiro (1961). Samples were taken from each heptane fraction for the determination of radioactivity and for the estimation of NEFA by titration.

RESULTS

In Fig. 1 the disappearance of NEFA, estimated on the basis of the fall in radioactivity in the incubation medium during a long incubation, is compared with the fall in titratable acidity. Both estimates indicate a progressive uptake with time, but the initial rate of fall of radioactivity exceeds that of titrated NEFA.

To exclude the possibility that the disappearance of radioactivity from the medium was due simply to the permeation of the intact albumin-palmitate complex into the extracellular space of the tissue, the following experiment was performed. Retinas were incubated for various time-intervals in the usual medium labelled by the addition of tracer amounts of ^{125}I -labelled albumin. After incubation, tissues were removed from the medium, rinsed in three changes of 0.9% sodium chloride and counted in a well-type scintillation counter. Samples of the incubation medium were similarly assayed for radioactivity along with control medium containing no tissue. Table 1 shows that, although a very small proportion (about 1%) of the radioactivity initially present in the medium was found in the tissue with perhaps a slight rise as incubation was

Fig. 1. Disappearance of NEFA from the medium during incubation with retina, estimated by titration (○) and by disappearance of radioactivity from the medium (●). Experimental details are given in the text.

Table 1. *Radioactivity in retinal tissue after incubation in medium containing ^{125}I -labelled albumin*

Experimental details are given in the text.

Incubation time (hr.)	Radioactivity in medium (counts/min.)	Radioactivity in tissue	
		(counts/min.)	(% of total)
1.5	224 762	1566	0.62
3.0	237 233	1988	0.79
4.5	242 258	2685	1.06
6.0	247 920	2829	1.11

Fig. 2. Relationship between $[^{14}\text{C}]$ palmitate uptake by retina ($\mu\text{equiv./g. wet wt. of tissue/90 min.}$) and total NEFA concentration in the medium (m.-equiv./l.). Experimental details are given in the text.

Fig. 3. Influence of glucose concentration in incubation medium on $[^{14}\text{C}]$ palmitate influx ($\blacktriangle$), incorporation of radioactivity into tissue lipids ($\bullet$) and glucose uptake by the tissue ($\circ$). Points represent the means of triplicate experiments, each employing two retinas. Experimental details are given in the text.

prolonged, it accounted for at most one-tenth of the demonstrated $[^{14}\text{C}]$ palmitate disappearance.

Factors affecting the uptake of non-esterified fatty acid. The rate of influx of $[^{14}\text{C}]$ palmitate into the tissue is closely related to the NEFA concentration in the incubation medium. Fig. 2 shows that, when the influx of total NEFA (calculated from the disappearance of radioactivity from the medium and the specific radioactivity of the preparation) was plotted against the total NEFA concentration in

Fig. 4. Uptake of $[^{14}\text{C}]$ palmitate and its incorporation into tissue fat esters over a prolonged incubation at two initial glucose concentrations: (a) 250 mg./100 ml.; (b) 25 mg./100 ml. Experimental conditions are given in the text. $\bullet$, Concn. of glucose in the medium; $\circ$, radioactivity in tissue esters; $\blacktriangle$, $[^{14}\text{C}]$ palmitate uptake.

the medium, over the range studied (0.2–1.8 m.-equiv. of NEFA/l.) the relationship was approximately linear, suggesting a first-order reaction.

$[^{14}\text{C}]$ Palmitate passes into the retina even in the absence of glucose, but, as Fig. 3 shows, the rate of influx increases as the glucose concentration in the medium is raised from 0 to 200–400 mg./100 ml. At higher glucose concentrations there is no further increase. Fig. 3 also shows the relation between glucose concentration in the medium and glucose uptake by the tissue.

The interrelation between changing glucose concentration and the duration of incubation is shown in Fig. 4. In Fig. 4(a), the uptake of $[^{14}\text{C}]$ palmitate is shown at various times of an incubation in which the starting concentration of glucose was 250 mg./100 ml.; in Fig. 4(b) the experimental arrangements were the same except that the starting glucose concentration was 25 mg./100 ml. In the latter case, the uptake stopped much earlier than in the former. However, uptake at the higher glucose concentration slowed before the glucose concentration had dropped to 25 mg./100 ml., which was the initial concentration in the other case.

Cellular metabolism of non-esterified fatty acid. Table 2 shows the results of a number of experiments in which the $[^{14}\text{C}]$ palmitate taken up by the tissue is set against the radioactivity found in tissue fat esters, free fatty acid and carbon dioxide.

Table 2. *Influx of [¹⁴C]palmitate into retinal tissue and recovery of radioactivity in tissue fat esters, non-esterified fatty acid fraction and carbon dioxide*

Experimental details are given in the text. Each result is the mean of triplicate experiments, each employing three retinas.

	10 ⁻³ × Radioactivity (counts/min./g. wet. wt. of retina)					Mean radioactivity (% of [¹⁴ C]palmitate influx)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	
[¹⁴ C]Palmitate influx	182.0	132.6	143.4	177.4	152.0	(100)
¹⁴ C in tissue fat esters	69.9	71.0	69.3	40.0	43.9	37.3
¹⁴ C in tissue NEFA fraction	24.5	19.1	22.4	18.0	14.7	12.5
¹⁴ C in CO ₂	10.6	17.1	15.3	22.8	14.8	10.2

Fig. 5. Time-course of changes in the specific radioactivities of medium NEFA (specific radioactivity/10³) (○) and tissue NEFA (specific radioactivity/10³) (▲). Experimental details are given in the text. The tenfold scale difference between medium and tissue NEFA specific radioactivities should be noted.

Fig. 6. Percentages of [¹⁴C]palmitate taken up from medium recovered in CO₂ (○), NEFA (●) and tissue esterified fatty acids (▲) at various concentrations of NEFA in the medium. Experimental details are given in the text.

After simple Dole (1956) extraction of the tissue, only half of the radioactivity disappearing from the medium was recovered in the heptane phase. Of this, one-quarter was found in the free fatty acid fraction and the rest in fat ester. About 10% of the ¹⁴C influx was recovered as ¹⁴CO₂. There are variations from experiment to experiment; the final column summarizes the general findings and shows that 40% of the radioactivity disappearing from the medium is unaccounted for.

The change in the specific radioactivities of medium and tissue NEFA over a 7½ hr. incubation is shown in Fig. 5. There was a rapid initial rise of the specific radioactivity of tissue NEFA over the first 1½ hr., followed by a much slower increase. The rise in specific radioactivity of the tissue NEFA was due entirely to increased labelling, for the tissue titratable acidity remained almost constant for the whole incubation period. Even after 7½ hr., however, the specific radioactivity of the tissue

NEFA was still much lower than that of the medium NEFA.

Figure 6 summarizes the effect of varying the concentration of medium NEFA on the metabolic fate of that part of it which entered the tissue. As the concentration of NEFA in the medium rose, the proportion of the radioactivity taken up that was found in the tissue fat esters increased. By contrast, the proportion of radioactivity recovered from tissue NEFA or carbon dioxide remained approximately constant. Fig. 3 shows the effect of the concentration of glucose in the incubation medium on the esterification of [¹⁴C]palmitate by retinas. The rate of esterification was enhanced by raising the glucose concentration from zero to 200 mg./100 ml., but higher concentrations did not lead to a further increase. The enhanced esterification was due to the increased NEFA uptake rather than a change in the proportionate distribution of the ingoing ¹⁴C label in the fractions studied.

DISCUSSION

The experimental results reported demonstrate that NEFA, in the form of complexes with albumin,

passed into the retinal tissue *in vitro*. We are confident that this passage was not due to simple permeation of the NEFA-albumin complex into the tissue or adsorption on to it, since, when ^{125}I -labelled albumin was incubated with retina, only about 1% of the radioactivity initially present in the medium was recovered from the tissue; further, much of the ^{14}C label disappearing from the medium was recovered in the fat ester and in carbon dioxide.

The rate of influx of [^{14}C]palmitate into the tissue was influenced by the presence of glucose possibly required to generate L- α -glycerophosphate. The NEFA was taken up even in the absence of glucose, and a substantial proportion of it, whether in the presence or the absence of glucose, was recovered in the non-esterified form in the tissue extract. The kinetics of accumulation of [^{14}C]palmitate in the tissue NEFA fraction suggests that it entered two compartments. One, freely accessible, probably comes rapidly to equilibrium with the medium NEFA; the other is probably much larger and exchanges more slowly, so that even after $7\frac{1}{2}$ hr. incubation it is far from approaching equilibrium.

The rate of NEFA influx was linearly related to the NEFA concentration in the incubation medium. A similar relationship, suggesting first-order kinetics, has been reported in other tissues (Kerpel *et al.* 1961; Shtacher & Shafrir, 1963). As the rate of NEFA influx rose, increasing proportions of the ingoing NEFA were found in the tissue fat ester. Since there was little or no change in the proportions represented in the tissue NEFA pool, or in the carbon dioxide, the rise of proportion in the fat ester must have been at the expense of the 40% of the NEFA taken up that was unaccounted for in the fractions so far examined.

In retina, the proportion of the NEFA taken up that was recovered as carbon dioxide is similar to that found in the adipose tissue of starved rats (Shapiro, Chowder & Rose, 1957) and higher than that found in muscle (Shtacher & Shafrir, 1963). The role of NEFA as an energy-producing substrate in retina is difficult to assess. We can make minimum estimates of the energy made available by fatty acid oxidation on the assumption that the proportion of [^{14}C]palmitate taken up and oxidized represents its accompanying unlabelled medium NEFA that undergoes the same processes. In a similar way, maximum estimates of energy production from glucose oxidation can be made. Thus, at a rate of uptake *in vitro* of 20 mg. of glucose/g. wet wt. of retina/hr., and assuming that about 20% of the glucose taken up is oxidized to carbon dioxide via the Krebs cycle and the rest is converted into lactate, then approx. 8.5 cal. will be produced/g. wet wt. of retina/hr. from glucose. On the basis of

the experimental results presented above, at a concentration of NEFA in the medium of 1 m-equiv./l. and at a rate of NEFA influx of 2 m-equiv. of NEFA/g. wet wt. of retina/hr., assuming that approx. 10% is oxidized to carbon dioxide then about 0.2 cal. will be produced/g. wet wt. of retina from NEFA taken up from the medium.

Since we do not know the total size of the oxidizable fat pool within the tissue, we cannot from these experiments draw any conclusions about the contribution of fatty acid to the total carbon dioxide production of the cell.

Indirect estimates of the importance of non-glucose oxidizable substrates have been made by a number of workers, either by molecular comparison of oxygen uptake with glucose oxidized or by measuring oxygen consumption in the absence of exogenous glucose. Reading & Sorsby (1962) found that 33–45% of the oxygen utilized by retinas from rats aged 21–60 days could not be accounted for by oxidation of glucose, an estimate in keeping with that of Cohen & Noell (1959–60), who set 68% of the oxygen uptake against glucose oxidation. Reading (1963) concluded that half of the oxygen taken up by retina was used to oxidize endogenous substrate, most probably fat, for, when glucose was absent, the rate of oxygen consumption fell to 50% of the level found in its presence. By contrast, Cohen & Noell (1959–60) reported a higher oxygen consumption in the absence of glucose than in its presence. In the face of these conflicting findings this approach to the estimate of the role of non-glucose substrates appears to be of doubtful value. In any case, the metabolic processes of retina incubated with and without glucose probably differ considerably.

In retina, as in adipose tissue (Kerpel *et al.* 1961; Raben & Hollenberg, 1960) and muscle tissue (Shtacher & Shafrir, 1963; Neptune *et al.* 1960), uptake and esterification of NEFA is enhanced by glucose. This regulating influence of glucose may depend on the provision for esterification of L- α -glycerophosphate, the only other likely source being free glycerol released by lipolysis and phosphorylated by glycerokinase.

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REFERENCES

- Borgström, B. (1952). *Acta physiol. scand.* **25**, 101.
- Chlouverakis, C. (1963). *Metabolism*, **12**, 936.
- Cohen, L. H. & Noell, W. K. (1959–60). *J. Neurochem.* **5**, 253.
- Dole, V. P. (1956). *J. clin. Invest.* **35**, 150.
- Dole, V. P. & Meinertz, H. (1960). *J. biol. Chem.* **235**, 2595.

- Fredrickson, D. S. & Gordon, R. S., jun. (1957). *J. clin. Invest.* **36**, 890.
- Fredrickson, D. S., McColester, D. L., Havel, R. J. & Ono, K. (1958). In *Chemistry of Lipides as Related to Atherosclerosis*, p. 205. Ed. by Page, I. H., Springfield, Ill.: Charles C. Thomas.
- Fritz, I. B. & Kaplan, E. (1961). *Amer. J. Physiol.* **198**, 39.
- Gordon, R. S., jun. (1957). *J. clin. Invest.* **36**, 810.
- Gordon, R. S., jun. & Cherkes, A. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 150.
- Hoffman, W. S. (1937). *J. biol. Chem.* **120**, 51.
- Keen, H., Field, J. B. & Pastan, I. H. (1963). *Metabolism*, **12**, 143.
- Kerpel, S., Shafir, E. & Shapiro, B. (1961). *Biochim. biophys. Acta*, **46**, 495.
- McCalla, C., Gates, H. S., jun. & Gordon, R. S., jun. (1957). *Arch. Biochem. Biophys.* **71**, 346.
- Neptune, E. M., Sudduth, H. C., Foreman, D. R. & Fosh, F. J. (1960) *J. Lipid Res.* **1**, 229.
- Raben, M. S. & Hollenberg, C. H. (1960). *J. clin. Invest.* **39**, 435.
- Reading, H. W. (1963). *Nature, Lond.*, **199**, 817.
- Reading, H. W. & Sorsby, A. (1962). *Vision Res.* **2**, 315.
- Reshef, L., Shafir, E. & Shapiro, B. (1958). *Metabolism*, **7**, 723.
- Shapiro, B., Chowder, I. & Rose, G. (1957). *Biochim. biophys. Acta*, **23**, 115.
- Shtacher, G. & Shafir, E. (1963). *Arch. Biochem. Biophys.* **106**, 205.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Tissue Metabolism*. Minneapolis: Burgess Publishing Co.
- Woeller, F. H. (1961). *Analyt. Biochem.* **2**, 508.