The Irreversible Disposal Rate of Free Fatty Acids in the Plasma of Fed and Starved Rats

By VINCENT J. CUNNINGHAM

Medical Research Council Toxicology Unit, Medical Research Council Laboratories,
Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

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1. The irreversible disposal rate coefficient for free fatty acids in the plasma of fed and starved rats was determined after a single intravenous injection of $[1-^{14}\text{C}]$ palmitic acid into each rat. The dose of labelled palmitic acid was given as a complex with ^{131}I -labelled albumin in rat serum. The total amount of $[1-^{14}\text{C}]$ palmitic acid remaining in the plasma was determined at short times after injection from the $^{14}\text{C}/^{131}\text{I}$ ratio in the injected serum and in the collected plasma. The rate coefficient was determined from the area under the curve that describes the disappearance of $[1-^{14}\text{C}]$ palmitic acid with time from the plasma. Possible sources of error in these determinations are discussed. 2. The irreversible disposal rate coefficient was significantly higher in fed rats (2.07 min^{-1}) than in rats which had been starved for 24 h (1.53 min^{-1}). The possible relationship between this difference and the processes whereby free fatty acids are removed from the plasma is discussed briefly. 3. An estimate of the irreversible disposal rate for free fatty acids in plasma was made from the concentration of free fatty acids in plasma and from the volume of distribution of ^{131}I -labelled albumin. The irreversible disposal rate was significantly lower in the fed state than in the starved.

Free fatty acids are transported in the plasma principally as complexes bound to albumin (Fredrickson & Gordon, 1958a), the uptake of these free fatty acids by the tissues probably involving a rapid transfer of free fatty acids from albumin to analogous tissue sites via an unbound form of free fatty acids (Spector, 1968). Over short times, the net transport of free fatty acids from the plasma into the tissues for oxidation and esterification may be considered as an irreversible disposal of free fatty acids from the plasma (Baker *et al.*, 1959; Baker, 1969). Over longer periods, recycling will result in some return of label to the plasma pool of free fatty acids (Baker & Schotz, 1967). The present paper is concerned with the irreversible process in the whole rat over short times. First, the approach described in an accompanying paper (Heath & Barton, 1973) is used to determine the irreversible disposal rate of free fatty acids in the plasma, and an assessment is made of the errors involved. Secondly, the method is applied to a comparison of fed and starved rats.

The irreversible disposal rate (R) of a substance in a pool is, under certain circumstances, related to the pool size of that substance (Q) by a coefficient (k) such that $R = kQ$ (Heath & Barton, 1973). Q for free fatty acids in plasma is the product of plasma volume and the concentration of free fatty acids in the plasma. A wide variety of evidence suggests that changes in the concentration of free fatty acids

in plasma stem principally from changes in the inflow of free fatty acids from adipose tissue to plasma (Armstrong *et al.*, 1961; Baker & Rostami, 1969), and it is often possible to compute variations in the outflow of free fatty acids from the plasma simply from variations in the concentration of free fatty acids, when a coefficient of proportionality has been established (Winkler *et al.*, 1969). However, such proportionality may vary with different states of the animal (Paul, 1970) indicating that effects in addition to a simple mass action determine the rate of outflow of free fatty acids.

The irreversible disposal rate coefficient (k) may be calculated for free fatty acids from the area (A_q) under a plot of q against time after a single injection of labelled free fatty acids (where q equals quantity of label in the free fatty acid pool in the plasma). The relationship between A_q and k has been dealt with from a theoretical standpoint, both generally and specifically for the case of free fatty acids in plasma, by Baker & Rostami (1969) and by Heath & Barton (1973). Important advantages of this relationship are its lack of dependence on any particular model and its applicability in the non-steady state under conditions that will be investigated in this paper. This allows a study to be made of outflow transport independently of changes in inflow transport which may occur during the experiment.

Values for R and for k have been determined for

free fatty acids in the plasma of rats in the starved state (Baker & Schotz, 1967) and in a comparison of the starved and glucose-re-fed states, when no variation in k was observed (Baker & Rostami, 1969). The present paper differs from the above principally in two ways; first, Baker & Rostami (1969) described the disappearance of label from the plasma as the sum of two exponential curves giving a best fit to all the points, the area under the curve being calculated from the slopes and intercepts of the components. Such an approach makes it difficult to calculate the error associated with the area from that associated with the determination of each datum point, and the calculated area is more sensitive to error in the curve at later times. These difficulties may be overcome, and the comparison of different groups made easier if the area under the curve is determined as described by Heath & Barton (1973). Secondly, methods used for the determination of k involve an estimate of total label remaining in the free fatty acid pool in the plasma from the amount present in a given sample of plasma. Errors may be increased if a mean plasma volume is applied to a group of rats, because of individual variations and because of incomplete mixing of the injected material at short times after injection. In the present study ^{131}I -labelled albumin was used as an internal distribution marker for each individual rat.

Materials and Methods

Animals

Male albino rats of the Porton strain were fed on M.R.C. diet 41B (Bruce & Parkes, 1956), and weighed 180–200g in the fed state. When starved rats were used, food was withdrawn at 09:30h on the day preceding the experiment. Experiments were performed between 09:30 and 11:15h.

Preparation of labelled serum

[1- ^{14}C]Palmitic acid (specific radioactivity 50 $\mu\text{Ci}/\mu\text{mol}$) and ^{131}I -labelled human serum albumin (specific radioactivity 20–80 $\mu\text{Ci}/\text{mg}$ of protein) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The albumin was first passaged through donor rats as a screen for any component of the label that might be removed rapidly from the plasma (Dewey, 1959). Labelled albumin (25–50 μCi in 0.2ml of aq. 0.85% NaCl) was injected into a tail vein of donor rats on the day preceding the experiment and blood was collected by aortic cannulation under ether anaesthesia shortly before the experiment. Serum was separated and samples (5ml) were complexed with the potassium salt of [1- ^{14}C]palmitic acid (25 μCi , 0.5 μmol) essentially as described by Baker & Rostami (1969) but on a proportionately decreased scale. The doubly labelled

serum-palmitic acid complex was passed through a glass-fibre filter (Whatman, G.F.A. 2.5cm) before use. For each experiment, the donor rats were fed or starved as were the experimental rats, so that the concentration of free fatty acids in the injected serum should be comparable with that in the plasma of experimental rats.

Injections and plasma sampling

Rats received labelled serum (0.2ml containing 1 μCi of [1- ^{14}C]palmitic acid) via a tail vein under ether anaesthesia. The syringe was weighed before and after injection. Rats were decapitated at specific times after injection while still under anaesthesia. Blood was collected immediately for 5s into a small amount of powdered heparin in polypropylene centrifuge tubes (5ml; M.S.E.). The centrifuge tubes were fitted with a small plastic conical funnel, cut so as to fit over the tube so that blood drained from the funnel into the tube. This procedure usually allowed the collection of more than 2ml of blood.

Samples of blood were stored in ice for periods of up to 1.5h until plasma was separated by centrifugation at 10000g for 20min.

Assays of radioactivity

Plasma was transferred to pre-weighed glass vials. Samples (1ml) were taken for extraction of free fatty acids (see below) and the vials were reweighed. ^{131}I was measured directly in the remaining plasma in a Panax Gamma one-sixty counter. Weighed samples of the labelled serum that had been used for injection were analysed concurrently for ^{131}I . At least 10000 counts above background were recorded for each sample.

Free fatty acids were extracted into heptane from samples (1ml) of plasma by the method of Dole & Meinertz (1960) as modified by Trout *et al.* (1960). ^{14}C -labelled free fatty acids were determined in the extract after adsorption and elution from an ion-exchange resin (Kelley, 1968). Amberlite resin IRA-410 (BDH Chemicals Ltd., Poole, Dorset, U.K.) was hydroxylated (Kelley, 1968) and samples (1ml) of the extract in heptane were shaken with 0.5g of the treated resin for 30min at room temperature in a scintillation vial. Heptane was removed by aspiration and the resin was washed four times with hexane (5ml), dried in air and then incubated for 1h at 37°C with 1ml of Soluene-100 (Packard Instrument Co. Ltd., Wembley, Middx., U.K.). Scintillant (10ml) was added and ^{14}C was counted in a Packard Tri-Carb liquid-scintillation spectrometer. The scintillant consisted of 5.3g of 2,5-diphenyloxazole and 0.4g of 1,4-bis-2-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene. [^{14}C]Toluene was used as an internal standard. Negligible

variation in counting efficiency occurred if samples were counted within 24h of the experiment. Over longer periods progressive coloration of the scintillant occurred, this colour deriving from the resin.

Weighed samples (approx. 0.2ml) of the labelled serum (for injection) were diluted with unlabelled serum (10ml) and ^{14}C -labelled free fatty acids and ^{131}I were assayed exactly as described above.

Determination of free fatty acids

Samples (1.8–2.0ml) of the extracts of plasma in heptane were assayed titrimetrically for free fatty acids by the method of Salaman & Robinson (1961) with an autoburette (Type ABU.1 Radiometer, Copenhagen, Denmark). The titrant was 5mm-tetra-*n*-butyl ammonium hydroxide in methanol (Kelley, 1965). The indicator was Thymol Blue in propan-2-ol (10mg/l) which had been adjusted to a yellow colour with 1M-NaOH. Palmitic acid (Sigma, London) in heptane was used as a standard. This procedure involves only a single phase in the titrating vessel.

Results and Discussion

Disappearance of labelled palmitic acid from the plasma

In the experiments described in Table 1, groups of rats were injected via a tail vein with a sample of serum labelled with ^{131}I and complexed with $[1-^{14}\text{C}]$ -palmitic acid (see the Materials and Methods section). At various times after injection, samples of plasma were obtained (one sample per rat) and a ratio was calculated in each case: ($^{14}\text{C}/^{131}\text{I}$) plasma sample/ ($^{14}\text{C}/^{131}\text{I}$) injected serum.

This ratio ($\times 100$) was taken to equal the % of the injected $[1-^{14}\text{C}]$ palmitic acid remaining in the plasma at the time of sampling, the rate of loss of ^{131}I -labelled albumin from the vascular system being negligible compared with that of labelled fatty acids (Dewey, 1959). The labelled albumin was therefore used as an internal distribution marker for the complexed $[1-^{14}\text{C}]$ palmitic acid in the plasma of each individual rat, and no independent calculation of plasma volume was required at this stage. Although the calculation was not dependent on the even distribution of either label throughout the circulation, it was assumed in further calculations that the ratio in the collected sample of plasma was representative of the mean ratio throughout the whole plasma at the designated sampling time. Possible errors arising from this assumption are discussed below.

In Table 1 the disappearance of $[1-^{14}\text{C}]$ palmitic acid from the plasma of rats that had been starved for 24h is compared with that of fed rats. A very rapid initial loss of label was observed. In agreement

Table 1. *Disappearance of injected $[1-^{14}\text{C}]$ palmitic acid from the plasma of fed and starved rats*

Groups of 20 rats, which were either fed or starved for 24h, were injected with serum containing ^{131}I -labelled albumin and $[1-^{14}\text{C}]$ palmitic acid. At each of five specific times the percentage of the injected dose of $[1-^{14}\text{C}]$ palmitic acid remaining in the plasma of four rats was determined. The area under the corresponding disappearance curve was calculated as described in the Results and Discussion section. The significance of the difference between the means was determined by Student's *t* test.

Time after injection	% of injected dose remaining (mean $\pm$ S.E.M.)	
	Fed rats	Starved rats
8s	67.31 $\pm$ 2.05	63.61 $\pm$ 3.02
26s	31.35 $\pm$ 2.01	38.34 $\pm$ 1.57
47s	18.32 $\pm$ 2.42	22.75 $\pm$ 1.56
1 min 45s	4.54 $\pm$ 0.97	8.84 $\pm$ 0.88
3 min	1.64 $\pm$ 0.32	4.50 $\pm$ 0.54
Area under corresponding activity-time curve (% $\times$ min) (Mean $\pm$ S.E.M.)	48.4 $\pm$ 2.2	65.5 $\pm$ 2.25 (<i>P</i> < 0.001)

with published results (Laurell, 1959; Baker & Ros-tami, 1969) this initial loss was followed by 'tailing' in which small amounts of the initial dose persisted for longer times. In separate experiments on fed rats, 0.41% of the injected dose remained at 5min, and 0.15% at 10min. In starved rats the corresponding values were 1.85% and 0.75%. 'Tailing' may be attributed to a recycling of label subsequent to its initial removal from the free fatty acid pool in plasma, and its significance can only be described in terms of a more complex study than is presented here (Baker & Schotz, 1967).

In the present study attention was directed towards the behaviour of label up to 3min after injection and to an extrapolation to infinite time therefrom (see below). The effect of inclusion of the observed 5min and 10min points in subsequent calculations is considered later.

Choice of sampling times

Because of the rapid disappearance of labelled palmitic acid from the plasma, it was necessary to collect samples of plasma very soon after injection of label for an accurate description of the disappearance curve. Further, the irreversible disposal rate coefficient is derived from the area under this curve. The choice of suitable times and the subsequent

calculation of the area was therefore made on the basis given by Heath & Barton (1973) [see eqns. (6.2) and (6.10)], a procedure which facilitates the determination of, and minimizes errors associated with the calculation of, this area. Preliminary experiments had indicated the general shape of the radioactivity-time curve for starved rats, the area under which was then divided in the proportion 1.5:1:1:1:1. Times were chosen approximately at the centroids of these areas (8s, 26s, 47s, 1 min 45s, 3 min). The division of the area into five equal parts would have resulted in an unacceptably short initial sampling time.

Comparison of fed and starved rats

In Table 1, the amount of injected [^{14}C]palmitic acid (mean $\pm$ s.e.m.) remaining at each of the above time-points is given, together with the area (mean $\pm$ s.e.m.) under the corresponding activity-time curve ($\% \times \text{time}$). The area under the curve for fed rats was significantly smaller than that for starved rats.

The determination of these areas may be subject to systematic errors. Time was measured from injection to decapitation and blood was collected for the next 5s. Again, because of the rapid disappearance of labelled palmitic acid, its concentration in the plasma and the ratio between it and the labelled carrier must vary significantly both throughout the circulation at a given time and also over a period of 5s at the point of collection of blood. It is therefore difficult to define exactly the time at which the ratio in the collected sample of plasma is representative of the true state in the plasma of the intact rat. If the definition of the time of sampling were in error by 3s, then a consequent error of about 6% would be involved in the determination of area under the disappearance curve for starved rats and of about 9% for fed rats. It is likely, however, that such effects would be parallel in the two groups of rats, such that the relative areas are more precise than the absolute.

Irreversible disposal rate coefficient

For the purposes of the following discussion it is assumed that the behaviour of [^{14}C]palmitic acid reflects that of the whole plasma free fatty acid pool. However, individual differences are observed between the species of free fatty acid and the limitations of the use of a single free fatty acid tracer are pointed out by Hagenfeldt *et al.* (1972). Palmitic acid was chosen as it is the predominant free fatty acid in rat plasma (Göransson & Olivecrona, 1964).

The irreversible disposal rate coefficient (k , min^{-1}) for free fatty acids in plasma is then given by $k = D/A_q$ where D = the initial dose of label = 100% and A_q is the area under the radioactivity-time curve (Heath & Barton, 1973). If this simple relationship

is to be applicable in the present case then k must be constant within each given rat throughout the experiment. No direct evidence for this is presented here, but variations in k which might be anticipated are (i) those associated with fluctuations in the concentration of free fatty acids, and (ii) progressive variation with time consequent upon disturbance of the animal.

When fed and starved rats were compared (Table 2), a significant difference of about 25% was observed in k , together with a 3-fold difference in the concentration of free fatty acids in the plasma. However, it is reasonable to suppose that over the range of free fatty acid concentrations observed within one experiment, any variation in k from this cause would be negligible. Further, the amount of label remaining in the circulation of a given rat at one time (q_x) is a function of k for that rat and within each experiment, there was no significant correlation between q_x and the corresponding concentration of free fatty acids at each time.

Conversely, any progressive change of k with time might be expected to be associated with a progressive change in free fatty acid concentration. When the concentrations of free fatty acids in the plasma at specific times after injection were compared no significant correlation of concentration with time was observed, suggesting no progressive changes in k with time.

The coefficient of error (standard error of the mean divided by the mean) of label remaining at each time, increases with time (Table 1). This would be the expected effect of variation in k between animals. Such variation does not invalidate the application of the above simple relationship between k and the area A_q (Heath & Barton, 1973) but contributes to the standard error of the mean values of k presented in Table 2.

Irreversible disposal rate

The irreversible disposal rate is given by the product of the coefficient k , and the pool size $C \times V$ where V is plasma volume and C is the concentration of free fatty acids in the plasma. In the present work, attention has been directed principally to the determination of k , this determination being independent of a knowledge of plasma volume. An estimate of the apparent plasma volume may, however, be made from the absolute amount of injected ^{131}I label. In the experiments reported in Table 2, the apparent volume of distribution of label at 8s was about 5% less than that at later times, suggesting that mixing was not complete. This error was considered negligible relative to the range observed in the concentration of free fatty acids in the plasma within each experiment and the mean apparent pool size of all rats is presented. These correspond to apparent plasma

Table 2. *Irreversible disposal of free fatty acids in the plasma of fed and starved rats*

These values are derived from the experiments presented in Table 1 as described in the Results and Discussion section. The pool size was calculated from the dilution of injected ^{131}I -labelled albumin in plasma and from the concentration of free fatty acids determined in samples of plasma from each individual rat (Materials and Methods section). Values are means $\pm$ S.E.M. and the significance of differences between means was determined by Student's *t* test.

	Concentration of free fatty acids in plasma ($\mu\text{equiv./ml}$)	Pool size of free fatty acids in plasma ($\mu\text{equiv./100g body wt.}$)	Irreversible disposal rate coefficient (min^{-1})	Irreversible disposal rate ($\mu\text{equiv./min per}$ 100g body wt.)
Fed	0.356 ± 0.018	1.54 ± 0.08	2.07 ± 0.09	3.19 ± 0.22
Starved	0.976 ± 0.026	4.14 ± 0.13	1.53 ± 0.05	6.33 ± 0.28
<i>P</i>	<0.001	<0.001	<0.001	<0.001

volumes of 4.34 ml/100g body wt. in the fed state and 4.29 ml/100g body wt. in the starved.

General discussion

When rats starved for 24h were compared with fed rats (Table 2) a significant difference in the irreversible disposal rate for free fatty acids in plasma was seen to derive largely from the difference in the concentration of free fatty acids in plasma. This result is, of course, consistent with and supports the generally accepted view that variations in the rate of utilization of free fatty acids are consequent upon an increased or decreased rate of release of free fatty acids from adipose tissue into the plasma (Scow & Chernick, 1970). However, the irreversible disposal rate coefficient was significantly lower in starved rats than in fed rats (Table 2), which suggests that further effects at the level of uptake of free fatty acids by the tissues are involved in the transition from the fed to the starved state in the rat. Early work by Fredrickson & Gordon (1958*b*) in man, and by Laurell (1959) in the rat also indicates differences in the fractional turnover rates of free fatty acids in plasma in different nutritional states, and recent work (Barter & Nestel, 1972) points to changes in man after glucose feeding. Two possible mechanisms for such changes are (i) an approach towards saturation of the tissue free fatty acid receptor sites at high plasma free fatty acid concentration and (ii) inhibition of uptake of free fatty acids by ketone bodies.

It is difficult to extrapolate from equilibrium studies on the receptor sites for free fatty acids on mammalian cells (Spector *et al.*, 1972) to a dynamic study in the whole animal as presented here, but if the rate of equilibration of free fatty acids between albumin and tissue sites is much greater than the subsequent utilization of free fatty acids by the cells, then a saturation phenomenon could occur at high plasma free fatty acid concentrations and would manifest itself as a fall in the value of *k*.

The higher concentration of ketone bodies in blood associated with the starved state (Berry *et al.*, 1965) might also account for a lowering of the value of *k* for free fatty acids in plasma. Inhibition of uptake of free fatty acids by ketone bodies has been observed in the perfused rat heart (Olson, 1962) and in the dog heart *in vivo* (Bassenge *et al.*, 1965; Little *et al.*, 1970). In addition butyrate and acetoacetate compete as fuels in rat kidney cortex (Weidemann & Krebs, 1969). However, despite inhibition of oxidation of free fatty acids by ketone bodies, no effect on their fractional removal rate in whole animals is apparent (Balasse, 1970; Little *et al.*, 1970). These reasons for a change in the value of *k* with nutritional status do not reconcile the results reported in this paper with those of Baker & Rostami (1969) who report a value for *k* of 1.25 in both starved and glucose-re-fed states, since rapid changes in the concentration of ketone bodies also occur after glucose re-feeding (Start & Newsholme, 1968). If 5 min and 10 min points had been used for the calculation of *k* in the present work, then values for *k* in fed and starved states would have been 1.99 and 1.36 respectively.

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References

- Armstrong, D. T., Steele, R., Altszuler, N., Dunn, A., Bishop, J. S. & de Bodo, R. C. (1961) *Amer. J. Physiol.* **201**, 9–15
- Baker, N. (1969) *J. Lipid Res.* **10**, 1–24
- Baker, N. & Rostami, H. (1969) *J. Lipid Res.* **10**, 83–90
- Baker, N. & Schotz, M. C. (1967) *J. Lipid Res.* **8**, 646–660
- Baker, N., Shipley, R. A., Clark, R. E. & Incefy, G. E. (1959) *Amer. J. Physiol.* **196**, 245–252
- Balasse, E. O. (1970) *Eur. J. Clin. Invest.* **1**, 155–160
- Barter, P. J. & Nestel, P. J. (1972) *J. Lipid Res.* **13**, 483–490

- Bassenge, E., Wendt, V. E., Schollmeyer, P., Blümchen, G., Gudbjarnason, S. & Bing, R. J. (1965) *Amer. J. Physiol.* **208**, 162–168
- Berry, M. N., Williamson, D. H. & Wilson, M. B. (1965) *Biochem. J.* **94**, 17c–19c
- Bruce, H. M. & Parkes, A. S. (1956) *J. Anim. Tech. Ass.* **7**, 54
- Dewey, W. C. (1959) *Amer. J. Physiol.* **197**, 423–431
- Dole, V. P. & Meinertz, H. (1960) *J. Biol. Chem.* **235**, 2595–2599
- Fredrickson, D. S. & Gordon, R. S. (1958a) *Physiol. Rev.* **38**, 585–630
- Fredrickson, D. S. & Gordon, R. S. (1958b) *J. Clin. Invest.* **37**, 1504–1515
- Göransson, G. & Olivecrona, T. (1964) *Acta Physiol. Scand.* **62**, 224–239
- Hagenfeldt, L., Wahren, J., Pernow, B. & Räf, L. (1972) *J. Clin. Invest.* **51**, 2324–2330
- Heath, D. F. & Barton, R. N. (1973) *Biochem. J.* **136**, 503–518
- Kelley, T. F. (1965) *Anal. Chem.* **37**, 1078–1079
- Kelley, T. F. (1968) *J. Lipid Res.* **9**, 799–800
- Laurell, S. (1959) *Acta Physiol. Scand.* **47**, 218–232
- Little, J. R., Goto, M. & Spitzer, J. J. (1970) *Amer. J. Physiol.* **219**, 1458–1463
- Olson, R. E. (1962) *Nature (London)* **195**, 597–599
- Paul, P. (1970) *J. Appl. Physiol.* **28**, 127–132
- Salaman, M. R. & Robinson, D. S. (1961) in *Enzymes of Lipid Metabolism* (Desnuelle, P., ed.), pp. 218–230, Pergamon Press, Oxford
- Scow, R. O. & Chernick, S. S. (1970) *Compr. Biochem.* **18**, 19–49
- Spector, A. A. (1968) *Ann. N.Y. Acad. Sci.* **149**, 768–783
- Spector, A. A., Ashbrook, J. D., Santos, E. C. & Fletcher, J. E. (1972) *J. Lipid Res.* **13**, 445–451
- Start, C. & Newsholme, E. A. (1968) *Biochem. J.* **109**, 37p–38p
- Trout, D. L., Estes, E. H. & Friedberg, S. J. (1960) *J. Lipid Res.* **1**, 199–202
- Weidemann, M. J. & Krebs, H. A. (1969) *Biochem. J.* **112**, 149–166
- Winkler, B., Steele R. & Altszuler, N. (1969) *Endocrinology* **85**, 25–30