Comparison of Guinea-Pig Serum Lipoproteins after Iodination by Two Different Methods

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1. Guinea-pig low-density lipoproteins were isolated by ultracentrifugation and iodinated either by the ICl method or by the chloramine-T procedure. 2. The efficiency of labelling by both methods was essentially the same. 3. When the two products were compared by ultracentrifugation, gel chromatography and immunodiffusion analysis, no significant difference in their properties was detected. 4. When they were compared by gradient-gel electrophoresis, aggregates were found in the product of the ICl method, but not in the lipoprotein iodinated by the chloramine-T process. 5. Both products were metabolized by the guinea pig with essentially the same fractional catabolic rate. 6. The fractional catabolic rate of lipoprotein iodinated by the chloramine-T method was not significantly different from that of lipoprotein biologically labelled in the protein moiety with [⁷⁵Se]selenomethionine. 7. It is concluded that the products of both methods of iodination are almost equally acceptable, provided that the optimum conditions for the chloramine-T reaction are carefully established.

The two most commonly used methods of labelling proteins by radioiodination are potentially damaging, because they use the powerful oxidizing agents ICl (McFarlane, 1958) or chloramine-T (Greenwood *et al.*, 1963). Both these methods have been used to label serum lipoproteins for metabolic studies, but McFarlane's (1958) procedure has been the more popular of the two. To minimize unwanted substitution of the lipid moiety, however, it is usually performed at a high pH, which may damage the lipoprotein. The chloramine-T method had been decried by several workers (Schonfeld *et al.*, 1974; Shepherd *et al.*, 1976), although others have used it with apparently satisfactory results (Eaton & Kipnis, 1969; Sundaram *et al.*, 1972). However, Sundaram *et al.* (1972) appear to be alone in comparing the two methods directly in a metabolic experiment. They used both methods to iodinate the same human high-density lipoproteins, and found the products to be almost indistinguishable in turnover studies. Unfortunately, no comparisons of this kind have been made on LD lipoproteins. We have therefore found it necessary to determine which procedure is the best for labelling guinea-pig LD lipoproteins for metabolic studies. Our findings are the subject of the present paper.

Abbreviation used: LD lipoprotein, low-density lipoprotein (density defined in the text).

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Experimental

Materials

Radioactive isotopes were from The Radiochemical Centre, Amersham, Bucks., U.K. Na¹²⁵I and Na¹³¹I were both carrier-free, and [⁷⁵Se]selenomethionine was in sterile solution containing 100 µCi/ml. 1,1,3,3-Tetramethylurea was from Schuchardt (Munich, West Germany), and acrylamide and *NN'*-methylenebisacrylamide were from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals were of the highest grade available from BDH Chemicals (Poole, Dorset, U.K.).

Methods

Blood samples. Randomly bred guinea pigs from a closed laboratory colony of the Hartley–Dunkin strain (wt. 350–750 g) were bled by heart puncture, under diethyl ether anaesthesia. The blood was left to clot at room temperature for 2 h and the serum isolated by centrifugation at about 800 g for 20 min at 4°C.

Isolation of lipoproteins. A lipoprotein fraction of density 1.007–1.100 g/ml was isolated by sequential centrifugation as follows. Undiluted serum was centrifuged at 30000 rev./min in the Spinco 30.2 rotor for 16 h at 11°C, after which the supernatant layer of very-low-density substances was aspirated off and discarded. The density of the remaining serum was raised to 1.100 g/ml by mixing with NaBr solution of density 1.286 g/ml in the ratio 2:1 by

volume. This mixture was then centrifuged as above, and the lipoproteins of density less than 1.100 g/ml were recovered by aspiration. They were washed once by centrifugation at ρ 1.100 g/ml. All solutions used contained 1 mM-EDTA and 0.01 % NaN_3 .

Labelling of lipoproteins by the chloramine-T method. The following solutions were freshly prepared. Phosphate-buffered saline: 0.15 M-NaCl/50 mM- Na_2HPO_4 /1 mM-EDTA, adjusted to pH 7.5 with HCl. KI solution: 0.05 mg/ml of phosphate-buffered saline. Chloramine-T (sodium *N*-chloro-*p*-toluenesulphonamide): 5 mg/ml of water. Sodium metabisulphite: 0.01 M in water. Sodium metabisulphite: 5 mg/ml of phosphate-buffered saline.

Before iodination by this method, the freshly isolated lipoproteins were dialysed against several changes of phosphate-buffered saline at 4°C. A preliminary study of the conditions of reaction was carried out by the method of Hunter (1970), by using essentially the same apparatus. The reaction mixture contained known quantities of lipoprotein, KI and Na^{125}I dissolved in 1 ml of phosphate-buffered saline. Measured quantities of chloramine-T were delivered into this from a micrometer syringe, while the solution was rapidly stirred. At 2 min after each addition, the redox potential was measured (Hunter, 1970) and a 10 μl sample withdrawn from the reaction mixture. Each sample was immediately diluted with 1 ml of 0.01 M-sodium metabisulphite solution in water, followed consecutively by 0.1 ml of bovine plasma and 0.7 ml of 20% (w/v) trichloroacetic acid solution. After a thorough mixing, the resulting suspension was left for 2 h and then centrifuged at 500g for 3 min. The supernatant was removed into another tube. The residue was resuspended in 1.5 ml of 6% trichloroacetic acid solution and centrifuged under the same conditions. The supernatant was removed as before. The radioactivity in the precipitate was considered to be lipoprotein-bound, and that in the combined supernatants as unbound. The precipitate was subsequently extracted twice with 1.5 ml of ethanol/diethyl ether (3:1, v/v) to remove radioactivity associated with lipids. The first extraction was left to continue overnight, the second for 4 h, both at room temperature (18–20°C). The radioactivity remaining in the precipitate was considered to be apolipoprotein-bound.

The optimum conditions for iodination were determined from these preliminary experiments, and were used in the following routine procedure. The reaction was carried out in a small vial equipped with a magnetic stirrer. To 1 ml of phosphate-buffered saline containing lipoprotein equivalent to 1 mg of protein [as determined by the method of Lowry *et al.* (1951)] were added 50 μl of KI solution and the required amount of Na^{125}I or Na^{131}I (0.25–1.0 mCi in 0.05–0.20 ml of solution). The mixture was gently stirred, with precautions to prevent it becoming warm.

Chloramine-T was delivered from a micrometer syringe, through a fine poly(vinyl chloride) tube that dipped under the surface of the solution. The rate of addition was about 50 $\mu\text{g}/\text{min}$, and continued until a total of 200 μg had been reached. During this process the rate of stirring was increased to ensure rapid mixing of the reagent. When addition was complete, stirring was stopped and the mixture left for 5 min. Chloramine-T was then neutralized by the addition of 50 μl of sodium metabisulphite in phosphate-buffered saline.

Unless otherwise stated, unbound iodide was removed in the following way: the reaction mixture was passed down a column (15 cm $\times$ 1.0 cm) of Sephadex G-50 in phosphate-buffered saline at about 12 ml/h. The position of the labelled protein was monitored with a Geiger counter. After elution, the protein was dialysed overnight against several changes of phosphate-buffered saline, at 4°C. This was followed by dialysis for 8 h against four changes of NaCl solution of density 1.100 mg/ml, after which the lipoproteins were recovered by ultracentrifugation as described above. The product, which contained an average of 0.45 g-atom of iodine/mol of lipoprotein, was finally dialysed against 0.15 M-NaCl at 4°C.

Labelling of lipoproteins by ICl method. The procedure was based on that of McFarlane (1958) and required the following reagents. Stock ICl solution: a solution of 0.66 g of NaIO_3 in 2 ml of water was added to 0.1 g of NaI dissolved in 4.2 ml of conc. HCl. After addition of 5.8 g of NaCl, the volume was made up to 40 ml with water, and the solution extracted three times with 5–10 ml portions of carbon tetrachloride. After excess solvent had been blown off with N_2 , the volume was made up to 50 ml. Working ICl solution: this was made by diluting the stock solution 25-fold with 2 M-NaCl. Glycine buffer, pH 10.0: 63 ml of a solution of 0.1 M-glycine in 0.1 M-NaCl was mixed with 37 ml of 0.1 M-NaOH.

After dialysis against several changes of 0.15 M-NaCl, freshly isolated lipoprotein was concentrated osmotically with solid poly(ethylene glycol) to give a protein concentration of 10 mg/ml. This solution was dialysed against 0.15 M-NaCl for 30 min and then against glycine buffer for 30 min. All these operations were carried out at 4°C.

The iodination was carried out as follows. To a polystyrene tube containing lipoprotein solution equivalent to 1 mg of protein was added 1 mCi of Na^{125}I in 0.05 ml of glycine buffer. To this mixture, 50 μl of working solution of ICl was quickly added, with thorough mixing. After reacting for 1 min, the mixture was diluted to 1 ml with glycine buffer. Unbound iodide was then removed as described for the chloramine-T method.

Biological labelling. LD lipoprotein that had been labelled biosynthetically with [^{75}Se]seleno-

methionine was used as a control in one series of metabolic experiments. It was considered that lipoproteins labelled in this way would differ minimally from the native compounds. They were prepared by administering 125 μ Ci of [⁷⁵Se]selenomethionine in 1.25 ml solution to each of two guinea pigs by intracardiac injection. After 5–6 h, the animals were killed by exsanguination, serum was prepared, and LD lipoproteins (ρ 1.007–1.100 g/ml) isolated by ultracentrifugation as described above. After washing once by ultracentrifugation at 1.100 g/ml, they were dialysed against frequent changes of 0.15 M-NaCl for 30 min at 4°C.

Ultracentrifugal analysis. Because of the relatively high density of guinea-pig LD lipoproteins (Mills *et al.*, 1972), analytical ultracentrifugation was carried out in NaCl/NaBr solution of density 1.100 g/ml, in a Spinco model E centrifuge at 52640 rev./min and 26°C. The procedure was essentially that of DeLalla & Gofman (1954), but the co-ordinates of the schlieren patterns were transferred from the original photographs on to punched paper tape by means of a digital data reader (PCD, Farnborough, Hants., U.K.) and were processed by a digital computer by a program like that described by Ewing *et al.* (1965). The refractive increment of LD lipoprotein in the solvent of density 1.100 g/ml was taken to be 0.000149 ml/mg, and the other parameters were assumed to be the same as for human lipoproteins.

Immunology. The double-diffusion method of Ouchterlony (1953) was used in 1% agarose gels, prepared in 0.05 M-veronal buffer, pH 8.6. Diffusion was left to proceed at room temperature and the gels were inspected after 24 and 48 h.

Antiserum was prepared against the fraction of guinea-pig LD lipoprotein that was insoluble in 4.2 M-tetramethylurea (Kane, 1973). The precipitated material was washed first with 4.2 M-tetramethylurea, and then with ethanol/ether (3:1, v/v), after which it was redissolved in 10 mM-sodium decyl sulphate. This antigen solution was administered to New Zealand White rabbits as described by Chapman *et al.* (1972).

Gel chromatography. Iodinated LD lipoproteins were chromatographed on Sephadex G-200 in 0.05 M-sodium barbital buffer, pH 8.6, containing 3% bovine serum albumin, essentially as described by Schonfeld *et al.* (1974). The column used was 114 cm $\times$ 1.0 cm diameter, and the eluate was collected in fractions of 1.0–1.1 ml.

Gradient gel electrophoresis. The procedure was a slight adaptation of that described by Pratt & Dangerfield (1969). The following solutions were freshly prepared for each batch of tubes. Solution A: 19 g of acrylamide and 1 g of *NN'*-methylenebisacrylamide in 100 ml of water. Solution B: 10% (w/v) ammonium persulphate in water. Solution C: 10%

NNN'-tetramethylenediamine in water. The following mixtures were then prepared. 'Light' solution: 2.5 ml of A + 0.04 ml of B + 0.04 ml of C + 0.75 ml of 1.0 M-H₃PO₄, made up to 25 ml with water. 'Heavy' solution: 6.5 ml of A + 0.06 ml of B + 0.06 ml of C + 2.5 ml of 50% (w/v) sucrose solution + 2.5 ml of 35% Tris solution + 1.75 ml of 1.0 M-HCl, made up to 25 ml with water.

Ten glass tubes (7.5 cm $\times$ 0.4 cm internal diameter) were filled simultaneously by a method adapted from that of Castle *et al.* (1975). With an MSE gradient maker 13 ml each of the 'light' and 'heavy' solutions were mixed to form a linear gradient. After the gel had polymerized, it was left to mature at 4°C for 2 days before use.

Before analysis, the lipoprotein solutions were mixed with an equal volume of stain, which consisted of a saturated solution of Sudan Black B in ethylene glycol, to which 1.0% (v/v) of acetone was added. After standing for 30 min, the mixture was applied to gels at the rate of about 50 μ g of protein/gel, in a volume not exceeding 100 μ l. The analysis was then carried out in a Shandon apparatus for polyacrylamide-gel electrophoresis (Shandon Southern Instruments, Camberley, Surrey, U.K.) by using the buffers prescribed by Pratt & Dangerfield (1969). The initial current was 1.8 mA/tube, and the run continued for 2 h at constant voltage.

Measurement of turnover rate in vivo. The first set of five experiments compared the metabolism of LD lipoprotein labelled by the two different iodination procedures. For each experiment, a preparation of guinea-pig lipoprotein was divided into two parts, one of which was labelled with ¹²⁵I by the Hunter (1970) procedure, and the other with ¹³¹I by using ICl. A mixture was then made that contained 0.30–1.8 mg of each labelled apo-lipoprotein (total radioactivity approx. 2 μ Ci).

In a second set of two experiments, mixtures were made that contained 10 mg of LD lipoprotein labelled with [⁷⁵Se]selenomethionine and about 75 μ g of lipoprotein, which had been labelled with ¹²⁵I with chloramine-T.

In each case, the mixture of lipoproteins was injected, via the heart, into a recipient guinea pig. Samples of blood (1.8 ml) were taken from the heart after 2 min, and then at intervals up to about 70 h. These samples were drawn into tubes containing 2 mg of EDTA, except for the last, when 10 ml of blood was taken and left to clot.

In the first series of experiments, the samples of plasma or serum were treated as follows: 0.4 ml was thoroughly mixed with 0.2 ml of 20% trichloroacetic acid solution and the precipitate washed twice with 1 ml of 6% trichloroacetic acid, followed by three extractions with 1 ml of ethanol/ether (3:1, v/v). The radioactivities of the two iodine radioisotopes were then determined either on a Wallac Decem GTL

300–500 counter (LKB Produkter, Bromma, Sweden) or on a Biogamma II counter (Beckman Instruments, Fullerton, CA, U.S.A.).

A different procedure was necessary in the second series of experiments because of the re-utilization of selenomethionine in the synthesis of serum proteins. The lipoproteins from each sample (0.5ml) were precipitated by the addition of heparin and MnCl_2 to final concentrations of 0.2% and 0.05M respectively (Burstein *et al.*, 1970). The precipitate was removed by centrifugation at 1500g for 20min, dissolved in 1ml of 0.002M-Tris buffer adjusted to pH7.7 with HCl, and re-precipitated by adding 2M- MgCl_2 to a final concentration of 0.05M. This precipitate was then treated once more in the same way before being extracted twice with ethanol/ether (3:1, v/v). The radioactivities of the two radioisotopes were then determined on the Biogamma counter.

In all the experiments the least radioactive samples were counted for radioactivity until the coefficient of variation was less than 1.9%. The values of serum apolipoprotein-bound radioactivity were expressed as percentages of the initial radioactivity and were plotted on a logarithmic scale against time. In general, the curves for the two radioisotopes could not be superimposed, but appeared to become parallel after the early period of equilibration. Straight lines were fitted to these later sections by the method of least squares and the parallelism for each pair was tested statistically (Crow *et al.*, 1960). These straight lines were also used to determine the fractional catabolic rate of the labelled apolipoprotein by the procedure of Matthews (1957).

Results

Trial iodinations of human LD lipoproteins by the Hunter (1970) procedure established that labelling was more efficient in 0.03mM-KI solution than at lower concentrations (McTaggart, 1974). Under these conditions, a maximum efficiency was achieved at a redox potential of 150–200mV. When guinea-pig LD lipoprotein was treated in the same way, the efficiency of iodination was related to the redox potential by a curve like that shown in Fig. 1. After a fairly rapid rise between 0 and 100mV, the efficiency of labelling became almost constant between 150 and 200mV. Under these conditions, up to 50% of the iodine entered the lipid moiety, whereas in the human LD lipoprotein the proportion was less than 10%. As shown in Table 1, this performance on guinea-pig LD lipoprotein was not significantly better than that obtained by the ICl method.

The effect of the two methods of iodination on the physical properties of the lipoproteins was tested as follows.

Fig. 1. Dependence of efficiency of labelling of guinea-pig serum LD lipoproteins on the redox potential during iodination by the chloramine- τ procedure

The curves are specimens of those obtained by the method of Hunter (1970), as described under 'Methods'. ----, Lipoprotein-bound radioactivity; —, apolipoprotein-bound radioactivity. The plateau region of the curve consistently began at 100 ± 10 mV, the efficiency of labelling the apolipoprotein ranging from 40 to 50%. Efficiency is quoted as bound radioactivity as a percentage of total radioactivity used in the reaction.

Table 1. Result of iodination of guinea-pig LD lipoproteins by either the chloramine- τ or the ICl procedure

Values are the mean ($\pm$ S.D.) for the numbers of experiments shown in parentheses in the first column. The determination of the radioactivity in lipoprotein and in apo-lipoprotein was as described under 'Methods'. 'Total radioactivity' refers to the radioactivity before removal of unbound iodide. The last column quotes the mean efficiencies of labelling the apo-lipoprotein, expressed as percentages of the total radioactivity in the reaction mixture.

Procedure	Lipoprotein radioactivity	Apo-protein radioactivity	Efficiency (%)
	Total radioactivity	Lipoprotein radioactivity	
Chloramine- τ (5)	0.80 ± 0.02	0.59 ± 0.07	47 ± 7.5
ICl (3)	0.80 ± 0.03	0.51 ± 0.05	41 ± 5.5

Analytical ultracentrifugation

After iodination and removal of unbound iodide, different preparations of labelled guinea-pig LD lipoprotein were dialysed against NaCl solution of density 1.100g/ml. The lipoproteins were then isolated by ultracentrifugation and analysed centrifugally as described under 'Methods'. Examples of the distributions obtained, both before and after iodination, are shown in Fig. 2. Neither labelling procedure caused any significant change in the distribution. In the cases illustrated, the peak flotation rate fell from 7.1 to 6.5S after iodination by using ICl, and was increased from 7.3 to 8.1S

Fig. 2. Effect of iodination on the ultracentrifugal distribution of guinea-pig serum LD lipoproteins

(a) Iodination by the chloramine-T procedure; (b) iodination by the ICl method. Both are representative of the experiment described in the text. The open histogram represents the distribution before iodination, and the shaded histogram that after labelling. Each step in the histogram represents the percentage of the total lipoprotein between the two relevant values of flotation rate.

after iodination by the Hunter (1970) procedure. In other experiments, however, changes were induced in the opposite directions, suggesting that no consistent alteration in centrifugal properties is brought about by either method of iodination.

Gel chromatography

The products of both methods of labelling gave a single main peak which was eluted at the void volume of the Sephadex G-200 column, with only a small amount of trailing. This was followed, at the included volume, by a small peak of low-molecular-weight material.

Fig. 3. Chromatography of radioiodinated guinea-pig LD lipoproteins on Sephadex G-200

A single preparation of lipoprotein was used for the experiments in (a) and (b). One part was iodinated with ^{131}I by using ICl, and another part was labelled with ^{125}I by using chloramine-T. The two products were mixed and chromatographed on a column of Sephadex G-200 in 0.05M-sodium barbital buffer, pH8.6, containing 3% bovine serum albumin. (a) Elution pattern of the lipoprotein labelled by using ICl; (b) elution pattern for the lipoprotein labelled by using chloramine-T. The arrows indicate the void volume. (c) Elution pattern given, in a separate experiment, by a different lipoprotein preparation after labelling by the chloramine-T procedure.

For the experiment illustrated in Figs. 3(a) and 3(b), a single preparation of LD lipoprotein was divided into two parts, one of which was iodinated with ^{131}I by using ICI and the other with ^{125}I by using chloramine-T. Both products were mixed and applied to the column. The material labelled by McFarlane's (1958) method (Fig. 3a) gave 85.5% of the recovered activity in the main peak and 7.4% at the included volume. After treatment by Hunter's (1970) method (Fig. 3b) 60.9% of the activity was recovered in the lipoprotein peak and 23.8% at the included volume. The total radioactivity between these two peaks amounted to 7.1% and 15.3% respectively.

Fig. 3(c) shows the result of iodination of a second lipoprotein preparation by the Hunter (1970) procedure. The activity recovered in the two peaks was 85.1% and 6.7% respectively, with 8.2% in the intermediate region. Similar results were obtained with a third preparation of guinea-pig lipoproteins, and with

two preparations of human LD lipoproteins. It is noteworthy that these results were obtained on freshly iodinated lipoprotein. In older specimens, there was evidence of the disaggregation and additional peaks reported by Eaton & Kipnis (1969) and Schonfeld *et al.* (1974).

Polyacrylamide-gradient-gel electrophoresis

Fig. 4 shows an example of the pattern obtained by gradient electrophoresis of iodinated guinea-pig LD lipoprotein (tube *a*, ICI method; tube *b*, untreated; tube *c*, chloramine-T method). There is clear evidence of large particles in the preparation treated with ICI, which were absent from the original material and from that treated with chloramine-T.

Immunodiffusion

As shown in Fig. 5, both preparations of iodinated lipoprotein reacted with antiserum to guinea-pig apo-lipoprotein B to give precipitin lines that fused completely with that produced by the native lipoprotein.

Measurement of metabolic turnover

As the main use of the labelled lipoprotein was to be the study of lipoprotein metabolism *in vivo*, the two procedures were compared in metabolic turnover experiments. Five separate tests were made, the results of one being summarized in Fig. 6. The form of the decay curve was like that described for other animals (Hay *et al.*, 1971; Langer *et al.*, 1972; Eisenberg & Rachmilewitz, 1973). A rapid fall over a period of about 24 h was followed by a slower exponential decay up to at least 75 h. During the latter period, the curves diverged by only 0.00097 radians, an amount that was not significantly different from zero (Student's *t* test). Thus, once equilibration with the extravascular pools had

Fig. 4. Polyacrylamide-gradient-gel electrophoresis of radioiodinated guinea-pig LD lipoproteins

Lipoproteins were prepared and iodinated as described under 'Methods' and were then pre-stained by mixing with an equal volume of Sudan Black B in ethylene glycol. The polyacrylamide gel gradient ranged from approx. 2% to approx. 5% and was prepared as described under 'Methods'. Lipoprotein equivalent to about 50 μg of protein was applied to each gel. (a) Lipoprotein after iodination by the ICI procedure; (b) native lipoprotein; (c) lipoprotein after iodination by the chloramine-T process.

Fig. 5. Immunodiffusion analysis of iodinated guinea-pig LD lipoproteins

The centre well contained antiserum to the fraction of guinea-pig LD lipoprotein that was insoluble in tetramethylurea. Well (a): LD lipoprotein that had been iodinated by the ICI procedure; well (b); the native lipoprotein; well (c): lipoprotein that had been iodinated by the chloramine-T method.

Fig. 6. Decay of radioactivity in the serum of a guinea pig, after injection of LD lipoproteins labelled with radioactive iodine

The native lipoproteins were obtained from a single guinea pig. A portion was iodinated by the ICl method ($\blacktriangle$) and the remainder by the chloramine-T process. The products were mixed and administered to a second guinea pig. The method of measuring the rate of decay of radioactivity in the serum is described in the text.

been established, the two differently labelled lipoproteins were metabolized at the same rate. Analysis of the data by the method of Matthews (1957) gave a fractional catabolic rate of 0.097 h^{-1} for the apo-lipoprotein labelled by Hunter's (1970) procedure, and 0.092 h^{-1} for that labelled by using ICl. The half-life was 15 h in both cases.

The mean divergence of the five pairs of curves studied in this way was 0.0019 radians (s.d. ± 0.0017), and in no case did the slopes of the fitted straight lines differ significantly. The mean fractional catabolic rate calculated from these curves was 0.0895 h^{-1} (s.d. ± 0.0148) for apo-lipoprotein labelled by using chloramine-T, and 0.0862 h^{-1} (s.d. ± 0.0101) for that labelled by using ICl.

In two additional experiments, the turnover of guinea-pig LD lipoprotein that was iodinated by using chloramine-T was compared with that which was biologically labelled with [^{75}Se]selenomethionine. One set of decay curves is shown in Fig. 7. The iodinated lipoprotein equilibrated less rapidly with the extravascular pool than that labelled *in vivo*, and the curves therefore crossed. Nonetheless, in the second exponential phase, they became almost parallel. In the example shown, the angular

Fig. 7. Decay of radioactivity in the serum of a guinea pig after injection of iodinated LD lipoproteins mixed with biologically labelled LD lipoproteins

LD lipoproteins labelled biologically in the protein moiety were obtained by administering [^{75}Se]selenomethionine to a guinea pig. These were mixed with LD lipoproteins that had been radioiodinated by the chloramine-T method and injected via the heart into a recipient animal. The rate of disappearance of the two radioisotopes from the serum was measured as described in the text: $\blacksquare$, ^{75}Se ; $\bullet$, ^{125}I .

divergence was 0.0055 radians, which was significantly different from zero ($P < 0.05$), but in the other case it was only 0.0017 radians, which was not significant. In these experiments the fractional catabolic rates for the iodinated apolipoprotein were 0.0536 and 0.0589 h^{-1} and for the biologically labelled protein they were 0.0465 and 0.0629 h^{-1} .

Discussion

We conclude from the above results that it is possible to iodinate guinea-pig lipoproteins by either the ICl method or the chloramine-T procedure without causing significant damage to the molecule. So far as the physical properties of the products were concerned, the ICl method consistently caused a small amount of aggregation that the chloramine-T process did not. However, this aggregation could not be detected with certainty in the ultracentrifuge.

It was notable that, when the preparations were fresh, neither method of iodination gave rise to the extra peaks on gel-permeation chromatography that were described by Eaton & Kipnis (1969) and Schonfeld *et al.* (1974). However, additional peaks appeared in our preparations after they had been stored for a short time at 4°C . This agrees with the observation, made by Eaton & Kipnis (1969) on rat LD lipoprotein that had been labelled by the chloramine-T method,

Few attempts have been made to compare *in vivo* the integrity of lipoproteins labelled by different methods. Sundaram *et al.* (1972), who studied high-density lipoprotein in man, are the only investigators to have made simultaneous comparisons of homologous lipoproteins in a single animal. They found the products of both methods of iodination to be metabolically almost indistinguishable, but they did not extend their experiments to include biologically labelled material. Shepherd *et al.* (1976) compared the metabolism of differently labelled human LD lipoproteins separately in rats and found that the clearance of material labelled by the ICl method obeyed first-order kinetics, whereas that of material labelled by the chloramine-T procedure did not.

In our experience, there was no consistent significant difference in the decay of plasma apo-lipoprotein-bound radioactivity from the products of either method of iodination. Nonetheless, the decay curves were not always superimposable. Although the reasons for this cannot be precisely identified, the following seem to be the most probable. (1) The preparations may have contained different amounts of slightly denatured material, which was rapidly removed from the circulation during the first phase. This source of uncertainty would be decreased if the labelled lipoproteins were 'screened' in another guinea pig before use. However, the small size of the animal makes this technically difficult to do. (2) The rate of equilibration with the extravascular pool is influenced by slight differences between the two preparations.

The analysis of the decay curves suggests that both these effects must be small, since there are no significant differences between the estimates of fractional catabolic rate. Moreover, any changes in the behaviour of the lipoproteins were unconnected with a particular method of iodination, because neither consistently led to a steeper decay curve than the other.

In the experiments on biologically labelled lipoprotein, it is necessary to consider the consequence of recycling of selenomethionine. It was not possible to determine how much radioactivity was incorporated into freshly synthesized apo-lipoprotein, but the growing radioactivity of the other plasma proteins showed that it must have occurred. Nonetheless, the relatively small pool of apo-lipoprotein must ensure that re-incorporation was small, although this would be partly offset by the rapid turnover of the apoprotein. It seems likely that the crossing of the decay curves in these experiments was at least partly due to re-utilization, although its influence on the estimates of fractional catabolic rate was evidently insignificant.

It was notable that the catabolic rate determined from these two experiments was substantially lower than that obtained when iodinated lipoproteins

were used alone. This is probably due to the low specific radioactivity of the biologically labelled lipoprotein, the dose of which had to be relatively large. As a consequence, the initial lipoprotein concentration in the plasma of these guinea pigs was raised to a value comparable with that of animals with a diet-induced hypercholesterolaemia (Mills *et al.*, 1972), in which the fractional catabolic rate of apo-(LD lipoprotein) has already been shown to be only about 0.05 h^{-1} (Mills & McTaggart, 1974). In the current experiments, therefore, the animals were not in a steady-state condition and the estimated fractional catabolic rates are unphysiological. Despite this reservation, the experiments show that the two differently labelled lipoproteins were dealt with metabolically at essentially the same rate.

Our experience shows that, if the reaction conditions are properly chosen, the chloramine-T method is at least the equal to that using ICl. Others (e.g. Schonfeld *et al.*, 1974; Shepherd *et al.*, 1976) have said that the method iodinate at low efficiency, and produces denatured products. It seems likely that these unsatisfactory results were the consequence of a poor choice of conditions of reaction, notably an inappropriate concentration of chloramine-T, or the use of too short a reaction time. Both these conditions have been discussed by Hunter (1970). The chloramine-T procedure has two advantages: (a) the rate of liberation of hypochlorous acid, which is the effective oxidizing agent, is relatively slow and the rate of iodination is also slow; (b) the exposure to the oxidizing agent can be precisely monitored by measuring the redox potential, and the addition of an unnecessary excess of chloramine-T can be avoided. It is significant that Hay *et al.* (1971) were able to iodinate rat LD lipoprotein with an efficiency greater than 70% by exposure to chloramine-T for 1.5–2 h at a redox potential of 250–300 mV. In many other cases, however, chloramine-T has been added in great excess, and the reaction allowed to continue for only a short time, presumably in an attempt to minimize the possibility of damage to the lipoprotein. It appears that this manoeuvre is largely unsuccessful and that superior results can be obtained if the method of Hunter (1970) is adhered to. It is also worth noting that this procedure may not only produce less aggregation of the lipoprotein than that of McFarlane (1958), but has the advantage that it can be applied with good results to solutions containing low concentrations of apolipoprotein. The ICl method is said to become progressively less efficient when the amount of protein present is less than 2–5 mg (Freeman, 1967).

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