Photolysis of Cholesterol during Biological Experiments

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1. When dilute aqueous emulsions of radioactive cholesterol are exposed to daylight, extensive photolysis may take place. This results chiefly in the formation of substances more polar than cholesterol, some of which are probably acidic. Substances less polar than cholesterol are formed to a smaller extent. Photolysis is greatest when the emulsions are strongly acidic or strongly alkaline. 2. Photolysis takes place rapidly if radioactive cholesterol stored in the dry state, either on glass or on filter paper, is exposed to daylight. 3. If precautions are taken to minimize exposure to daylight during analysis and storage of the samples, the amount of non-enzymic alteration of cholesterol in biological experiments may be negligible.

During a study of the metabolism of radioactive cholesterol by cell-free preparations of liver, we found that in some experiments almost as much cholesterol underwent chemical change in the presence of boiled mitochondria as in the presence of living mitochondria. Investigation of this anomaly showed that the amount of cholesterol altered chemically in the absence of living mitochondria depended mainly on the extent to which light was admitted to the samples during their analysis after incubation. Since these findings have a practical bearing on the design of biological experiments with cholesterol *in vitro*, we are reporting them here. A brief summary of this work has been published (Hais & Myant, 1963).

METHODS

Liver mitochondria were prepared from male Wistar rats, weighing about 250 g., essentially by the method of Whitehouse, Staple & Gurin (1959). [$^{26-14}\text{C}$]Cholesterol was incubated with mitochondria by a modification of the method of Whitehouse *et al.* (1959). The incubations were carried out in stoppered Warburg flasks with hyamine base [*p*-(di-isobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide] as CO_2 absorber. The hyamine (0.2 ml. of a 1 M solution in methanol) was contained in a glass cup (capacity 0.4 ml.) with a stem fitting loosely into the centre well of the flask. For acidification at the end of the incubation, 0.3 ml. of 20% (w/v) trichloroacetic acid or 2 N-HCl was placed in the side arm.

A portion (20 $\mu\text{l.}$) of a solution of [$^{26-14}\text{C}$]cholesterol (1.6 $\mu\text{g.}$, with 0.1 μC of ^{14}C) in benzene was pipetted into the flask and the benzene evaporated under a stream of N_2 . Tween 20 (polyethylene glycol sorbitan monolaurate) (16 $\mu\text{g.}$

in 0.2 ml. of methanol) was pipetted on the area covered by the cholesterol and the methanol evaporated under N_2 . To each flask were added: 0.5 ml. of a suspension in 10% (w/v) sucrose of the washed mitochondria from 0.3 g. wet wt. of liver, and 1.0 ml. of incubation mixture similar to that used by Whitehouse *et al.* (1959), with the omission of AMP and the 'soluble cofactor'. The flasks were incubated for 6 hr. in duplicate at 37° in a water bath, with air as the gas phase, with shaking. At the end of the incubation the acid was tipped in and shaking continued for a further 12 hr. The flasks were then opened and the hyamine-containing cups removed. The outside of each cup was washed by dipping it into two lots of toluene to remove any contamination from the incubation mixture. The contents of the main compartment were filtered through Whatman no. 1 filter paper after the addition of 1.5 ml. of ethanol, and the filtrate was extracted four times with 4 vol. of light petroleum (b.p. $60\text{--}80^\circ$; described below as 'petroleum') equilibrated with aq. 50% (v/v) ethanol. In some of the experiments in which no incubation with mitochondria was carried out, the extraction procedure was modified as explained in the text.

Radioassay. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer, with a mixture of 10 ml. of 0.3% 2,5-diphenyloxazole in toluene and 2 ml. of ethanol as scintillating liquid. The cups containing the hyamine were dropped into the vials containing the scintillating liquid, the hyamine was dissolved by gently shaking the vials and the samples were assayed without removing the cups (preliminary tests showed that the presence of the glass cups did not alter the counting rate). Radioactivity was assayed in 0.2 ml. samples from the combined petroleum extracts and in 0.1 ml. samples from the aqueous-ethanol fractions after their extraction with petroleum. Radioactivity on the paper chromatograms was located by radioautography, and the amount of activity in each spot was measured by cutting the paper into segments 0.5 cm. wide and placing each segment flat on the bottom of a counting vial with 12 ml. of the scintillating liquid.

Paper chromatography. The petroleum extracts were concentrated under a stream of N_2 and chromatographed on Whatman no. 3MM filter paper by the method of Kodicek &

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Ashby (1954). The sample was applied as a round spot and the solvent allowed to dry. The end of the paper nearest to the solvent front was then dipped in a 5% (v/v) solution of kerosene in petroleum so that the solution wetted the paper as far as the centre of the spot. The petroleum was allowed to evaporate and the chromatogram developed in the ascending direction with propan-1-ol-methanol-water (15:82:3, by vol.) mixture as the mobile phase. In a few cases the mobile phase was 80% (v/v) acetic acid and the stationary phase was prepared from a 10% (v/v) solution of kerosene in light petroleum. The chromatography tanks were protected from light by covering them with black paper. During the chromatography with propanol-1-ol-methanol-water the rear margin of the kerosene moved towards the solvent front to a line corresponding to R_f about 0.2.

Materials. [26-¹⁴C]cholesterol and [4-¹⁴C]cholesterol were obtained from The Radiochemical Centre, Amersham, Bucks., and were stored in the dark as benzene solutions. On reversed-phase paper chromatography 93-98% of the activity in different batches behaved as cholesterol; traces of activity were always present between cholesterol and the solvent front, and on the chromatograms from some samples there were traces at the origin and along the rear margin of the stationary phase. When samples of the stock solutions of [26-¹⁴C]cholesterol were partitioned between aq. 50% ethanol and petroleum, more than 99% of the activity was recovered in the combined petroleum extracts.

Tween 20 was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.), hyamine from Nuclear Enterprises (G.B.) Ltd. (Edinburgh), kerosene from Hopkin and Williams Ltd. (Chadwell Heath, Essex) and Parafilm from A. Gallenkamp and Co. Ltd. (London).

RESULTS

In most experiments in which [26-¹⁴C]cholesterol was incubated with living mitochondria, 2-5% of the total radioactivity was recovered as ¹⁴CO₂ in the hyamine and 10-25% remained in the aqueous-ethanol fraction after extraction with petroleum. In some of our earlier experiments, up to 10% of the total activity remained in the aqueous-ethanol fraction from incubations with boiled mitochondria, though significant activity in the hyamine was never found unless living mitochondria were used. Since these experiments were done during the early summer, when some of the samples were exposed to bright daylight while they were being fractionated, the effect of light was tested at various stages of the experimental procedure.

Exposure to light during incubation. When precautions were taken to minimize the amount of light reaching the samples during their analysis after incubation, activity was negligible in the hyamine and aqueous-ethanol fractions obtained from incubations with boiled mitochondria. Light admitted during the incubation had no effect on the distribution of radioactivity between the hyamine, aqueous-ethanol and petroleum fractions. Table 1 shows the results of an experiment in which half the flasks were covered with a black cloth during the

Table 1. *Radioactivity recovered in hyamine, aqueous ethanol and light petroleum from an incubation of [26-¹⁴C]cholesterol with living and boiled mitochondria*

During the incubation half the flasks were exposed to light and the other half were kept in the dark. Other experimental details are given in the Methods section. The results are given as percentages of the total activity recovered from the flasks.

Fraction	Light during incubation		Dark during incubation	
	Living mitochondria	Boiled mitochondria	Living mitochondria	Boiled mitochondria
Hyamine	2.2	0	2.0	0
Aqueous ethanol	22.5	< 1.0	23.3	1.0
Petroleum	75.3	> 99.0	74.7	99.0

incubation and the equilibration after the addition of acid, and the other half were exposed to daylight, and to light from a fluorescent tube placed about 5 ft. above the water bath. The samples were extracted as rapidly as possible immediately after the flasks were opened. The extractions were carried out during the day, about 20 ft. from the nearest window and without fluorescent lighting.

Exposure to light during fractionation. To test the effect of light during fractionation of the samples, emulsions or solutions of [26-¹⁴C]cholesterol were subjected to conditions relevant to those used in the biological experiments, either in complete darkness or in full daylight.

Samples of the stock [26-¹⁴C]cholesterol solution (1.6 µg. in 10 µl. of benzene) were pipetted into Pyrex test tubes and the benzene was evaporated under nitrogen. Tween 20 (16 µg. in 0.2 ml. of methanol) was added to each tube and the methanol evaporated under nitrogen. The cholesterol was then emulsified or dissolved in the solutions shown in Table 2. One complete set of tubes was wrapped in tin foil to exclude light and another set was left uncovered. All the samples were then placed by a closed window for 3 days at room temperature. During the exposure, the sun shone for about 4 hr. each day. After exposure, the contents of half the 'dark' and half the light-exposed tubes were made alkaline by addition of 0.4 ml. of 10 N-sodium hydroxide. Enough water or ethanol was added to each tube to bring the concentration of ethanol to 50%, and the samples were extracted as rapidly as possible with petroleum.

The results of this experiment (Table 2), and of others, showed that photolysis of cholesterol was greatest in strongly alkaline or strongly acidic emulsions. As judged by the amount of activity in the aqueous-ethanol fraction, hardly any chemical alteration took place when the cholesterol was dis-

Table 2. *Effect of light on emulsions or solutions of [26-¹⁴C]cholesterol, as shown by the partition of radioactivity between aqueous ethanol and light petroleum*

The radioactive cholesterol was emulsified or dissolved in various solutions (1.6 μ g. in 1.5 ml.) and the samples were kept in the dark or exposed to daylight for 3 days. The concentration of ethanol was then adjusted to 50% and the samples were extracted with petroleum. In half the tubes from each set, the contents were made alkaline before the extraction with petroleum. Other experimental details are given in the Results section. The results are given as percentages of the total activity remaining in the aqueous ethanol after extraction with petroleum.

Medium during exposure	Kept in dark		Exposed to light	
	No NaOH	NaOH added before extraction	No NaOH	NaOH added before extraction
Tris buffer (pH 8.5)	0.2	0.05	1.6	1.4
Water (pH 6.0)	0	0	4.5	3.8
Ethanol	0	0	0.7	0.2
2N-NaOH	0.05	—	11.7	—
5% Trichloroacetic acid	0.4	0.17	4.9	17.7
5% Trichloroacetic acid + N ₂ *	—	—	0	12.9

* In this pair of tubes, the air above the liquid was flushed through with N₂ for 5 min. and the tubes were sealed with Parafilm.

solved in ethanol during exposure to light. Replacement of the gas phase by nitrogen made little difference to the amount of cholesterol degraded in acidic emulsions exposed to light. In emulsions exposed to light in the presence of trichloroacetic acid, the percentage of the total activity remaining in the aqueous ethanol was always greater if the emulsion was made alkaline before extraction with petroleum.

Experiments in which the radioactivity in the petroleum was analysed by paper chromatography suggest that the amount of cholesterol degraded in the light-exposed samples, as judged by the proportion of the total activity in the aqueous-ethanol fraction (Table 2), may have been underestimated.

In one experiment, an emulsion of [26-¹⁴C]-cholesterol in tris buffer, pH 8.5, was exposed to daylight for 3 days, with a control sample kept in the dark at the same time. After exposure, the samples were acidified with 0.2 vol. of 25% (w/v) trichloroacetic acid and enough ethanol was added to give a concentration of 50%. The samples were extracted with petroleum and the pooled extracts chromatographed on the same day, together with a sample of the stock solution of [26-¹⁴C]cholesterol. Radioautographs of chromatograms run in the propanol-methanol-water system showed that most of the activity in the 'dark' control and stock samples was due to cholesterol (R_F 0.55–0.65), though a faint trace of blackening could be seen between cholesterol and the solvent front. With both samples, there was also a horizontal band of blackening (R_F 0.15–0.20) corresponding to the position of the rear margin of the kerosene, a faint band just behind this, and a zone of blackening at the origin. In the samples exposed to light, the

Table 3. *Effect of light on an emulsion of [26-¹⁴C]cholesterol, as shown by the distribution of radioactivity on a paper chromatogram*

Emulsions of radioactive cholesterol in tris buffer, pH 8.5 (1.6 μ g. in 1.5 ml.), were kept in the dark or exposed to daylight for 3 days. Petroleum extracts from the emulsions were then submitted to reversed-phase paper chromatography with 5% kerosene in petroleum as the stationary phase and propanol-methanol-water (15:82:3, by vol.) as the mobile phase (see Fig. 1). Results obtained with the stock solution of [26-¹⁴C]cholesterol are shown for comparison. Other experimental details are given in the Results section. Results are given as percentages of the activity recovered in each zone on the chromatograms.

Zone on chromatogram	Stock solution	Emulsion kept in dark	Emulsion exposed to light
Origin	0.2	0.2	2.1
R_F 0.1–0.25	0.3	1.2	5.5
Cholesterol	93.8	92.2	43.9
Between cholesterol and solvent front	3.5	4.5	42.6

intensity of blackening in the cholesterol zone was diminished and that in all the other regions was increased, especially that between cholesterol and the solvent front (Fig. 1). Quantitative analysis of the paper chromatograms (Table 3) showed that less than half the activity in the petroleum fraction from the light-exposed sample was due to cholesterol.

In another experiment, in which the cholesterol emulsion was exposed to daylight for 7 days, less than 10% of the activity in the petroleum fraction

Fig. 1. Effect of light on emulsions of radioactive cholesterol in tris buffer, pH 8.5. The Figure shows radioautographs of reversed-phase paper chromatograms of stock solution of $[26-^{14}\text{C}]$ cholesterol (S) and of petroleum extracts made from emulsions kept in the dark (D) or exposed to daylight (L) for 3 days. The stationary phase was 5% kerosene in petroleum and the mobile phase propanol-methanol-water (15:82:3, by vol.). F, Solvent front; O, origin; arrow shows position of rear margin of stationary phase at the end of the run.

behaved as cholesterol when the sample was chromatographed in the propanol-methanol-water system. A sample of this fraction was also chromatographed with 80% (v/v) acetic acid as the moving phase. On the radioautograph prepared from the chromatogram at least four confluent zones of blackening could be seen between cholesterol and the solvent front (Fig. 2).

Exposure to light during storage after evaporation of solvent or on chromatography paper. Emulsions of $[26-^{14}\text{C}]$ - or $[4-^{14}\text{C}]$ -cholesterol were made up in the standard incubation mixture containing the usual additions, including mitochondria. The contents of the flasks were immediately acidified and enough ethanol was added to bring the concentration to 50%. The samples were then extracted with petroleum, and the petroleum extracts from each flask combined in 25 ml. glass measuring cylinders. Each sample was then treated in one of two ways: (1) The petroleum was evaporated to dryness under nitrogen and the measuring cylinder was sealed with Parafilm. Half the cylinders were wrapped in tin foil and the other half left uncovered. They were then placed near a closed window for 4 days at room temperature. After exposure, the inside of each cylinder was washed down with 0.4 ml. of petroleum, followed by 0.2 ml. of ethanol. The petroleum and ethanol washings were spotted separately on to chromatography paper and chromatographed at

Fig. 2. Effect of light on an emulsion of $[26-^{14}\text{C}]$ cholesterol in tris buffer, pH 8.5. The Figure shows a radioautograph of reversed-phase paper chromatograms of petroleum extract made from emulsion exposed to daylight for 7 days (L) and a radioautograph of stock solution of $[26-^{14}\text{C}]$ cholesterol (S) run in parallel with the light-exposed sample. The stationary phase was 10% kerosene in petroleum and the mobile phase aq. 80% (v/v) acetic acid. F, Solvent front; O, origin.

Fig. 3. Effect of light on $[26-^{14}\text{C}]$ cholesterol deposited on the inner surface of a glass measuring cylinder. The Figure shows radioautographs of paper chromatograms prepared from petroleum washing (1) and ethanol washing (2) of samples exposed to daylight for 4 days, and from petroleum washing of sample kept in darkness (3). The stationary phase was 5% kerosene in petroleum and the mobile phase propanol-methanol-water (15:82:3, by vol.). F, Solvent front; O, origin; arrow shows position of rear margin of solvent front. Experimental details are given in the Results section.

once in the propanol-methanol-water system. The distribution of radioactivity in the samples left in the dark did not differ significantly from that in samples of the stock solutions of radioactive cholesterol put on the paper immediately before the

Table 4. *Effect of light on samples of [26-¹⁴C]cholesterol on chromatography paper, as shown by the distribution of radioactivity along the chromatogram*

After the samples were applied to the chromatography paper the solvent mixture was run either immediately or after the papers had been kept in the dark or exposed to daylight for 4 days. The stationary phase was 5% kerosene in petroleum and the mobile phase propanol-methanol-water (15:82:3, by vol.). Other experimental details are given in the Results section.

Zones on paper chromatogram	Conditions before chromatography...	Radioactivity (% of total)		
		Run immediately after application	Kept in dark	Exposed to light
Origin		0.2	0.1	1.9
Between origin and cholesterol		0.3	0.2	3.3
Cholesterol		94.1	96.1	63.0
Between cholesterol and solvent front		5.4	3.6	31.8

chromatography. In the light-exposed samples, however, less than half the total activity was in the form of cholesterol (Fig. 3). In both the petroleum and the ethanol washings most of the activity not due to cholesterol was in one or more fractions more polar than cholesterol, but there was also an increase in the amount of activity at the origin and at the rear margin of the stationary phase (Fig. 3). The results obtained with [4-¹⁴C]cholesterol did not differ significantly from those obtained with [26-¹⁴C]cholesterol. (2) Portions of each of the unevaporated petroleum extracts were spotted on three pieces of chromatography paper. One paper was submitted at once to chromatography. The other two were kept for 4 days by a closed window, after wrapping one of them in black paper. After exposure, both papers were submitted to chromatography. The distribution of activity on the paper kept in the dark did not differ significantly from that on the paper submitted to chromatography immediately after applying the sample; on the paper exposed to light more than 30% of the total activity was present in the zone between cholesterol and the solvent front, and the amount of activity in the zones with R_f less than that of cholesterol was also increased (Table 4). Results obtained with [4-¹⁴C]cholesterol did not differ significantly from those with [26-¹⁴C]cholesterol.

The whole of this experiment was completed during January, when the sun shone weakly for less than 1 hr. each day.

DISCUSSION

The exposure to daylight in most of these experiments was greater than any likely to occur in practice. Nevertheless, our results show that, unless care is taken to minimize exposure to light during the analysis and storage of the samples, chemical alteration of cholesterol due to the action of light could easily exceed that due to enzymic action

during incubations with living mitochondria. Table 1 shows, however, that if this precaution is taken, it is possible to study the enzymic degradation of cholesterol *in vitro* without interference from the effects of photolysis. Exposure to light during incubation for up to 18 hr. in a water bath gives rise to little or no photolysis. This may be due to the fact that the incubation mixture contains protein and a buffer at pH 8.5, both of which may protect the cholesterol from the effects of light. It is also possible that light of the relevant wavelength is absorbed to some extent by the water in the water bath.

Schulze & Winterstein (1904, 1906) have shown that cholesterol in the crystalline state is chemically altered after prolonged exposure to light, and Neher (1958) has mentioned the need to exclude light during paper chromatography of steroids. Acker & Greve (1963) have also shown that the cholesterol in egg-containing foods forms autoxidation products when exposed to light. However, no one seems to have made a special study of the effect of light on dilute aqueous emulsions of cholesterol in conditions similar to those of a biological experiment.

Dauben & Payot (1956) have shown that [¹⁴C]-cholesterol is oxidized, mainly at the 5-, 6- and 7-positions, when stored for many months in the crystalline state with access to air. If air is excluded, little decomposition takes place. These changes were much less conspicuous in samples of non-radioactive cholesterol. They concluded that [¹⁴C]cholesterol undergoes radiation-induced oxidation and that oxygen is required for this process. In our experiments, exclusion of air made little difference to the amount of degradation caused by light (Table 2). However, we cannot exclude the possibility that oxygen is required for the action of light on emulsions of cholesterol, since no attempt was made to remove all traces of oxygen.

Bergström & Wintersteiner (1941, 1942) have shown that dilute emulsions of cholesterol undergo

autooxidation at 37°, resulting in the formation of 7 α -hydroxycholesterol, 7 β -hydroxycholesterol and 7-oxocholesterol. More recently, 3 β ,5 α ,6 β -tri-hydroxycholestane (Mosbach, Nierenberg & Kendall, 1953) and 25-hydroxycholesterol (Fieser, 1954) have been detected among the autooxidation products of cholesterol emulsions. The former substance has also been demonstrated in extracts from suspensions of cholesterol incubated with a boiled liver preparation (Danielsson, 1960). In our experiments, little or no degradation took place when samples of cholesterol, either as emulsions or in the solid state, were left in the dark for several days, although air was not excluded. This raises the possibility that autooxidation of cholesterol is dependent on, or at least enhanced by, exposure to light. If light does stimulate autooxidation, differences in exposure to light may explain the wide variations in the amount of non-enzymic breakdown of cholesterol noted in biological experiments by different workers [compare, for example, Danielsson (1960) with Fredrickson (1956)].

Since the chief object of this work was to find conditions in which non-enzymic breakdown of cholesterol does not occur, we made no attempt to identify the products of photolysis. The radioactive substances produced from [26-¹⁴C]- and [4-¹⁴C]-cholesterol by the action of light were similar in quantity and quality; this makes it unlikely that light disrupts the side chain of cholesterol. Some of the polar substances formed in emulsions of cholesterol exposed to light (Fig. 2) may be hydroxylated derivatives, similar to those formed during autooxidation, but we have no evidence for this. The observation that the amount of activity in the aqueous-ethanol fraction from light-exposed emulsions was increased if the emulsions were made alkaline before the extraction (Table 2)

suggests that some of the substances formed during the exposure to light were acidic. The formation of substances less polar than cholesterol is more difficult to understand. Some of the radioactivity moving with R_f 0.2 or less may have been due to esters derived from the breakdown products of cholesterol at some stage during the analysis of the samples. In favour of this, the intensity of the horizontal band of blackening at R_f 0.2 was always much greater after acidification with trichloroacetic acid than after acidification with hydrochloric acid.

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REFERENCES

- Acker, L. & Greve, H. (1963). *Fette, Seifen, Anstrichmittel*, **65**, 1009.
Bergström, S. & Wintersteiner, O. (1941). *J. biol. Chem.* **141**, 597.
Bergström, S. & Wintersteiner, O. (1942). *J. biol. Chem.* **143**, 503.
Danielsson, H. (1960). *Acta chem. scand.* **14**, 846.
Dauben, W. G. & Payot, P. H. (1956). *J. Amer. chem. Soc.* **78**, 5657.
Fieser, L. F. (1954). *Science* **119**, 710.
Fredrickson, D. S. (1956). *J. biol. Chem.* **222**, 109.
Hais, I. M. & Myant, N. B. (1963). *Biochem. J.* **87**, 8p.
Kodicek, E. & Ashby, D. R. (1954). *Biochem. J.* **57**, xii.
Mosbach, E. H., Nierenberg, M. & Kendall, F. E. (1953). *J. Amer. chem. Soc.* **75**, 2358.
Neher, R. (1958). *J. Chromat.* **1**, 205.
Schulze, E. & Winterstein, E. (1904). *Hoppe-Seyl. Z.* **43**, 316.
Schulze, E. & Winterstein, E. (1906). *Hoppe-Seyl. Z.* **48**, 546.
Whitehouse, M. W., Staple, E. & Gurin, S. (1959). *J. biol. Chem.* **234**, 276.