COMPARISON OF EFFECTS OF D_2O AND X-RAY IRRADIATION ON LIPID COMPOSITION OF TISSUE CULTURE CELLS†

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INTRODUCTION

At the time of its discovery in 1932 the importance of the biological implications of mass effects of deuterium was recognized[1, 2]. Immediately following the discovery and preparation of deuterium oxide a few biological experiments were carried out on the effects of deuterium oxide on growth of cells[3], enzyme reactions[4] and tumour development[5]. In recent years, with the availability of large quantities of deuterium oxide, the influence of deuterium mass effects in biology has begun to come under intensive study.

The interest of the present authors originally centred on the effects of D_2O upon the chemical composition of various strains of cells grown in tissue culture. Two phenomena became evident; growth in D_2O resulted in enlargement of the cells accompanied by an appearance of lipid-containing vacuoles (this was true with several lines of cells)[6] and under certain conditions the deuterated cells supported the growth of poliomyelitis virus to a greater extent than did control cells[7, 8]. Since X-ray irradiation of cells also results in enlargement of cells[9] and increase in virus yield when infected[10], we compared the two methods of treatment[11]. For these experiments it was necessary to use Monkey kidney (MK) cells which can be grown in Petri dishes and thus can be irradiated more easily than can strains of cells grown in suspension. It was found that at elevated temperatures (40°) poliovirus (CHAT strain) would multiply in cells grown in D_2O (25 per cent) but not in X-ray irradiated (500–2850r) cells. Overlay of the infected, X-ray irradiated cells with D_2O promoted virus growth, indicating that the X-ray irradiated cells are competent at 40° but are incapable of providing a milieu suitable for growth of CHAT virus. One possible area of difference between D_2O-treated and X-ray irradiated cells was in the lipid spectrum of the cells and a comparison of the lipids of these two types of cells is the subject of this report.

MATERIALS AND METHODS

In early experiments several lines of cells were used. HeLa and L cells were grown as monolayers on the surface of glass containers. In studies involving L-5178Y, a stable cell line derived from a spontaneous murine

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leukaemia of thymic origin, cells were grown in suspension in agitated culture. The cells were grown in medium containing 30 per cent of redistilled D_2O (>99·4 per cent) since earlier work had shown that good cell yields could be obtained at this concentration of D_2O. The medium used was a balanced salt solution supplemented with 2 per cent horse serum. The horse serum used in this study was taken from a single lot in order to minimize lipid changes induced by variability in the serum lipids. Cultures were checked periodically to ensure absence of contaminating pleuropneumonia-like organisms.

To overcome variations in growth of cells within individual flasks, cells were grown in a large number of small flasks using a standard inoculum. At 12 hour intervals, several flasks were pooled and the cells harvested by centrifugation.

Lipid determinations were carried out on cells which had been incubated at 35° for 12, 24 and 36 hours. The cells used as controls had been continuously passed in conventional medium; cells grown in D_2O had previously been passed daily in 30 per cent D_2O for one week. The results of duplicate determinations at each of these times were averaged in order to obtain an average cellular lipid value over the logarithmic phase of growth.

Monkey kidney cells were grown in Petri dishes. Monolayer cultures were prepared from primary trypsinized monkey kidneys[12]. Cell number was determined by trypsinizing cells 10 days after deuterium treatment or X-ray irradiation. Cell volume was found by centrifugation of known numbers of cells in 10 ml graduated centrifuge tubes.

X-ray irradiation was done by using a Keleket X-ray tube operating at 200 kV and 20 mA at a distance of 30 cm. No filters were used. The rate of irradiation was monitored and dosage was varied by altering the time of exposure to X-ray.

For lipid analysis[13] pooled cells, taken 7–8 days after treatment (25 per cent D_2O or 2000r X-ray), were collected, lyophilized and extracted

Table 1. Solvent systems used for chromatography of cell lipids on silicic acid

Fraction no.	*Solvent*	(ml)	*Lipid eluted*
1	Hexane–benzene 94:6	150	Hydrocarbons
2	Hexane–benzene 80:20	250	Sterol ester
3	Hexane–benzene 40:60	250	Triglyceride
4	Benzene	200	Sterol
5	Benzene–chloroform 25:75	250	Diglyceride
6	Chloroform	200	Monoglyceride
7	Chloroform–methanol 90:10	200	Phospholipid A
8	Chloroform–methanol 75:25	200	Phospholipid B
9	Chloroform–methanol 33:67	250	Phospholipid C
10	Methanol	—	

continuously in a Soxhlet extractor for 18 h using methylal–methanol 4 : 1. The lipid extract was taken to dryness under nitrogen at 50–60°. The lipid extracts were subjected to chromatography on activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.). The chromatographic scheme is presented[14] in *Table 1.* All solvents were reagent or spectroquality grade and when required were distilled prior to use. Squalene was determined by the method of Rothblat *et al.*[15]; sterols by the Mann modification[16] of the ferric chloride method of Zlatkis, Zak and Boyle[17]; glycerides by the method of Van Handel and Zilversmit[18]; free fatty acids by the procedure detailed by Albrink[19], and phospholipid phosphorus by the method of Fiske and Subbarow[20]. All data represent the average of at least three different experiments.

RESULTS

The inhibitory effect of large concentrations of deuterium oxide is well documented. The effects of varying concentrations of D_2O upon the yields of HeLa, L and L-5178Y cells is shown[21] in *Tables 2* and *3.* While even

Table 2. Effect of D_2O on yield of HeLa and L cells

% D_2O *in growth medium*	*Total yield of cells*†	
	HeLa	L
0	100	100
10	87	85
20	64	54
30	49	34
40	23	16
50	13	4

† As % of control.

Table 3. Effect of D_2O on mass doubling time, wet weight and dry weight of L-5178Y cells

D_2O *in growth medium* (%)	*Mass doubling time* (h)	*Wet wt.* (mg/10^6 cells)	*Dry wt.* (mg/10^6 cells)
0	11·5	0·71	0·12
5	12·3	0·75	0·12
10	13·0	1·20	0·15
20	14·2	1·20	0·18
30	19·0	1·50	0·15
40	29·0	1·50	0·15
50	56·0	—	

10 per cent D_2O will affect cell growth, concentrations as high as 30 per cent can be used and still yield reasonable cell concentrations.

Early experiments on the lipid composition of these cells (HeLa, L and L-5178Y) grown in D_2O indicated that the major change was an increase in total glyceride concentration[22]. A more careful study of the effects of 30 per cent D_2O upon the lipids of L-5178Y cells[14] showed that the major changes were increases in total glyceride and sterol ester content (*Table 4*).

Table 4. Lipid content (mg/g dry wt.) of L-5178Y cells grown in conventional or D_2O- containing (30%) medium†

Cell fraction	Medium	
	H_2O	30% D_2O
Squalene	3·37 ± 0·68[a]	2·74 ± 0·83
Cholesterol ester	5·02 ± 0·53	12·89 ± 1·94[c]
Free cholesterol	5·59 ± 0·25	7·12 ± 0·51[d]
Total cholesterol	10·60 ± 0·60	20·00 ± 1·40[e]
Triglyceride	1·80 ± 0·30	4·73 ± 1·11[d]
Diglyceride	3·88 ± 0·53	3·47 ± 0·64
Monoglyceride	1·73 ± 0·33	1·21 ± 0·30
Phospholipid[b] A	0·35 ± 0·05	0·30 ± 0·05
Phospholipid B	0·57 ± 0·04	0·61 ± 0·06
Phospholipid C	1·36 ± 0·07	1·47 ± 0·07
Total phospholipid	2·28 ± 0·06	2·38 ± 0·06

† All values represent the average of six determinations.
[a] Standard error of the mean.
[b] Expressed as lipid phosphorus.
[c] $0·01 > p > 0·001$.
[d] $p = 0·02$.
[e] $p < 0·001$.

A comparison of the effects of X-ray irradiation and D_2O (25 per cent) upon the size and volume of MK cells indicated that the average number of cells was reduced by 41·5 per cent after D_2O treatment, by 33·7 per cent after X-ray irradiation with 500r and by 68·9 per cent after 2000r. Average cell volume (ml $\times$ 10^{-8}) was increased by 48 per cent after D_2O treatment and by 218 per cent after X-ray treatment (2000r). These results are presented in *Tables 5* and *6*.

Analysis of the lipids of the control MK cells, the deuterium oxide treated cells and X-ray irradiated cells is detailed in *Table 7*. Both types of treated

Table 5. Effect of D_2O and X-ray irradiation on average number of cells in monkey kidney monolayers

Exp. No.	*Treatment*	*Average no. of cells*† (%)
1	Control	100
	D_2O (25%)	86·3
	X-ray 500r	78·1
	2000r	36·8
2	Control	100
	D_2O (25%)	43·9
	X-ray 500r	54·4
	2000r	27·2
3	Control	100
	D_2O (25%)	40·4
	X-ray 2000r	26·5
4	Control	100
	D_2O (25%)	63·3
	X-ray 2000r	33·8

† All values represent the average of six determinations.

Table 6. Volumes of monkey kidney cells 8 days after treatment with D_2O (25%) or X-ray (2000r)

Exp no.	*Treatment*	*No. cells per monolayer* ($\times 10^6$)	*Total cells* ($\times 10^7$)	*Vol.* (ml)	*Average cell vol.* (ml $\times 10^{-8}$)
1	Control	3·47	1·25	0·073	0·58
	D_2O	1·54	0·66	0·051	0·77
	X-ray	0·92	0·20	0·037	1·85
2	Control	3·08	1·09	0·058	0·53
	D_2O	1·95	0·34	0·030	0·88
	X-ray	1·04	0·24	0·041	1·71

Table 7. Lipids of control MK cells and MK cells grown in D_2O (25%) or X-ray irradiated (2000r)

	Control	D_2O	*X-ray*
% Lipid	35·3 ± 3·4[a]	49·4 ± 4·0	50·0 ± 15·3
Squalene (γ)	4·8 ± 0·9[a, b]	12·0 ± 1·4	7·6 ± 0·5[a]
Cholesterol ester (mg)	1·5 ± 0·7	2·8 ± 1·4	2·2 ± 1·1
Triglyceride (γ)	10·7 ± 0·1[a, b]	13·7 ± 0·3	5·1 ± 1·5[a]
Cholesterol (mg)	14·2 ± 0·8	14·9 ± 0·8	13·7 ± 2·1
Diglyceride (γ)	5·2 ± 0·2[a]	10·0 ± 1·6	6·2 ± 0·4[a]
Monoglyceride (γ)	1·2 ± 0·1[a, b]	7·5 ± 1·2	3·2 ± 0·6[a]
Phospholipid (mg)	0·14 ± 0·03	0·13 ± 0·02	0·13 ± 0·02
Total glyceride (γ)	17·1 ± 1·5	31·2 ± 0·7	14·5 ± 1·1

[a] Statistically different from D_2O.
[b] Statistically different from X-ray.

cells contained much more lipid than did the controls. The major portion of the lipid of all the cells was in the cholesterol and cholesterol ester fractions. Deuteration or X-ray treatment increased the squalene and cholesterol ester content of the cells. X-ray irradiation has been shown to increase cholesterol biosynthesis in rats[23–25] and D_2O has been shown to enhance cholesterol and fatty acid synthesis by mouse liver homogenates[26]. Our results indicate a similar enhancement of sterol synthesis in tissue culture cells. There was an increase in total cholesterol content of the cells but not of free cholesterol which may reflect differences in utilization or metabolism of this sterol This point requires further experimental clarification. The effect of D_2O upon exchange of cholesterol between cells and medium has not been determined. The total glycerides of the D_2O-treated cells were higher than those of the X-rayed or control cells. Both treated groups exhibited higher monoglyceride levels, the diglycerides of the control and X-rayed cells were equal and both were significantly lower than those of the deuterated cells. The triglyceride content of the D_2O-treated cells was higher than that of the controls which, in turn, was higher than that of the X-ray irradiated cells. These findings in the D_2O-treated cells are consistent with findings in the other tissue culture cell systems[14, 22] and may reflect increased fatty acid synthesis[26]. In the X-ray irradiated cells we may be observing either inhibition of the conversion of monoglycerides to triglycerides or enhancement of a lipolytic system. The former possibility is the most reasonable.

This provocative finding requires further investigation. No changes in phospholipid content were observed but the possibility that the spectrum of phospholipids may be different in the control and treated MK cells exists. Gas chromatographic analysis of the fatty acid composition of the cholesterol ester, glyceride and phospholipid fractions of the control, deuterated and X-rayed cells is under way.

The findings in MK cells treated with D_2O reflect results obtained in our earlier experiments[14, 22]. *Table 8* summarizes the changes in lipid composition observed in the experiments with MK and L-5178Y cells.

Table 8. Percentage change (from control) of lipids of D_2O grown and X-rayed cells

	D_2O *grown*		*X-rayed*
	L5178Y	MK	MK
Squalene	− 19	+150	+ 58
Cholesterol ester	+157	+ 87	+ 47
Free sterol	+ 27	+ 5	− 3
Total sterol	+ 89	+ 12	+ 1
Triglyceride	+163	+ 28	− 52
Diglyceride	− 11	+ 92	+ 19
Monoglyceride	− 30	+525	+175
Total glyceride	+ 27	+ 83	− 15
Phospholipid	+ 4	− 7	− 7

SUMMARY

Studies of two different tissue culture cell lines (L5178Y and MK) grown in medium containing 20–30 per cent D_2O show the following as compared with control cells:

(*a*) Increased generation time;
(*b*) Increased cell size;
(*c*) Increased total lipid content;
(*d*) Increased cholesterol ester and total glyceride, the glyceride increase being primarily in the triglyceride fraction;
(*e*) Little or no change in free cholesterol;
(*f*) No change in phospholipid.

X-ray irradiation (2000r) of the MK cells results in increased cell size and total lipid with major lipid changes being increased squalene, cholesterol ester, and monoglyceride; no changes in free cholesterol, diglyceride and phospholipid and a drop in triglyceride content.

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