Effect of Microwaves at X-Band on Guinea-pig Skin in Tissue Culture

2. Effect of the Radiation on Skin Biochemistry

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Small pieces of guinea-pig skin were exposed to a uniform field of microwaves at X-band (8,730 MHz). Measurements showed that 26% of the incident energy was reflected, 34% was absorbed, and the remaining 40% was transmitted. Absorbed energy was converted to heat, causing a rise in the temperature of the skin. After exposure to microwaves the skin was maintained *in vitro* on a nutrient medium. Uptake of radioactive substances from the medium into skin constituents was measured. A graded reduction in the uptake of sulphate ions into chondroitin sulphate, proline into collagen, and of phosphate into phospholipid, nucleic acid, and phosphoprotein fractions was found. The incident energy density causing 50% reduction of all these biochemical activities was approximately 4,750 mJ./sq. cm. under the thermal conditions of the experiment. The cooling rate of the tissue is important in determining the effect of microwaves.

In Part I an apparatus was described (Lawrence, 1968) for exposing small pieces of skin to microwaves of known power density at an X-band frequency of 8,730 MHz. The skin was heated by the radiation, and over an appropriate range of power density there was a graded reduction in respiration of the skin cells. This showed that metabolism was to some extent continuing in the affected cells. In previous work on the effects of heat (Lawrence and Ricketts, 1957; Carney, Lawrence, and Ricketts, 1962; Carney, Lawrence, and Ricketts, 1965) methods were developed for measuring various aspects of skin metabolism through the addition of radioactive tracer substances to the tissue culture medium. This paper reports on the uptake of ^{32}P -phosphate, ^{35}S -sulphate and ^{14}C -proline by the cells of skin exposed to microwaves and subsequently maintained *in vitro* on a nutrient medium.

Methods

Slices of guinea-pig ear skin were weighed and exposed to microwaves under sterile conditions as described (Lawrence, 1968). The skin slices were then incubated for 24 hours at 37°C. on a culture medium containing one of the following radioactive substances: $20\mu\text{C}$

^{32}P disodium hydrogen phosphate (Carney *et al.*, 1962), $150\mu\text{C}$ ^{35}S sodium sulphate (Lawrence and Ricketts, 1957), or $5\mu\text{C}$ ^{14}C -L-proline per 10 ml. of medium (Carney *et al.*, 1965). All measurements of radioactivity (see below) were related to the fresh weight of each skin slice.

Phosphate Skin slices were fractionated by the trichloroacetic acid method of Schneider (1945) into fractions containing phospholipids, nucleic acids, and phosphoproteins. The radioactivity of each fraction was measured using a Geiger counter for aqueous solutions.

Sulphate The cells in skin slices were killed by freezing to -79°C . ^{35}S -sulphate ions were removed by dialysis of the skin slice against sodium sulphate solution, and the remaining ^{35}S -sulphate was liberated by hydrolysis and precipitated as barium sulphate, as described by Lawrence and Ricketts (1957). Radioactivity was measured by a Geiger counter with a mica end-window.

L-Proline Skin samples were homogenized in ethanol to extract free proline and autoclaved to convert collagen to gelatine as described by Carney *et al.* (1965). The radioactivity of the gelatine extract was measured using a liquid scintillation counter and NE 220 scintillator.¹

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Measurement of Energy Absorbed by Skin An attempt was made to determine the amount of energy absorbed by skin exposed to microwaves at X-band. The instruments for measuring reflected and transmitted energy were arranged as shown in Fig. 1 of Part 1 (Lawrence, 1968). The area marked w-w on the specimen carrier (Fig. 2 of Part 1) was covered with skin; this was then inserted into the modified waveguide (Fig. 3 of Part 1) and exposed to microwaves at a total power of 0.2 watt. This relatively low power output was chosen to avoid undue heating of the skin whilst the amounts of power reflected and transmitted were measured. In practice about 95% of the area of the waveguide was covered with skin; if the specimen is taken up to the edge of the waveguide there is a possibility of causing a short circuit.

Results

In the experiments on the absorption of microwave energy by skin slices it was found that 26% of the incident energy was reflected by standard slices of about 0.15 mm. thickness, a further 34% was absorbed by the skin, and the remaining 40% was transmitted. Filter paper moistened with saline, thickness 0.125 mm., behaved similarly to skin in respect of electrical properties in the waveguide; 20% of the power was reflected and 28% was absorbed. By building up several layers of filter paper it was found that three layers was the limit of penetration; rather more than 30% of the power was then being absorbed and the remainder reflected. Measurements with a copper-constantan thermocouple placed under specimens of skin, or wet filter papers, in the waveguide indicated that these rapidly heated, reaching 100°C., if sufficient power was available, and remained at this temperature until the specimen had dried.

Mr. J. Roberts, of the Royal Radar Establishment, Malvern, carried out experiments using the same apparatus with filter paper moistened with saline to simulate skin in the waveguide. In general, temperature rise is given by the equation

$$\theta = \frac{A}{B} \left[1 - \exp(-Bt) \right]$$

where θ = temperature rise

A = heating rate °C. per second, proportional to microwave input power

B = cooling coefficient °C./sec./°C. temperature rise

t = time in seconds

For 1 watt per sq. cm., A was found by experiment to be 3.4°C. per second. For the sample mounted in the waveguide B was found to be 0.15 sec.⁻¹. The thermal time constant is defined as 1/B and is

therefore approximately 6 seconds. The maximum time used in the experiments with skin was 4 seconds; during this period the temperature rise of the specimen is approximately linear with time. For a specimen in free air, B was found to be 0.36 sec.⁻¹, the higher value of the cooling coefficient being attributable to unrestricted cooling when the specimen was not enclosed in the waveguide.

The incorporation of ³²P from orthophosphate into phospholipid, nucleic acid, and phosphoprotein fractions from guinea-pig skin exposed to microwaves was measured. Seven skin explants were exposed at each energy density and a further seven were unexposed controls. The mean radioactivity and standard error of the mean are shown in Table I. Exposure to microwaves resulted in a reduced incorporation of ³²P into all fractions. This reduction was statistically significant at the higher powers as shown in the Table. For convenience the mean value for the controls was taken as 100% incorporation of ³²P, and other values were expressed as percentages of this.

As explained in Part 1, exposure times were 1, 2 or 4 seconds at 970, 1940 or 3890 mW./sq. cm. giving the energy densities shown in Table I in millijoules per square centimetre.

Skin in tissue culture takes up ³⁵S-sulphate from the medium and incorporates it into chondroitin sulphate and other polysaccharide sulphates (Barker, Cruickshank, and Webb, 1965). Fixation of sulphate in non-dialysable form by skin exposed to microwaves was measured. Table II shows the radioactivity due to ³⁵S fixed in skin and resistant to dialysis. Exposures were made over a wider range of energy densities than in the phosphate experiment, and a larger number of skin explants was used. Mean values and their standard errors are shown in Table II. Differences from the control values for unexposed skin were highly significant. There was a graded reduction in sulphate uptake with increasing energy density.

¹⁴C-L-proline in the tissue culture medium is taken up by skin cells and incorporated into newly synthesized collagen (Carney *et al.*, 1965). The radioactivity of a gelatin extract provides a measure of the radioactivity of the collagen in skin. Table III shows the radioactivity of gelatin extracts from skin exposed to microwaves at various power densities. At the three highest power densities there was a significant reduction of proline uptake into collagen. At the lowest power density there appeared to be a stimulation of proline uptake, but this was not statistically significant.

The release of soluble substances from skin slices, total weight 25 mg., exposed to microwaves of X-band at a power density of 7,780 mW./sq. cm.

TABLE I
PHOSPHATE UPTAKE BY VARIOUS FRACTIONS FROM SKIN EXPOSED TO MICROWAVES IN THE X-BAND

	<div>o (controls)</div>	Energy Density (mJ./sq. cm.) 3,880 7,760 15,560		
Phospholipids				
Mean count per min./mg. fresh weight	38.4	23.0	8.34	2.78
± S.E.M.	± 5.02	± 5.43	± 1.76	± 0.98
% uptake relative to control	100	59.9	21.7	7.24
Statistical comparison with control	—	NS	S	S
		0.05 < P < 0.1	P < 0.001	P < 0.001
Nucleic acids				
Mean count per min./mg. fresh weight	92.0	60.7	33.4	11.5
± S.E.M.	± 5.5	± 15.0	± 6.6	± 1.8
% uptake relative to control	100	65.4	36.3	12.5
Statistical comparison with control	—	NS	S	S
		0.05 < P < 0.1	P < 0.001	P < 0.001
Phosphoproteins				
Mean count per min./mg. fresh weight	157.5	93.7	41.1	10.5
± S.E.M.	± 8.4	± 21.0	± 9.7	± 2.9
% uptake relative to control	100	59.5	26.1	6.66
Statistical comparison with control	—	NS	S	S
		0.01 < P < 0.02	P < 0.001	P < 0.001

TABLE II
INCORPORATION OF SULPHATE BY SKIN SLICES EXPOSED TO MICROWAVES AT X-BAND

	o (controls)	Energy Density (mJ./sq. cm.)				
		970	1,940	3,880	7,760	15,560
Counts per minute/mg. fresh weight	24.5	21.1	19.9	13.8	5.8	2.2
± S.E.M.	1.5	1.6	1.0	1.1	1.0	0.2
No. of explants	19	4	7	19	15	11
% uptake relative to control	100.0	86.0	81.0	56.0	23.0	9.0
Statistical comparison with control	—	NS 0.2 < P < 0.3	NS 0.05 < P < 0.1	S P < 0.001	S P < 0.001	S P < 0.001

TABLE III
PROLINE UPTAKE INTO COLLAGEN OF SKIN EXPOSED TO MICROWAVES AT X-BAND

	o (controls)	Energy Density (mJ./sq. cm.)				
		970	1,940	3,880	7,760	15,560
Counts per minute/mg. fresh weight	1235	1363	1074	397	242	85
± S.E.M.	± 155	± 474	± 384	± 69	± 96	± 54
No. of explants	13	3	6	13	9	6
% uptake relative to control	100	110	86.9	32.1	19.6	6.9
Statistical comparison with control	—	NS	NS	S P < 0.001	S P < 0.001	S P < 0.001

FIG. 1. Ultra violet light absorption spectrum of saline solution (1 ml.) in contact for 5 minutes with guinea-pig skin (25 mg.) immediately after exposure to microwaves in X-band at an energy density of 7,780 mJ./sq. cm. Control skin not exposed.

was investigated by transferring the exposed skin to 1 ml. of saline for 5 minutes to allow diffusion from the skin to occur. After removal of the skin the ultra violet absorption spectrum of the solution, shown in Fig. 1, was measured. A control experiment was performed in exactly the same way with skin which had not been exposed to microwaves. One of the effects of exposure to microwaves appears to be the release of soluble and rapidly diffusible substances with an ultra violet light absorption maximum at 265 mμ, possibly related to the 'burn toxin' released from skin by heating (Jones and Lawrence, 1964).

Discussion

Skin cells in tissue culture respire and take up nutrients from the medium. Radioactive substances in the medium become incorporated into skin constituents; measurement of the radioactivity provides a measure of their formation. Phosphate is incorporated mainly into intracellular substances. Low molecular weight phosphate esters are difficult to separate from the large amount of ^{32}P inorganic phosphate present, and it was not found practicable to make a measurement representative of these. Macromolecules containing phosphate are more easily separated from inorganic phosphate, and measurements of the incorporation of phosphate into fractions representative of the main types, phospholipid, nucleic acids, and phosphoproteins,

have been made. As Table I shows, exposure to microwaves reduced the incorporation of phosphate into these substances and similar results were obtained for these three macromolecules.

Some of the sulphate taken up by skin cells enters the intracellular adenosine pyrophosphate sulphate, from which it is transferred to the mucopolysaccharide sulphates (Brimacombe and Webber, 1964), heparin, the chondroitin sulphates, and keratosulphate. These are extracellular substances of high molecular weight and consequently do not diffuse out during dialysis of the skin to remove ^{35}S -inorganic sulphate. Chondroitin sulphate labelled with ^{35}S has been recovered from skin treated in this way (Lawrence, 1961). Thus the ^{35}S found in skin provides a measurement of the formation of one group of extracellular substances, the mucopolysaccharide sulphates. It was found that exposure to microwaves reduced the formation of mucopolysaccharide sulphates. This measurement was much the easiest to make and the most reproducible of those reported in this paper.

L-proline is taken up from the medium by skin cells in tissue culture and incorporated into various proteins but particularly into collagen which contains a high proportion of this amino acid. Some proline is converted to hydroxyproline, an amino acid characteristic of collagen. In previous work we have demonstrated the appearance of ^{14}C -L-hydroxyproline in collagen from skin in tissue culture (Carney *et al.*, 1965). Collagen, which is an insoluble intracellular constituent of skin, becomes labelled with ^{14}C . Autoclaving converts collagen to gelatin which on account of its solubility is readily removed from skin by repeated extraction (Carney *et al.*, 1965). Thus measurements of the radioactivity of gelatin extracts provide an indication of the incorporation of ^{14}C -L-proline into skin collagen. As Table III shows, exposure to microwaves reduced proline incorporation into collagen. At minimal exposures there was an apparent stimulation of proline uptake. Although this was not statistically significant, it is of interest since minimal exposures to laser light beams (Carney, Lawrence, and Ricketts, 1967) or to heat (Carney *et al.*, 1965) have shown a similar stimulation.

Previous work on the effect of thermal damage on the uptake of these radioactive substances by skin in tissue culture showed that when the percentage uptake was plotted against the temperature (37° to 47°C. maintained for 30 minutes) a sigmoid curve was obtained (Lawrence and Ricketts, 1957; Carney *et al.*, 1962; Carney *et al.*, 1965). A possible explanation for the shape of such a curve is that the

population of skin cells has a normal or Gaussian distribution of sensitivity to heat damage. Such a curve may be converted to a straight line by plotting the percentage on a probability scale. In more recent work on the effects of a laser beam (notional temperatures of the order of $1,800^{\circ}\text{C}$. for times of the order of a millisecond) on skin it was found that these percentages were approximately linear when plotted on a probability scale against the logarithm of energy density in joules per square millimetre. The particular value of a straight line relationship is that it enables the energy density corresponding with a 50% effect to be determined more accurately. In this work with microwaves it was logical therefore to plot percentages on the probability scale against energy density in millijoules per square centimetre.

Figure 2 shows the effect of microwaves on phosphate uptake into the phospholipids, nucleic acids, and phosphoproteins of skin cells plotted in this way. The energy density for 50% effect is seen

to be close to $4,750 \text{ mJ./sq. cm.}$, taking all the observations together. Similarly, in Fig. 3 the effect of microwaves on sulphate uptake into inter-cellular polysaccharide sulphates is shown. Four of the points are very close to the line, and it may be seen that the energy density corresponding with 50% effect is $4,370 \text{ mJ./sq. cm.}$ In the case of proline uptake the experimental points (Fig. 4) are more widely distributed, but nevertheless the 50% effect clearly occurs at a similar energy density. In Part I the respiration measurements showed 50% reduction at $4,750 \text{ mJ./sq. cm.}$ This then is about the energy density for 50% reduction of all these biochemical activities under the thermal conditions of experimental exposure in a waveguide.

If the exposures to microwaves had been made in free air, where cooling was unrestricted, there is reason to believe that the microwave doses would be approximately doubled for the same biological effect.

In applying this information *in vivo* the efficient

Fig. 2

Fig. 3

Fig. 4

FIG. 2. Phosphate uptake into fractions from guinea-pig skin exposed to microwaves. Percentage phosphate uptake relative to unexposed control skin plotted on a probability scale against the logarithm of energy density in mJ./sq. cm. Exposure times 1 to 4 seconds. Seven explants exposed in each energy density.

● phospholipid fraction; ○ nucleic acid fraction; × phosphoprotein fraction. The point of 50% reduction is at $4,750 \text{ mJ./sq. cm.}$

FIG. 3. Sulphate uptake into polysaccharide sulphates of guinea-pig skin exposed to microwaves. Percentage sulphate uptake relative to unexposed control skin plotted on a probability scale against the logarithm of energy density in millijoules per square centimetre. Exposure times 1 to 4 seconds. Numbers in brackets are the numbers of skin explants exposed at each energy density. The point of 50% reduction is at $4,376 \text{ mJ./sq. cm.}$

FIG. 4. L-Proline uptake into collagen of guinea-pig skin exposed to microwaves. Percentage uptake relative to unexposed control skin plotted on a probability scale against the logarithm of energy density in millijoules per square centimetre. Exposure times 1 to 4 seconds. Compared with Figs 2 and 3 experimental points are more widely distributed but consistent with 50% reduction at about $4,750 \text{ mJ./sq. cm.}$

heat transfer provided by circulating blood and the physiological mechanisms for body cooling must also be taken into account since clearly these play an overwhelming part in considerations of total thermal load on the body. However, in tissues with relatively poor circulation, notably in the lens of the eye, the thermal experience of cells responsible for maintaining transparency may be nearer to that of cells in isolated skin described in this paper. Although microwaves at X-band seem unlikely to reach the lens in appreciable intensity, since their penetration appears to be limited to about 0.5 mm. depth, microwaves of longer wavelength undoubtedly do so.

The absorption of microwave radiation is critically dependent on the physical condition of the specimen. Thus, if and when the surface layer becomes dried, it ceases to absorb and the energy penetrates to the next layer. This process is continuous and could result in damage to a comparatively great depth if the radiation were intense and prolonged.

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