Search for Millimeter Microwave Effects on Enzyme or Protein Functions

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Recent observations of nonthermal, resonant biological responses to weak millimeter microwave irradiation have led us to investigate whether similar influences exist on enzymatic functions *in vitro*. We chose (i) the reduction of ethanol in the presence of alcohol dehydrogenase and (ii) the cooperative binding of oxygen on hemoglobin. Using an irradiation intensity near 10 mW/cm² the frequency was continuously varied from 40 to 115 GHz with a resolution of a few MHz. No microwave influences were detectable within our experimental sensitivity of about 0.1% of the reaction rate in (i), or of the amount of bound oxygen at half saturation in (ii).

Millimeter microwave irradiation has been reported to influence a variety of organisms in a most striking manner [1–5]. For example changes of metabolism and growth rate were seen, of both positive and negative sign, which depended strongly on the frequency. The irradiation intensity was thereby very weak (<10 mW/cm²). In some cases heating effects were clearly ruled out as a possible origin of the irradiation effects. Specific absorption of biological entities has however not been found at millimeter wave frequencies, probably because of the strongly dominating water absorption [6] in this region. In view of the possibility that millimeter microwaves might specifically interact with subunits of living organisms, we investigated here whether such irradiation had an influence on the function of enzymes and proteins *in vitro*.

Alcohol dehydrogenase is a well-known enzyme [7] with a molecular weight of approximately 144 000. In our experiment, it catalyzes the reaction of ethanol with NAD^+, resulting in acetic aldehyde and NADH. NAD^+ and NADH are the oxidized and reduced forms, respectively, of the coenzyme nicotinamide-adenine-dinucleotide. The molecular weight of NAD^+ is 656. NADH shows a strong UV absorption band centered at 340 nm where NAD^+ is transparent, so that measuring at 340 nm provides a convenient way of monitoring the progress of the reaction [8].

We employed a constant-flow technique out of two thermostatted flasks (25 °C) containing the two reagents. These were (a) 60 ml ethanol added up to 1 l with 0.05 m sodium pyrophosphate buffer of pH 10.5, and (b) 0.5 g/l NAD^+ (Sigma) and 7 to 50 mg/l (depending on the flow rate) yeast alcohol dehydrogenase (Boehringer) in the same buffer but at pH 8.5. A common peristaltic pump was employed and mixing of (a) and (b) occurred at a T-junction provided in the silicone rubber tubing of 0.5 mm inner diameter. Here the reaction started and the coenzyme was converted from the non-absorbing to the absorbing form, at the high rate of approx. 400 μ/mol per min per mg enzyme under the given saturating condition. 10 cm downstream, the solution entered a measuring cuvette constructed of black plexiglass where the flow expanded along a taper section into a rectangular cross section of 1 cm × 0.6 mm, for a length of 1 cm, after which it was compressed back again. The UV transmission was measured by looking sideways through this straight section, *i. e.* the UV beam passing through quartz windows had an aperture of 1 cm × 0.6 mm and a pathlength of 1 cm in the liquid. We were thus observing a volume of 0.06 cm³ which was separated from the point of mixing by a volume of about 0.05 cm³. The flow rate was varied between 0.02 and 0.4 cm³/sec giving characteristic sample exchange times between 3 and 0.15 sec. The optical employed a 70 W incandencent lamp system which after filtering was imaged through the measuring cuvette. For filtering we used a combination of a 1 cm saturated $CuSO_4$ solution, a blocking filter UG 11 (Schott) and an interference filter at 337 nm with a bandwidth of 3 nm (Oriel). The transmitted light was

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imaged on a silicon photodiode. This signal showed a monotonic decrease from its initial value S_1 when, at a given flow rate, more and more enzyme was introduced in solution (b). Conditions were usually set so that a constant signal of about $S_1/2$ was observed. The noise on the photodiode signal, about 10^{-3} to $10^{-4}\,S_1$ in a 10 Hz bandwidth, was dominated by effects connected to the liquid motion, probably Schlieren effects. Detector and source noise together were only $2\times10^{-5}\,S_1$.

Microwaves were now brought to the measuring cell to irradiate the 1×1 cm^2 surface of the straight section at normal incidence. Since the absorption coefficient of water varies between 45 cm^{-1} at GHz and 90 cm^{-1} at 120 GHz [6], a high attenuation factor between 15 and 220 occurs for this frequency range in the 0.6 mm thick water layer. The microwaves were generated in backward wave tubes tunable either from 40 to 60, 60 to 90 or 75 to 115 GHz (Siemens), which were driven from a model 702/703 C sweep power supply (Micro-Now). Standard rectangular waveguide about 40 cm in length was used to connect the tube to the experiment. The waveguide contained a 20 db (1%) coupler for power monitoring purposes. Just before the measuring cuvette the guide was tapered up to a 1×1 cm^2 cross section and fixed to the plexiglass wall. The latter had a thickness of 3 mm and was highly transparent to microwaves. According to the tube's supplier, up to 80 mW of power can be generated in the central regions of the tuning ranges, so that with the strong reflection at the plexiglass/water interface the absorbed power in the liquid can be estimated to have been between 10 and 50 mW throughout the spectral range investigated. To estimate the upper limit of microwave heating we assume 80 mW and thus get 1.3 W/cm^3 absorbed power density in our sample. At the slowest liquid flow rate used, corresponding to an integration time of 3 sec, this amounts to a maximum possible heating by 0.9 °C. In order to better distinguish possible microwave effects we periodically switched the power on and off, at a frequency corresponding to the liquid exchange time in the cuvette, and detected the synchronous component of the diode signal using a PAR 124 A lock-in amplifier set at a time constant of 1 sec. For each irradiation period we thus assured a fresh sample to be present. The microwave frequency could be tuned by hand or slowly swept electronically. At least two runs were made for each frequency range, where the liquid exchange rate and the chopping rate were 2.5 sec^{-1}, and the microwave tuning rate was only 3.5 MHz/sec. No effect could be observed on the noise background which amounted to less than $6\times10^{-4}\,S_1$ in each case. The conditions were thus that even a very narrow-band resonance of 10 MHz width [4] would not have escaped our attention. We conclude from this experiment that the radiation could not have changed the turnover rate of alcohol dehydrogenase by more than 0.1%.

Hemoglobin and its function of oxygen transport have been extensively investigated [9 – 11]. It has a molecular weight of 67 000 and consists of four subunits. Each of these can bind an oxygen molecule, and the affinity of binding at a given subunit depends on the oxygen binding status of the other three subunits (cooperativity), as is manifested in the shape of the oxygenation curve. Visible red light absorption provides a convenient means of measuring the amount of bound oxygen. For example, at 633 nm the absorption coefficient of human hemoglobin at full oxygenation is only 73% of that in the deoxygenated state.

In our experiment we used human hemoglobin in its half-oxygenated status. While the oxygen partial pressure (approx. 30 Torr) was kept constant we tried to find out whether microwave irradiation could change the amount of bound oxygen. Starting from citrated blood, erythrozytes were first prepared by centrifugation ($15\,000\times g$, 15 min) and washing in sodium chloride solution, then lysed by dialysis against water (24 h). Then the stroma was removed by centrifugation ($48\,000\times g$, 20 min) and the resulting solution containing about 70 g/l hemoglobin was equlibrated to 0.05 m sodium phosphate buffer at pH 7.0 by dialysis. 30 ml of this solution was then constantly stirred in a thermostatted (25 °C) flask, with one drop of octanol added to prevent foam development. Either nitrogen or air could be bubbled through the solution in order to change the partial pressure of dissolved oxygen. Measurements were done in a thermostatted cuvette (25 °C) providing a pathlength of 0.5 mm and a cicular aperture of 2 cm in diameter, with 4 mm thick sapphire windows. One of the windows had two bores to adapt liquid flow in and out through silicone rubber tubing of 0.75 mm internal diameter. A closed-cycle flow connecting to the oxygenation flask was activated by a peristaltic

pump. The optical absorption was measured in the same setup as in the enzyme experiment before. This time a combination consisting of a blocking filter OG 519 (Schott) and an interference filter at 633 nm with a bandwidth of 3 nm (Oriel) was used. The light beam was inclined a small angle (10 °C) away from the normal on the sapphire windows and the beam aperture was 7 mm at the cuvette. The detector signal could now be observed as the hemoglobin solution was constantly pumped through the cuvette. It was seen to decrease by about 10% as the oxygen was admitted to the flask and vice versa, with full reversibility. Let in the following S_2 denote the signal change corresponding to this step. To prepare half-oxygenated hemoglobin we stopped the bubbling when a change of $S_2/2$ was reached. After experimentation we regularly checked whether the system had possibly drifted away from this status, by further oxy- and deoxygenating procedures. From tis we found it necessary to keep a flow of about 0.08 cm^3/sec through the measuring cuvette, since for a resting solution some slow changes of the oxygenation status were observed in the cuvette. Under these conditions the noise on the detector signal was again dominated by flow effects, most probably from some turbidity due to denaturated hemoglobin. In the 3 Hz bandwidth used here the noise varied from 5×10^{-4} to $2 \times 10^{-3}\, S_2$ for the different runs.

Microwaves were applied using a similar waveguide as above, but with the taper section (horn) leading to a final 2×2 cm^2 cross section, so that the irradiated area was four times that of the preceeding enzyme experiment. The horn was also inclined at 10 °C from the normal, but in the direction opposite to that of the red light beam. The latter in fact passed through one side wall of the horn which was made out of thin metal mesh having 8 wires per mm, thus providing 100% reflectivity for the microwaves. The microwave reflectivity at the sapphire surface is about 25%. Since the absorption in sapphire is negligible, we can estimate the absorbed intensity reaching the hemoglobin solution to be between 3 and 13 mW/cm^2 throughout the spectral region investigated. Maximum microwave heating could have been 20 mW/cm^2 corresponding to 0.4 W/cm^3, which could from a simple heat conduction estimation have led to a maximum temperature difference of only 0.03 °C between solution and sapphire. The irradiation experiment was now performed again by modulating (at 0.5 sec^{-1}) and synchroneous detection. No effects were seen in the noise limits stated. We furthermore tried a much higher microwave modulation frequency of 600 sec^{-1} restricting thus the experiment to probe irradiation effects which were reversible within a millisecond. Although the noise level was thereby greatly reduced to $10^{-5}\, S_2$ no effect was detected in the range from 60 to 90 GHz. Altogether, we thus conclude that the irradiation is not able to shift the equilibrium oxygen constant of half-oxygenated hemoglobin by more than 0.4%.

We can not conclude from these two *in vitro* experiments that millimeter microwaves could not at all change the function of the two molecules tested, since we do not know the amount of possibly nonlinear amplification with which such changes could manifest themselves in an active biological environment. It might well be that *e. g.* the 20% growth rate changes of yeast observed in [4] originate frora much smaller changes in some key molecules or processes. In this view it might bc advisable to repeat the experiments at increased signal-to-noise ratios. A longer exposure time seems, however, not necessary since we feel a small system like an isolated macromolecule should adapt to new environmental conditions well within one second. We might further speculate that possible interaction frequencies of the molecules tested exist but lie outside our present frequency range (40 to 115 GHz), possibly above. Larger molecules or entities might then have resonance frequencies in our range and should be candidates for future testing. This view is supported by the theoretical concept of H. Fröhlich [12] which tends to give larger biological entities a greater likelihood to be susceptible to irradiation effects, because of their greater likelihood to make use of (microwave frequency) oscillations for biological control purposes. Practical suggestions of such interdisciplinary cooperation will be most welcome.

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