Enkephalin-like immunoreactivity in human gastrointestinal tract

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5 AUTHORS, INCLUDING:

Stephen Sullivan
Vancouver Island Health Authority
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round granules with a mean diameter of approximately 260 nm and a closely attached membrane, which thus were considerably smaller than those of the N cell. The VIP-positive cells varied in shape and contained much smaller granules with a mean diameter of 120 nm and a closely attached membrane.

Thus neurotensin is confined to a single type of endocrine cell which has previously been overlooked in ultrastructural studies because of confusion with the EG(L) cells[21]. With the application of electron immunocytochemical methods it can now be seen that the cell contains granules of a type distinct from those of the other endocrine cells in the ileal mucosa.

Neurotensin immunoreactivity was detected by radioimmunoassay in high concentration in the ileal mucosa with lesser quantities in the jejunal mucosa (Table 1). Very much smaller quantities were found in whole thickness extracts of the wall of the stomach, duodenum and colon. It was undetectable in the pancreas and gall bladder.

Table 1 Neurotensin immunoreactivity in various tissues

	Neurotensin (pmol per g wet weight)
Stomach (6)* full thickness	0.27±0.03
Duodenum (7) full thickness	0.24±0.10
Jejunum full thickness (2)	0.80 (1.19, 0.42)
mucosa (3)	2.82 (4.62, 1.78, 2.07)
Ileum full thickness (2)	12.0 (7.57, 16.4)
mucosa (3)	16.2 (23.1, 19. 0, 6.5)
Colon (4)	0.50±0.19
Gall bladder (3)	< 0.03
Pancreas (2)	< 0.03

Values are means ± s.e.m.
*No. of determinations.

Neurotensin was originally extracted from the hypothalamus[1], and its function there is no more clear than in the gastrointestinal tract. The morphological features of the neurotensin cells, which have microvilli and secretory granules located at the vascular pole, suggest they are sensitive to changes in the gut lumen which may thus provide the stimulus for the release of the peptide, presumably, because of the basal distribution of the neurotensin granule, directly into the circulation. This view is supported by the finding of neurotensin in rat plasma[11]. The restricted localisation of the peptide suggests that it may play a precise part in the sequence of post-digestive processes.

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JULIA M. POLAK
S. N. SULLIVAN
S. R. BLOOM
A. M. J. BUCHAN
P. FACER
M. R. BROWN
A. G. E. PEARSE

Departments of Histochemistry and Medicine, Royal Postgraduate Medical School, London W12, UK and Laboratories for Neuroendocrinology, The Salk Institute, San Diego, California

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Hydration effects on the photocycle of bacteriorhodopsin in thin layers of purple membrane

THE purple membrane of *Halobacterium halobium* acts as a light-driven proton pump, producing a transmembrane proton gradient which is coupled to ATP synthesis[1], and to phototaxis[2] in the intact bacteria. It contains a single type of protein, bacteriorhodopsin (BR) which spans a 45-Å membrane. The isolated purple membranes are flat oval sheets with an average diameter of 0.5 μm (refs 3, 4). Bacteriorhodopsin contains a retinal molecule (all-*trans* and 13-*cis*)[5] which is covalently bound via a protonated Schiff base to a lysine residue. It undergoes a photocycle described by the following scheme[6–8]:

$$BR^{570}_{\text{all-}trans} \rightarrow \text{'590'} \rightarrow \text{'550'} \rightarrow \text{'412'} \ (\text{'660'})$$

where proton ejection to the bulk solution occurs on the route '550' → '412' (refs 9, 10), whereas protonation of the bacteriorhodopsin takes place parallel to the '412' → $BR^{570}_{\text{all-}trans}$ process[11]. It has been reported that the reconstituted $BR^{548}_{13\text{-}cis}$ undergoes a cycle which involves the 'X' and the '610' intermediates[12]. It was demonstrated that proton transfer is a vectorial process where the proton is ejected from one side of the purple membrane and reprotonation takes place on the other side[13]. We present here results on the effects of the specific hydration of the purple membrane on the relaxation times of '412' and on the formation of the '660' and '610' intermediates. The results demonstrate that the full photocycle of bacteriorhodopsin can be observed in thin purple membrane layers even at the lowest hydration state and that the amount of absorbed water is rate limiting for the molecular process of the cycle.

Purple membranes were isolated from *H. halobium* (mutant NRL R_1M_1)[5]. Thin layers of purple membrane were prepared by drying concentrated suspension (3.5×10^{-4}M) of the purple membrane in water (*p*H 7.2), on a glass slide. The drying was performed at atmospheric pressure, 45% relative humidity and 25 °C. The average thickness of the preparations was 1–3 μm as determined by scanning electron microscopy. Variable degrees of hydration of the purple membrane were obtained by equilibrating the samples with different relative air humidities produced by saturated salt solutions[14]. The glass slide with the preparations was inserted in a 1×1 cm cuvette and incubated in a desiccator at the required specific humidity for about 24 h. Before measurement the cuvette was immediately sealed within the desiccator with a Teflon plus Parafilm cover. The limit of dryness, obtained by drying the membrane at 10^{-3} torr for approximately 4 h, was defined as the 0% relative humidity state.

Flash photolysis spectroscopy of thin-layer preparations of purple membrane show that bacteriorhodopsin undergoes a complete photocycle, involving the same intermediates characteristic of suspended purple membrane fragments in water. But the decay time of the '412' intermediate as well as the formation of '660' and '610' intermediates are determined by the specific hydration state of the membrane. Preparations equilibrated with 94% relative humidity show the same kinetics and

include the same transients as in purple membrane fragments suspended in water (Table 1). The relaxation times for '412' decay are slowed down by lowering the hydration state of the preparation (Fig. 1). The kinetics of '412' decay are analysed in terms of the sum of two or three exponentials (Fig. 2). The relaxation times and their relative amplitudes, at different hydration states, are given in Table 2. The initial values for the relaxation times and their corresponding amplitudes were obtained first graphically and then were optimised by a least square program (Harwell Subroutine Library, VCO5A). Essentially the same results were obtained when the initial values were calculated by a computer program (K. H. Mueller and T. Plesser, unpublished), within the frame of a non linear approximation[15,16]. The thermal decay kinetics of '412' could be enhanced by a factor of 15 when photobleached with 412-nm light at 0% relative humidity, in accordance with earlier experiments carried out with suspended purple membrane[17].

The '660' and '610' intermediates are not formed at relative humidity lower than 90%. Moreover, the 660-nm intermediate cannot be detected at *p*H higher than 8 (in a solution of 4M NaCl), or when suspending purple membrane in water–glycerol mixtures. In addition it has been shown that the formation of the '660' intermediate is temperature dependent[18]. Thus, the formation of this intermediate can serve as an internal probe of bacteriorhodopsin to environmental conditions, such as temperature, *p*H or relative humidity. The hydration effects were found to be fully reversible and each sample was recycled through the various hydration states several times without noticeable irreversible changes.

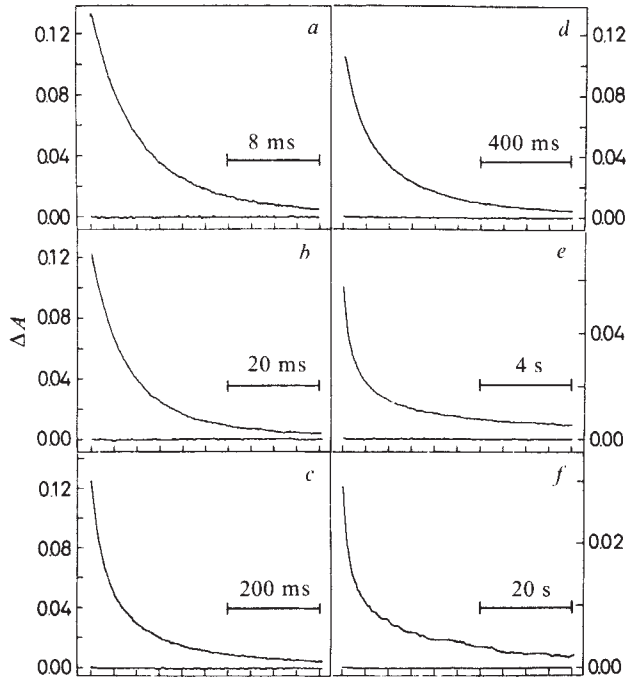

Fig. 1 Oscilloscope traces obtained in flash photolytic measurements of thin purple membrane layers, kept in various hydration states. The traces represent the decay kinetics of '412' nm intermediate formed by a linearly polarised light pulse from a rhodamine 6G laser at 585 nm of 1 μs duration. The samples were excited at right angles to the analysing light, where the plane of the thin purple membrane layers formed an angle of 45° both with the exciting and the analysis light axes. The amplified output of the photomultiplier passed through a variable RC filter to a digital scope, where the filter setting was such as to provide filter relaxation time equal to 0.05% of the oscilloscope's total time sweep width. The transient changes of '412' were recorded directly in absorbance units by the use of a lin-log converter. The flash experiments were performed with thin purple membrane equilibrated with the following relative humidities: *a*, 94%; *b*, 90%; *c*, 83%; *d*, 75%; *e*, 43%; *f*, 7%.

Table 1 Relaxation time constants (τ_i) of the '412', '660' and '610' intermediates

Intermediate	Purple membrane in water* (τ, ms)	Thin purple membrane layers† (τ, ms)
'412'	1.7±0.6 (0.21±0.13) 5.0±0.8 (0.79±0.13)	1.8±0.2 (0.3±0.07) 5.4±0.9 (0.69±0.07)
'660'	5.4±0.4	5.6±0.6
'610'	48±2.1	46.0±3.4

The relaxation time constant is defined by $A(t) = A_i e^{-t/\tau_i}$, where the absorbance change is the sum of exponential terms. The relative amplitudes are given in brackets near the corresponding relaxation time constants. The experiments were done at room temperature (22±1 °C).

*Suspended purple membrane in water at *p*H 7.2.

†Thin purple membrane preparation equilibrated with 94% relative humidity.

The results show that the amount of adsorbed water on the purple membrane determines the relaxation times of the '412' decay. The changes in hydration cause a change of the relaxation times over four orders of magnitude (Table 2). It should be added that the rate of the photoactivated formation of the intermediates in the pathway leading to '412' is only changed to a minor degree as a function of the state of hydration. The observed three relaxation times suggest that bacteriorhodopsin undergoes either a three-step conformational change along with the '412' → BR^{570} transition or that '412' populates three conformations which decay to that of BR^{570}. A similar interpretation was suggested earlier for the biphasic decay of '412' (refs 17, 19).

These effects could in principle be accounted for by a rate-limited diffusion of protons due to the amount and structure of the conducting water layers on the purple membrane or by a reversible conformation change induced by the hydration

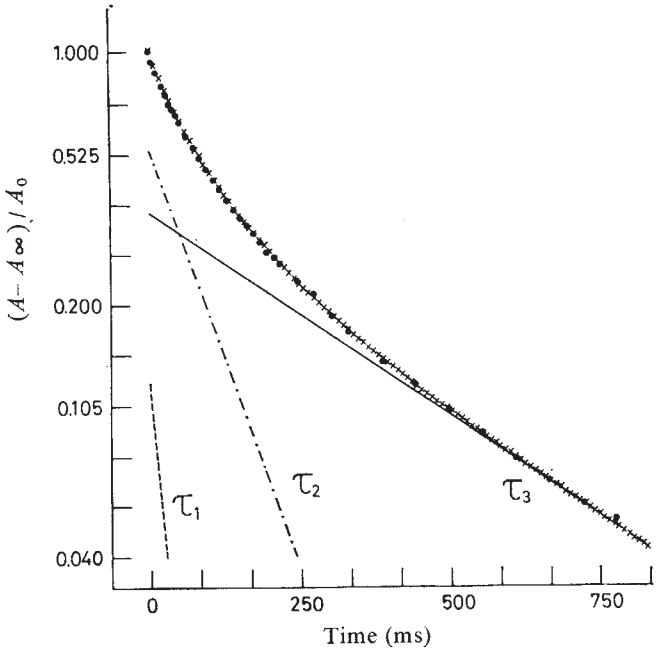

Fig. 2 Evaluation of data from Fig. 1*d*. The transient absorption $A(t)$ is expressed as a sum of exponentials by $A(t) = A_i e^{-t/\tau i}$, where A_i is the amplitude of the τ_i relaxation time constant. Closed circles represent the original data points and crosses represent the calculated data points using τ_1, τ_2 and τ_3 as the relaxation time constants.

Table 2 Relaxation time constants and amplitudes of the decay kinetics of '412' intermediate in thin layer preparations of purple membrane equilibrated in various relative air humidities

Relative air humidity (%)	τ_1	τ_2	τ_3
90	3.5 ± 0.4 ms; (0.63 ± 0.05)*	13.1 ± 1.1 ms; (0.37 ± 0.03)*	—
83	10.2 ± 2.4 ms; (0.18 ± 0.03)	45 ± 3.3 ms; (0.52 ± 0.02)	252 ± 26 ms; (0.31 ± 0.05)*
75	30.5 ± 7.0 ms; (0.11 ± 0.04)	124 ± 33 ms; (0.56 ± 0.08)	503 ± 111 ms; (0.33 ± 0.08)
43	244 ± 100 ms; (0.30 ± 0.06)	1.25 ± 0.4 s; (0.42 ± 0.04)	10.1 ± 3.0 s; (0.28 ± 0.06)
10	0.9 ± 0.2 s; (0.28 ± 0.11)	4.0 ± 1.8 s; (0.35 ± 0.06)	28 ± 8 s; (0.36 ± 0.09)
0	2.3 ± 0.7 s; (0.28 ± 0.03)	15.7 ± 4.5 s; (0.32 ± 0.03)	154 ± 21 s; (0.40 ± 0.4)

*Amplitudes are shown in parentheses.

degree of the purple membrane. The possibility of a diffusional rate-limited proton transfer could be disregarded, however, as it would have imposed a continuous spectrum of relaxation times which is inconsistent with the observed data. The possibility of extreme *p*H changes due to changes in the amount of adsorbed water can be ruled out, since no spectral shifts of the absorption spectrum of the thin purple membrane layers were observed at different hydrations where a blue shift of the spectrum is expected at high *p*H and a red shift at low *p*H (ref. 20 and our unpublished data). A contribution of surface electrical potential changes of the purple membrane suspended in water was analysed by using high ion concentrations (1–4M NaCl) and increasing the *p*H up to 9. In this extreme case the decay of the '412' was only slowed down by a factor of 50.

Although changes in hydration were shown to control the conformation of proteins[21], X-ray diffraction studies[4] of wet and dry specimen of the purple membrane have not shown any significant differences, possibly due to the fact that local changes might escape the sensitivity of the method applied with a 7-Å resolution. Indeed, hydration-induced conformational changes might be expected to occur in much smaller dimensions. Hydration can act either on the immediate environment of the retinal or through inducing a conformational change in the protein which is then transmitted to the retinal–protein interaction region. A possible induced conformational change may also involve an increased formation of internal hydrogen bonding on dehydration, competing for proton transfer, or a conformational induced *p*K shift of the terminal amino acids. It should be noted that the dehydration process may bring about a large change in the dielectric constant in the vicinity of the proton acceptor. Such a change can shift the *p*K by two to four units[22] and thus change the kinetics by orders of magnitude. Variation of the hydration state of the purple membrane may change the microviscosity of the lipids or even bring to phase separation. The influence of the lipid–protein interaction on the photocycle must be of a secondary importance, however, because no significant difference was found in the kinetics and in proton uptake of proteoliposomes prepared from lipid-depleted protein and from native purple membrane[23]. The conformation change is further supported by the observation of a decoupling of the *cis–trans* isomerisation process as a function of hydration[24], as well as by the photochemical bleaching of the '412' intermediate even at the lowest humidity state. In addition, dehydration of vertebrate rhodopsin was found to stop the meta I ⇌ meta II transition[25]. Thus in both bacteriorhodopsin and in rhodopsin those conformational changes which involve both protonation–deprotonation reactions and large enthalpy change are those which are most effected by dehydration.

These results stress the importance of the state of hydration in bacteriorhodopsin, presumably as a conformation-controlling event, which might be of general significance for energy transducing membranes especially in the case where deprotonation and protonation processes, coupled to conformation changes are an inherent part of the energy transformation process. The results also indicate that the photochemical reaction is independent from the state of the bulk solution and is only influenced by the microenvironment of the reaction system which operates in a state of quasi isolation.

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RAFI KORENSTEIN
BENNO HESS
Max-Planck-Institut für Ernährungsphysiologie,
Rheinlanddamm 201,
4600 Dortmund-1, FRG

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Detection of carcinogen–DNA adducts by radioimmunoassay

COVALENT binding of carcinogen to nucleic acids is believed to be an essential component of the carcinogenic process[1], so it is desirable to have highly sensitive and specific methods for detecting such adducts in cells and tissues exposed to known and suspected carcinogens. We describe here a radioimmunoassay (RIA) capable of detecting nanogram amounts of DNA adducts resulting from the covalent binding of the carcinogen *N*-2-acetylaminofluorene (AAF). AAF and its activated derivative *N*-acetoxy-AAF (*N*-Ac-AAF) are potent carcinogens[2] and mutagens[3,4], and transform cells in culture[5,6]. DNA obtained from rat liver following *in vivo* exposure to AAF, and DNA exposed *in vitro* to *N*-Ac-AAF contain as the major (80%) adduct *N*-(deoxyguanosin-8-yl)-acetylaminofluorene (dG-8-AAF)[7–9] and a minor (20%) adduct 3-(deoxyguanosin-N^2-yl)-acetylaminofluorene (dG-N^2-AAF)[10]. These two types of modification produce markedly different conformational effects on the DNA helix[2,11,12]. The major adduct, recognised by