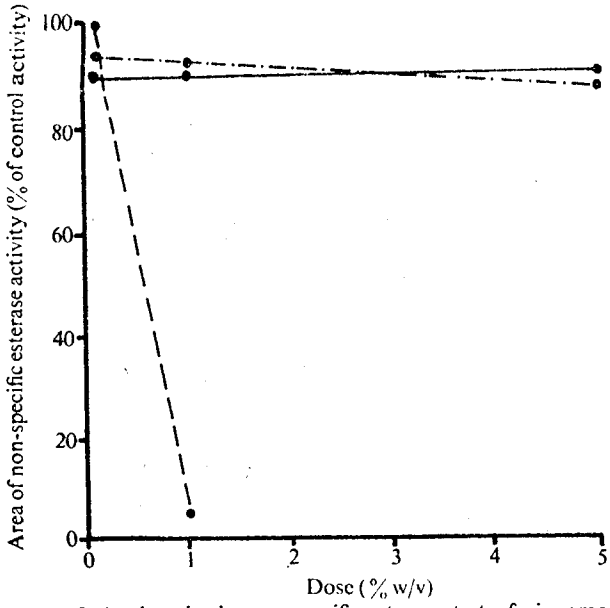

Fig. 1 Behaviour in the non-specific esterase test of cinnamonitrile (●—●), cinnamaldehyde (●— · — · —●), and CS (● – – –●).

three consecutive days with either cinnamonitrile, CS or cinnamaldehyde at 5%, 1% or 0.1% concentrations in acetone. A control group of sixteen mice were treated with acetone alone. Three days after the final application all the mice were killed and a piece of skin from the centre of the painted area was excised and frozen in hexane pre-cooled to −75° C.

The skin samples were mounted on cryostat tissue holders, and three 22 μm transverse sections at 110 μm intervals were cut from each skin sample on a 'Bright's' cryostat at a cabinet temperature of −25° C. The sections were air-dried at 6° C and fixed in formol calcium gum sucrose. Non-specific esterase activity was demonstrated in the sections by the method of Holt[13] using 5-bromoindoxyl acetate as substrate.

Sections of skin from every group were included in each incubation procedure to allow subsequent correction for incubation variation. The sections were then mounted in 90% (w/v) aqueous solution of polyvinylpyrrolidone. The area of non-specific esterase activity in the sebaceous glands, as indicated by the reaction product, was measured in each skin sample using a 'Quantimet B' image analyser (Image Analysing Computers Ltd, Melbourne, Royston, Herts).

The results (Fig. 1) show that of the three αβ-unsaturated compounds examined only CS suppressed non-specific esterase activity. Because the relatively reactive cinnamaldehyde did not suppress the enzyme activity, reactivity with thiol groups alone does not explain the marked suppression caused by CS when applied to mouse skin at 1% and 5% (w/v) concentrations. In other comparative experiments the extent of suppression caused by CS was similar to that produced by 7,12-dimethylbenz[*a*]anthracene treatment.

The reason why known carcinogens, or indeed CS, suppress non-specific esterase activity, at present, remains unknown. But considering the results of previous experiments using the sebaceous gland test and despite the obvious drawbacks of the predictive value of such bio-assay systems, CS should be fully investigated for carcinogenic activity.

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Interaction of Hallucinogenic Drugs with Encephalitogenic Protein of Myelin

Immunochemical techniques involving humoral antibody have long been used in elucidating the structural relationships of proteins and small antigenic molecules. Development of a quantitative technique for sensitized lymphocytes with high discriminatory capacity in respect to closely related antigens has made it possible to use these cells to assess structural requirements for antigenicity[1-3]. When sensitized lymphocytes are brought into contact with specific antigen a material is liberated which lowers the electrophoretic mobility of normal guinea-pig macrophages. The change in cell mobility can be measured in a Zeiss cytopherometer. This assay has been used in the study of several experimental and human autoimmune diseases to give an accurate and quantitative assessment of the cellular immune response[1,4].

Lymphocytes from patients with multiple sclerosis and other degenerative neurological diseases have been shown to be sensitized to the encephalitogenic basic protein from human myelin (EF)[5]. Patients with malignant neoplasia also possess lymphocytes able to respond to EF and to a synthetic peptide Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg which corresponds to the main encephalitogenic determinant in the human protein[6], that region of the protein which induces experimental autoimmune encephalomyelitis in guinea-pigs. Details of the method are given elsewhere[3] and specimens are randomized and estimated blind.

Carnegie[7] drew attention to the striking resemblance between the postulated structure for the encephalitogenic determinant and that required by a serotoninergic receptor as calculated by Smythies, Benington and Morin[8]. However, the structure proposed by Carnegie did not completely agree with the structural requirements for encephalitogenicity set out by Westall *et al.*[9]. In particular in Carnegie's model glutamic acid was a key residue while Westall *et al.* thought this was unimportant. To resolve this conflict Smythies *et al.*[10,11] proposed an alternative model where the above peptide was placed in an anti-parallel-β-pleated sheet conformation. Field *et al.*[6] have demonstrated that serotonin can block the reaction between sensitized human lymphocytes and encephalitogenic protein (EF) or the above peptide provided it is added before the antigen. Serotonin had no effect in systems where the antigen was structurally unrelated to EF. Moreover, acetylcholine, succinylcholine, histamine, adrenaline, noradrenaline and (in the present study) dopamine were without effect.

We describe here the effect of indole derivatives related to serotonin on the interaction of human encephalitogenic protein with lymphocytes from patients with degenerative neurological disease. Some hallucinogenic drugs which are thought to react with serotoninergic receptors in the central nervous system have also been studied.

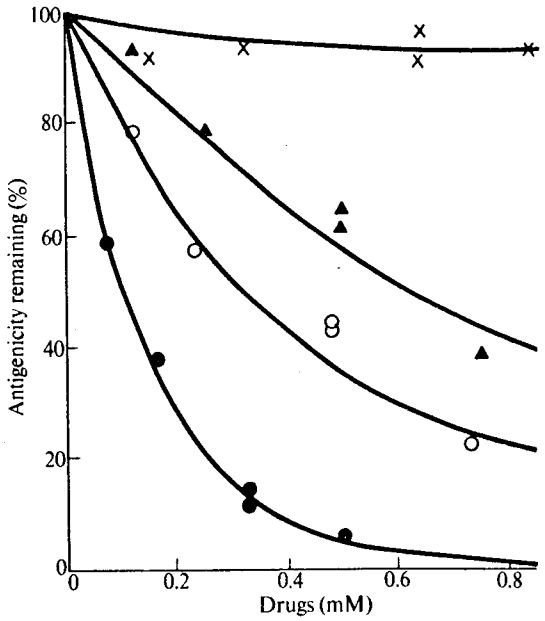

Fig. 1 Effect of indoles on the antigenicity of encephalitogenic protein of myelin. Antigenicity was determined in cytopherometer assay with lymphocytes from a patient with multiple sclerosis. Solutions of the drugs, 0.8 mg/ml., in tissue culture medium 199 were prepared, and 125, 250, 500 or 750 µl. were mixed with 0.5×10^6 lymphocytes from a patient with multiple sclerosis and 10^7 guinea-pig macrophages in a total volume of 3 ml. medium 199. Encephalitogenic protein (EF, 100 µg in 100 µl.) was then added. The change in electrophoretic mobility of macrophages was then measured at 23° C after the customary incubation for 90 min. In the above system EF, in the absence of blocking agents, usually caused a decrease of around 0.25 µm $s^{-1} V^{-1} cm^{-1}$ in the electrophoretic mobility of the macrophages. Results are expressed as relative mobility where the result with EF in the absence of any blocking agent is taken as 100%. Calculation[3] showed that differences in mobility of 20% and 15% were significant at the 0.001 and 0.01 levels respectively. ×, Tryptamine; ▲, 5-CH_3O-N,N-dimethyltryptamine; ○, 5-Cl-N,N-dimethyltryptamine; ●, 5-OH-tryptamine.

Lymphocytes, macrophages and encephalitogenic protein were prepared and used in the cytopherometer assay as previously described[3].

Increasing concentrations of serotonin and drugs blocked the reaction between EF and lymphocytes (Fig. 1). All drugs showed no direct effect on the mobility of macrophages. The drugs are either reacting with the antigenic determinant on EF or with the complementary antibody-like molecule on the surface of specifically sensitized lymphocytes. Lymphocytes incubated with serotonin, washed and then used in the test react in exactly the same manner as do untreated cells indicating that serotonin is without effect on the lymphocytes. Also, the type of curve[8] of Fig. 1 would not be produced if there was a direct effect on the cell. In preliminary experiments no significantly greater uptake of ^{14}C-serotonin by reactive lymphocytes was observed. Thus we suggest that serotonin is interacting with EF. As the tryptophan region of EF interacts with the lymphocytes[6] and as this region fulfils the structural requirements of a binding site for serotonin we assume that the loss of antigenicity results from the drug binding to this region of the protein. From a dose response curve for EF with lymphocytes[12] we estimated the amount of free EF required to elicit the particular response for all points on the curves (Fig. 1). The amount of bound EF was taken as the total EF (1.78 µM) less the free EF taking the molecular weight of EF as 18,500[13]. In comparison the amount of bound drug was assumed to be negligible with the free drug concentration on a molar basis. From reciprocal plots of the drug concentration and the ratio of bound to total EF the apparent association constant K^1 was calculated from the slope of the regression line from the experimental points. Similar data were obtained for the binding of serotonin to a synthetic encephalitogenic peptide of only 9 amino-acids, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg[9]. The values of K for the association of serotonin to the peptide and EF were found to be 9.8×10^4 M^{-1} and 7×10^4 M^{-1} respectively. Similarly K^1 values for 5-chlorodimethyltryptamine and 5-methoxydimethyl-tryptamine were 4.3×10^4 M^{-1} and 2.9×10^4 M^{-1}. Because of the complexity of the assay system and the assumptions involved these values should be considered as approximate and confirmation by more rigorous techniques should be sought.

With the other drugs, tested at only one concentration (130 µg $ml.^{-1}$), we can estimate their relative ability to interact with EF by comparing the molar concentration of the drug with that of serotonin required to block the antigenicity of EF to the same extent (Table 1).

From the interaction of the hallucinogenic drugs with EF (Table 1) it is clear that apart from LSD they have less effect than serotonin. Tryptamine and simple indoles did not appear to interact with EF. The 5-methoxytryptamine derivatives are of interest because the 5-methoxy and dimethyl-5-methoxy derivatives had significant interaction but the bulkier diethyl and N-methyl-N-cyclopentanyl derivatives were without effect.

Table 1 Blocking by Indole Derivatives and Hallucinogenic Drugs of Antigenic Determinant of Encephalitogenic Protein in Reaction with Lymphocytes from Patients with Neurological Diseases

Compound	Relative antigenicity in presence of drug: Patient A (stroke)	Patient B (multiple sclerosis)	Relative molar ability to block antigenicity
None	100%	100%	
Significant blockage ($P < 0.001$)			
5-hydroxytryptamine (serotonin creatinine sulphate)*	12.5	12.4	1.0
N,N-dimethyltryptamine*	37.0	38.0	0.4
5-chloro-N,N-dimethyltryptamine HCl	—	43.0	0.6
D-lysergic acid diethylamide (LSD) D-tartrate	48.0	—	1.0
5-hydroxy-N,N-dimethyltryptamine (bufotenine)†	52.5	48.0	—
5-methoxy-N,N-dimethyltryptamine HCl	61.0	62.0	0.4
5-methoxytryptamine	63.5	65.0	0.3
3,4,5-trimethoxyphenethylamine HCl (mescaline)*	64.0	62.0	0.3
N,N-dimethyl-2,5-dimethoxy-4-methylamphetamine HCl (dimethyl DOM)	66.0	72.0	0.3
Insignificant blockage			
Tryptamine HCl*	92.0	—	0.0
Indole*	91.0	—	0.0
Hydroxyindole*	91.0	—	0.0
N-isopropyltryptamine maleate	102.0	—	0.0
Dehydrobufotenine HCl	98.0	—	0.0
5-methoxy-N,N-diethyltryptamine HCl	90.0	95.0	0.0
1-methyl-5-methoxy-N,N-dimethyltryptamine HCl	97.0	—	0.0
5-methoxy-N-methyl-N-cyclopentanyltryptamine†	98.0	—	0.0
5-benzyloxy-N,N-di-n-butyltryptamine HCl†	98.0	—	0.0
3-(β-N-piperidinoethyl) indole HCl	89.0	—	0.0
3-(β-N-pyrrolidinoethyl) indole HCl	93.0	—	0.0

All measurements were made in the cytopherometer assay with lymphocytes sensitized to encephalitogenic protein. In the incubation mixture 0.5×10^6 human lymphocytes were mixed with 10^7 guinea-pig peritoneal macrophages. The drugs, 400 µg (except LSD 150 µg), were added to the incubation mixture a few minutes before the addition of 100 µg of the encephalitogenic protein and made to a total volume of 3.1 ml. with tissue culture medium 199.

Drugs marked † were not completely soluble in the incubation mixture.

Drugs marked * were from Sigma Limited. LSD was from Spofa United Pharmaceutical Works, Praha. Others were kindly given by Drs R. B. Barlow, F. Benington and R. D. Morin.

Moreover a methyl group at the 1 position blocked the ability of the indole to bind to EF.

Drugs known to be hallucinogenic are shown to partially block the reaction of the protein with lymphocytes (Table 1). Indole derivatives without hallucinogenic activity had no effect. LSD, mescaline and dimethyltryptamine[14] are thought to interact with receptors for serotonin. However, correlation between hallucinogenic potency and blockade in this test is not complete. For example hallucinogenic potency runs d-LSD 5 - methoxy - N,N - dimethyltryptamine N,N - dimethyltryptamine[14] whereas the order for the last two is reversed in the current test. Moreover, N,N-dimethyl-2,5-dimethoxy-4-methylamphetamine (N,N-dimethyl-DOM), inactive as a hallucinogen[14], shows some blocking activity. Therefore it appears that the receptors concerned must be similar but not identical.

The failure of tryptamine to block the antigenicity and the ability of serotinin to react suggests that charge-transfer reactions may be of prime importance. In addition, since 5-hydroxyindole was inactive the amine group is also important. From physical measurements EF appears to have an essentially random conformation in aqueous solution[15], but in the presence of lipids changes to a mixture of α-helix and β-structure[16]. The anti parallel-β-pleated sheet conformation for the tryptophan region would provide a binding site which satisfies spatial requirements to accommodate the smaller hydroxytryptamine derivatives. Lymphocytes would be expected to recognize an ordered form of EF rather than a random structure and perhaps in our test system EF may associate with cell membranes and assume an ordered structure "recognized" by the drug and by the lymphocytes. Preliminary experiments in a purely aqueous medium have failed to show the same degree of association of (^{14}C)-5-hydroxytryptamine for EF. Experiments with model membranes which incorporate EF may be necessary to confirm the binding we have demonstrated.

The association constant of 7×10^4 M^{-1} found for serotonin with EF is lower than that (10^7 M^{-1}) determined by Marchbanks[17] for serotonin with nerve ending particles. There is evidence for the binding of serotonin by preparations of synaptosomal membranes[18,19]. However, in Alivisatos *et al.*[18,19] the equilibrium was displaced by tryptamine and hydroxyindole which do not interact with EF in our system and which are not hallucinogenic. Although the concentration of serotonin in brain is only 0.5 to 5 μM[20], the local concentration, at least in cytoplasm of certain invertebrate cells[21], can be as high as 4 mM. Thus, if serotonin were released in high concentration in close proximity to myelin protein significant interaction could occur.

Myelin fractions have been shown[22] to contain between 12%[23] and 23%[22] of the serotonin in brain[23]. Alivisatos *et al.*[18] has also demonstrated binding of ^{14}C-serotonin to myelin. During rapid myelination in the rat between (7 and 14 days) there is a striking sigmoidal increase in the activity of associated enzymes[26]. While tryptophan hydroxylase[25] activity, serotonin concentration[26] and myelin development showed a similar pattern with age during this critical period, the concentration of noradrenaline increased in a linear manner between birth and 42 days[26].

Monoamine oxidase binds serotonin prior to exerting its oxidative activity. This binding is blocked by pargyline (N-benzyl-N-methylpropynyl-amine HCl) which attaches to the active site of the enzyme[27], but is without effect on the antigenicity of EF. Thus the binding site on the oxidase for serotonin must be different from that on EF. Serotonin also interacts with the postsynaptic and presynaptic membranes in certain synapses. Imipramine is known to block the re-uptake of serotonin by presynaptic membranes[28] and also blocked the antigenicity of EF giving an association constant of approximately 6×10^4 M^{-1}.

It is surprising that a myelin protein can interact with serotonin, as hallucinogenic drugs in general are usually assumed to influence serotonin receptors at synapses[14,29,30] though there is little direct evidence for this. Myelin is considered to be derived from and maintained by oligodendroglial cells which respond to serotonin in culture[31,31] by changing their pulsation. Perhaps these cells have a more active role in the CNS and the drugs may be affecting their function.

As there is approximately 500–1,000-fold molar excess of EF compared to serotonin in the adult rat brain there cannot be one molecule of serotonin associated with each molecule of EF. A possible site for *in vivo* interaction is at the nodes of Ranvier in the central nervous system. Dickinson *et al.*[33] have suggested that the basic protein is localized in the intra period line of myelin which may be exposed, free of lipid, in this region. Alternatively from the imipramine result the encephalitogenic determinant may merely be mimicking a synaptic receptor for serotonin which would have a similar structure. We can speculate that hallucinogenic drugs may exert their action along the course of the myelin sheath (perhaps in the vicinity of the nodes of Ranvier) rather than specifically at synapses. Indeed differential interference with conduction might lead to temporal dispersion of impulse volleys with consequent functional disturbance—both sensory and motor.

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