and during certain parts of their life cycles and, possibly, as suggested by Morris *et al.* (*15*), to extend the time between insect outbreaks.

The long-term and possibly more significant effect of such heavy predation on already low densities of Lepidoptera larvae must be evolutionary. By acting as a strong selective force, bird predation can influence many of the prey's morphological, behavioral, and life-history traits and, in turn, their role in ecosystem processes. For instance, the heavy midsummer bird predation that we document here may not only select for patterns of crypsis in Lepidoptera larvae, but also for their choice of substrates on which to hide, their feeding schedules, and even the timing of their life cycles. We suggest that any or all of these effects will influence when or where a particular species of caterpillar feeds and thus its pattern of leaf consumption. The evolutionary effects of predation therefore have ecological consequences for the prey and their host plants.

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6. This understory plant was chosen because it is host to relatively high and less variable insect densities than are saplings of the dominant tree species (J. C. Schultz, in preparation), it can be easily enclosed, and the insects on its large leaves are readily observed and counted.
7. This visual inspection method permits continuous, nondestructive sampling and is more accurate than other methods (J. C. Schultz *et al.*, in preparation). Other insect censuses conducted simultaneously on the same plant species, as part of a larger research program on forest insects, yielded densities and faunal composition similar to those in this experiment.
8. R. T. Holmes, unpublished data.
9. Representatives of the families Geometridae, Noctuidae, and Torticidae comprised more than 90 percent of the larvae observed.
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Experimental Phenylketonuria: Replacement of Carboxyl Terminal Tyrosine by Phenylalanine in Infant Rat Brain Tubulin

Abstract. *In the brains of newborn rats, about half of the tubulin molecules are modified posttranslationally by the addition of an aromatic amino acid at the carboxyl terminus of the α chain. Of the added residues, 96 percent are tyrosine and 4 percent are phenylalanine. After induction of hyperphenylalaninemia, the proportion of tubulin molecules containing carboxyl terminal phenylalanine increases up to eightfold and the pool of tyrosine-containing molecules decreases by an equivalent amount.*

Human phenylketonuria is a disease in which a brain dysfunction is associated with an inherited deficiency of phenylalanine 4-hydroxylase (E.C. 1.14.16.1) in the liver (*1*). The metabolic sequela of this deficiency is an excess of phenylalanine, which accumulates in the blood and spinal fluid and leads to elevated brain phenylalanine. Experimentally, hyperphenylalaninemia has been produced in animals (*2, 3*) to study the biochemical abnormalities associated with the disease. The studies in animals have indicated that the biochemical characteristics of phenylketonuria can be induced, provided the treatment is administered early during development (*2, 3*). However, efforts to explain the pathogenesis of the brain damage have had no success.

In an unrelated study in chick brain we showed that a unique modification of tubulin, the posttranslational addition of aromatic amino acid residues to the carboxyl terminal end of the α chain (*4*), was enhanced in early development (*5*). A tubulin-specific ligase catalyzed the reaction (*6*) and tyrosine and phenylalanine competed for the binding site on tubulin (*7*). Direct quantitative analysis of the carboxyl terminal aromatic residues of purified tubulin from normal chick brain showed that tyrosine was the main modifier residue; phenylalanine modified no more than 2 percent of the tubulin molecules in young animals (*5*). This was consistent with the kinetic data for the reaction in vitro and the concentration in brain of each amino acid. Although the biological significance of this ligase reaction, which seems to be specific for α tubulin, is not known, the presence or absence or even the identity of an aromatic residue at the carboxyl terminal end of the tubulin molecule could conceivably affect the interrelationship between microtubules and other elements of the cell. Since microtubules appear to be essential for neuronal growth and function, a possible link between the phenylalanination of tubulin and the phenylketonuric condition seemed to be worth exploring.

We now report experiments in which we determined the state of modification of cytoplasmic brain tubulin in normal and hyperphenylalaninemic immature rats. Carboxyl terminal tyrosine and only small amounts of carboxyl terminal phenylalanine were present in about half of the molecules of tubulin in normal rats. In tubulin from the experimental animals phenylalanine substituted for a large proportion of carboxyl terminal tyrosine, although the availability of free carboxyl terminal sites for incorporation of amino acid in vitro was not affected.

Elevation of brain phenylalanine content was induced in albino male rats (Holtzman) by one of two procedures:

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(i) single intraperitoneal injection of phenylalanine (1 g per kilogram of body weight) (Mann Research) in 0.42 percent NaCl (*8*) or (ii) induction of chronic hyperphenylalaninemia as described by Greengard *et al.* (*3*). In the first case, 7-day-old rats were separated from their mothers, placed in a box kept at 30° ± 2°C and, after approximately 30 minutes, injected with the dose of phenylalanine. Weight-matched control animals were injected simultaneously with an equal volume of 0.9 percent NaCl. The animals were returned to the box and killed after 120 or 180 minutes. For the induction of chronic hyperphenylalaninemia, daily injections of both DL-α-methylphenylalanine (ICN Pharmaceuticals) and phenylalanine were given from age 3 or 4 days to age 7 days. Control and experimental animals were kept with their mothers and were killed 2 hours after the injection on the last day. The concentration of phenylalanine in brain extracts was determined by the method of McCaman and Robins (*9*) and a 20- to 40-fold elevation in phenylalanine concentration was observed.

Tubulin was purified from extracts of brain tissue obtained from control and experimental rats under the conditions described in Table 1. The procedure was based primarily on ion-exchange chromatography (*10*) and is presumed to purify bulk cytoplasmic tubulin because it does not select for molecules on the basis of their ability to assemble into microtubules. Electrophoretic analysis (*11*) of the purified material indicated that the tubulin was 95 to 98 percent pure. Enzymatic digestion of the protein with pancreatic carboxypeptidase A was shown to release only tyrosine and phenylalanine, presumably from the carboxyl terminal end of the α subunit of tubulin (*5*). Automated amino acid analysis of the material, processed as described in the legend of Table 1, was used to determine the proportion of carboxyl terminal aromatic residues.

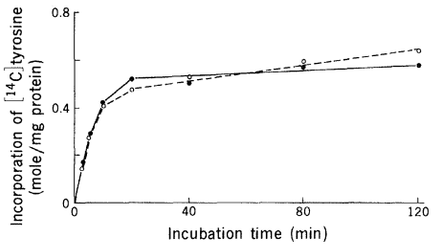

Fig. 1. Kinetics of incorporation of [^{14}C]-tyrosine into tubulin of the brain extracts from (●) normal and (○) hyperphenylalaninemic 7-day-old rats. Extracts were prepared as described in Table 1 and desalted in a column of Sephadex G-25 (coarse). Assay conditions were as previously described (*4*). Each point is the average of duplicate determinations.

The analysis of the carboxyl terminal end of tubulin molecules as isolated from brain tissue, by means of the nonselective procedure described above, indicated that in the case of the normal rats the main residue was tyrosine, although a small proportion of the molecules yielded phenylalanine (Table 1). Assuming a molecular weight of 110,000 for the tubulin dimer (*12*), the stoichiometry was 0.54 mole of tyrosine and 0.02 mole of phenylalanine per mole of tubulin dimer. The remaining tubulin molecules apparently lacked added residues, and hence contained a glutamate residue at the carboxyl terminal ends (*13*). These could be partially tyrosinated in vitro under optimal conditions (see below). We previously reported (*5*) a similar situation for tubulin from chick brain during development. After induction of hyperphenylalaninemia in 7-day-old rats, the minor proportion of brain tubulin molecules containing a phenylalanine residue at the carboxyl terminal end significantly increased about five- or eightfold depending on the length of the treatment (Table 1).

To rule out the possibility that the observed change was due to in vitro incorporation of phenylalanine into the carboxyl terminal position of α tubulin during the process of isolation of the protein, the amino acid was added to the brain homogenate to obtain a final concentration equivalent to that in the experimental samples. Analysis of tubulin from normal brain tissue prepared under these conditions gave a result equal within the experimental error to that of the control in Table 1.

In some experiments with the treated groups of animals the stoichiometry for the carboxyl terminal amino acids relative to tubulin was determined. The values for the tyrosine and phenylalanine residues were, respectively (in moles per mole of tubulin dimer): 0.45 and 0.10 for the group receiving a single intraperitoneal injection and 0.36 and 0.15 for the group with induced hyperphenylalaninemia. These should be compared with the corresponding values for the normal group shown above. In all cases the proportion of modified tubulin molecules was in the range 0.51 to 0.56. Apparently the size of the pool of modified tubulin molecules in the brain tends to be conserved even after a substantial change in the size of the pool of the precursor amino acid substrate for this type of modification reaction. To test this, the capacity of the residual unmodified tubulin to undergo tyrosination in vitro (*4*) was determined in extracts of normal brain tissue and of brain tissue from both experimental groups. Essentially the same kinetic pattern was obtained in all cases and, for simplicity, the results for the control and the hyperphenylalaninemia group are shown in Fig. 1. No significant changes in incorporation of [^{14}C]tyrosine (Amersham/Searle) were observed either at very short times of incubation, indicative of an unmodified ligase enzyme activity, or extended times of incubation, from which information about the availability of free sites for amino acid binding may be obtained. Although the incorporation reaction did not absolutely plateau (note the second, slow slope in the curves in Fig. 1), our best estimate of the net incorporation of [^{14}C]tyrosine by the ligase in these prepa-

Table 1. Analysis of the amino acids released from purified brain tubulin by digestion with carboxypeptidase A. Animals were treated as indicated in the text, killed by decapitation, and brains were removed. Brain tissue (5 to 8 g) was homogenized with one volume of buffer solution containing 0.1*M* piperazinediethanesulfonic acid (*p*H 6.5), 1 m*M* guanosine triphosphate, 1 m*M* ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.5 m*M* $MgCl_2$, 1 m*M* 1,4-dithiothreitol, in a motor-driven Teflon-in-glass homogenizer. Samples were centrifuged at 20,000 rev/min for 1 hour in a type 65 rotor (Beckman) at 3°C. Tubulin was isolated from extracts by ion-exchange chromatography and hydrolyzed with carboxypeptidase A (Worthington) as previously described (*5*). After digestion, samples were chilled to 0°C and deproteinized by ultrafiltration through UM-10 ultramembranes in an Amicon apparatus. Filtrates were evaporated to dryness and solubilized in a small volume of the amino acid analyzer buffer (sodium citrate, *p*H 2.2, 0.2*N* Na^+; Pierce). Occasionally, a blank tube was run in which purified tubulin and carboxypeptidase were combined and immediately filtered at 0°C. Values were expressed as the percent area of the peaks in the amino acid analyzer (Durrum model D 500) chromatogram after correction for the background of the blank sample. Values were the means of three separate determinations. The standard deviation (S.D.) shown corresponds to both sets of values.

Treatment	Amino acid released by carboxypeptidase A from purified brain tubulin (%)		
	Tyrosine	Phenylalanine	S.D.
Control	95.9	4.1	1.40
Single intraperitoneal injection of phenylalanine	79.6	20.4	4.02
Induction of chronic hyperphenylalaninemia	68.7	31.3	3.95

rations indicated that the capacity of tubulin to bind additional amino acids at the carboxyl terminal end was about 0.3 mole per mole of dimer for both control and experimental animals. This result, besides strengthening the assumption of the unchangeability in size of the pool of modified tubulin molecules, establishes that the molecules unmodified in vivo in control and experimental animals maintain the same reactivity toward tyrosine in vitro. It also demonstrates that a certain proportion of the rat brain tubulin neither contained nor accepted aromatic amino acids at the carboxyl terminal position.

We attempted to compare the capacity of tubulin for assembling at 37°C in the brain extracts of normal and experimental animals by following the kinetics of the development of viscosity. Extracts were prepared from 7-day-old rats (9 to 11 mg/ml) as described in Table 1 and viscometry was monitored as described by Olmsted and Borisy (*14*). Although the analysis of viscosity development in the extracts was complicated by low initial rates and high values of viscosity after the first 10 or 15 minutes of incubation at 37°C, apparently due to gelation, no difference was observed between the samples from experimental and control animals. We conclude that the kinetics of polymerization as measured by viscometry did not substantially change after the induction of hyperphenylalaninemia. By using a sedimentation assay, it was recently demonstrated (*15*) that tubulinyl-[^{3}H]phenylalanine and tubulinyl-[^{14}C]tyrosine prepared in vitro were randomly assembled into microtubules when the mixture was incubated at 37°C. Those results agree with the idea that neither the removal nor the addition of an aromatic amino acid to the carboxyl terminal end of α-tubulin affects the polymerizability of the microtubule protein (*15, 16*).

The results of this study indicate that cytoplasmic brain tubulin from animals in which the biochemical characteristics of phenylketonuria were induced unambiguously differed from tubulin of control animals in the nature of the noncoded aromatic amino acid residue that could be released by carboxypeptidase digestion. Hyperphenylalaninemia resulted in enhanced substitution of phenylalanine for tyrosine. Although the addition of an aromatic residue to the carboxyl terminal glutamate of the α chain of tubulin seemingly does not affect the assembly of microtubules in vitro, the presence of such a residue could alter the configuration of that region of the polypeptide. Moreover, the chemical difference between tyrosine and phenylalanine could affect the capacity of the protein or of the resultant microtubules to interact with other cellular elements. Our results do not permit us to say whether the pathogenesis of brain dysfunction in phenylketonuric individuals is dependent on the modification of tubulin; however, the existence of this modification suggests that the possibility of a link between the phenylalanination of tubulin and phenylketonuric condition needs to be investigated further.

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Classification of Opioids on the Basis of Change in Seizure Threshold in Rats

Abstract. *Twenty opioids have been subdivided into four classes by using flurothyl-induced seizures in rats to measure dose-response relationships, stereospecificity, naloxone sensitivity, and tolerance-cross-tolerance. The data support current theories of multiple opiate receptor types. Since the receptors involved mediate effects that are antagonized, enhanced, or unaffected by naloxone, the model is uniquely suitable for detecting novel narcotic antagonists that can then be used to differentiate opiate receptors in other systems.*

Morphine and related compounds have recently been subdivided into at least three different classes on the basis of subjective effects in humans (*1*), different sensitivities towards the narcotic antagonist naloxone (*2*), and dissimilar pharmacological profiles both in vitro (*3*) and in vivo (*4, 5*). We have extended the in vivo profile approach to include altered seizure threshold as a measure and have classified 20 opioids by comparing qualitative effects, dose-response curves of enantiomers, sensitivities toward naloxone, and tolerance-cross-tolerance properties. In light of these experiments, we now report that opioids can be classified into at least four groups in vivo. Three of the groups show good correspondence to a classification obtained from the chronic spinal dog preparation (*4*). The fourth group represents a new category; meperidine and pentazocine are the prototype analgesics.

Groups of 10 to 20 male Sprague-Dawley albino rats (300 to 350 g; Zivic-Miller) were studied. Rats were given only one injection; they received the vehicle or one of at least three doses of test agent (*6*) subcutaneously 30 minutes before being exposed to flurothyl (Indoklon), a volatile convulsant. The flurothyl was given as a 10 percent solution in 95 percent ethanol (volume to volume) to rats placed individually in 1-gallon glass jars (*7*). A constant rate of infusion of 0.10 ml per minute was maintained by a Harvard pump. The time interval between the start of the infusion and the onset of a clonic convulsion (almost invariably with loss of posture) represented the seizure threshold. Testing took place between 1000 and 1300 hours. Mean seizure thresholds for rats injected with saline were routinely in the range of 350 to 380 seconds.

Dose-related anticonvulsant effects were associated with the so-called sigma (σ) receptor agonists (*4*) *N*-allylnormetazocine (SK & F 10,047) and cyclazocine (Table 1). Both of these psychotomimetic benzomorphans caused behavioral activation; specific features seemed to be determined by the environment. In the novel and restricted envi-

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