was 3.0 Å for carboxypeptidase, residues 263 through 295. This region is definitely not an EF hand. By contrast the R (for EF in comparison with lysozyme) is 1.9 Å.

In order to test the discriminating power of our sequence scoring scheme we have programmed six scoring schemes for the detection of homologies (*6*). For example, in Dayhoff's (*7*) scheme random sequences would generate the alignment score $A \geq 2.6$ once per hundred and $A \geq 3.3$ once per thousand comparisons. In Table 1 we list average A values for each of the nine regions of the muscle proteins compared with each other; they are detectable as homologs by such statistical tests. For T4 lysozyme compared with these nine regions, A ranges from 1.1 to 4.1 with $\overline{A} = 2.0$. This would hardly be considered significant. Tentatively we conclude that our special scoring scheme is marginally more sensitive. A more stringent evaluation will be provided by the 15 sequences that scored 9/16 on our test (Table 2). We do not suggest that these contain EF hands, even though their $\overline{A}$ values range from 0.4 to 2.6. Eight of these 15 regions definitely are not EF hands. This is seen in their x-ray structures or is inferred from structures of their homologs.

Concerning the question of possible T4 lysozyme homology with the muscle proteins—MCBP, TN-C, and ALC—Kretsinger (*5*) proposed that calcium control proteins are homologous. Conversely we might suspect that all proteins that contain an EF hand are involved in calcium mediated processes. There is no evidence that T4 lysozyme even binds calcium, let alone that it is regulated by calcium ions. However, there is really no evidence that either the alkali or the DTNBLC bind calcium either. It is possible that one of the postulated EF hands of ALC evolved from a calcium binding hand but subsequently lost its calcium binding affinity. For instance, the ALC-δ region has five oxygen atoms as potential calcium ligands but only one carboxyl group. This is also the case in T4 lysozyme. This argument, of a noncalcium binding EF hand, is weakened by the fact that in the three nonhomologous proteins—concanavalin A (*13, 14*), thermolysin (*15*), and *Staphylococcus* nuclease (*16*)—the six calcium ions are six coordinate with oxygen but the number of carboxyl groups ranges from one (*16*) to four (*17*). We suggest that T4 lysozyme derived from a calcium modulated enzyme of the host bacterium.

Alternatively, the EF hand to T4 lysozyme may not be homologous to those of the muscle proteins. It may be simply a thermodynamically preferred conformation which has been arrived at by an alternative evolutionary route. After all, the existence of a pair of α helices does not imply homology. The spatial relation of the T4 lysozyme E and F helices may be fortuitous, or the EF hand may have an inherent stability that is not yet understood.

In conclusion, the strong suggestion of three calcium binding EF hands in alkali light chains from rabbit skeletal muscle supports the theory of a common evolutionary origin for the calcium control proteins. The prediction and discovery of an EF hand in T4 lysozyme again poses the question of convergent versus divergent evolution at the molecular level (*18*).

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17. We thank J. H. Collins as well as B. W. Matthews and S. J. Remington for sending us unpublished results and for subsequent discussions.
18. *Note added in proof*: (i) A. Weeds and A. McLachlan have kindly sent us [*Nature (Lond.)*, in press] a draft of their paper in which they have interpreted the ALC sequence in terms of four EF hands (1,2,3,4) as opposed to our interpretation of three (α,γ,δ). In order to align the β region with the EF hand they have effectively postulated that it contains three, single amino acid insertions. (ii) B. W. Matthews and S. J. Remington (*12*) have reinterpreted the T4 lysozyme electron density in the "loop" region, residues 50 to 56. Although the α helices of E and F remain unchanged, several of the loop atoms shift up to 5 Å. The revised lysozyme loop fits less well to the EF hand loop than does the structure used in their original publication. This revision seems to strengthen the interpretation of convergent evolution and to weaken the prediction of calcium binding and of homology.
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Daily Rhythm in Human Urinary Melatonin

Abstract. *The melatonin in urine samples from six healthy adult volunteers was concentrated on Amberlite XAD-2 resin, eluted with organic solvents, and quantitated by use of a bioassay technique (the dermal melanophore response of larval anurans to melatonin in their bathing medium). The melatonin content of samples collected between 11 p.m. and 7 a.m. was, in each case, several times higher than that of samples collected between 7 a.m. and 3 p.m. or between 3 p.m. and 11 p.m.*

Exogenous melatonin modifies sleep, locomotor activity rhythms, the electroencephalogram, the serotonin content of the brain, and, via a central action, secretion of pituitary gonadotropins (*1*). The rate at which the pineal organ normally synthesizes this hormone in vivo has been estimated indirectly by measuring pineal melatonin concentrations (*2*) and the in vitro activities of pineal enzymes that catalyze its biosynthesis (*3*). Data from such studies have been interpreted to show that melatonin synthesis in rats varies with a 24-hour rhythm, attaining maximum rates soon after the onset of the daily dark period. Studies on pineal constituents cannot, of course, be performed on material from human subjects. Thus, it has not been possible to determine whether melatonin synthesis also varies diurnally in human pineals and, if so, whether the rhythms in diurnally active humans and nocturnally active rats are in phase.

Barchas and Lerner (*4*), using a countercurrent isolation technique and a frog skin bioassay, demonstrated that melatonin could be detected in a 48-hour specimen of human urine. While

Table 1. Melatonin content of the urine during consecutive 8-hour periods. Values are nanograms of melatonin per 8-hour urine specimen. Subjects' initials are followed by age and sex in parentheses.

Time interval	Melatonin (ng) from subject					
	H.J. (18, F)	D.S. (26, F)	R.H. (23, M)	L.C. (18, F)	M.H. (27, F)	H.L. (45, M)
11 p.m.– 7 a.m.	15.6	8.5	3.9	3.6	0.9	1.1
7 a.m.– 3 p.m.	0	1.5	0.6	0	0	0.6
3 p.m.–11 p.m.	0	0.9	0.7	0.6	0	0
11 p.m.– 7 a.m.	22.8	26.4	3.6	2.7	1.8	0.9
7 a.m.– 3 p.m.	0	2.6	0.7	0.9	0	0.7
3 p.m.–11 p.m.	1.7	1.0	0.8	0	0.6	0.6
11 p.m.– 7 a.m.	24.0	13.9	1.6	2.5	2.7	2.1

this observation provided evidence that melatonin actually is secreted by the human pineal, the method used was not sufficiently sensitive to allow quantitation of melatonin in samples of urine collected during intervals shorter than a day. Recently, Pelham *et al.* (*5*), working with a more sensitive bioassay method and a simple chloroform extraction procedure, succeeded in demonstrating a 24-hour cycle in the concentration of a melatonin-like substance in plasma from human males.

While bioassay approaches based on the amphibian dermal melanophore response provide the most sensitive and specific analytical methods currently available for the measurement of melatonin from biological sources, their utility is limited by various technical obstacles: extraction of the melatonin into organic solvents and subsequently returning it to an aqueous medium for assay involves repeated steps requiring substantial volumes of organic solvents. Such procedures are cumbersome and time-consuming, imposing practical limits on the number of samples that can be analyzed. Moreover, the presence of even minute traces of organic solvents (or their derivatives) in the extract to be assayed can markedly alter melanophore responses to melatonin (*6*). To circumvent these problems, we used the nonionic, polymeric adsorbent, Amberlite XAD-2, for the primary isolation of melatonin from urine. Melatonin is eluted from the resin with a small volume of organic solvent; this is purified by a series of washes, and the melatonin is then returned to an aqueous phase for bioassay. The assay is calibrated with known concentrations of authentic melatonin that have been subjected to a similar extraction procedure.

Urine specimens obtained from healthy adult volunteers during 8-hour periods (11 p.m. to 7 a.m., 7 a.m. to 3 p.m., and 3 p.m. to 11 p.m.) were pooled in separate containers and kept under refrigeration. Specimens were made 1*M* in sodium chloride, adjusted to *p*H 10 with sodium hydroxide, degassed under vacuum, and filtered through Whatman No. 1 filter paper. Each filtrate was divided into three aliquots which were separately applied to prehydrated Amberlite XAD-2 columns (*7*). Each column was eluted with two 4-ml portions of chloroform and isopropanol (3 : 1), and then with 4 ml of a saturated solution of sodium chloride. The eluates from each column were pooled in centrifuge tubes. The aqueous phase was aspirated and discarded, and the organic phase was washed successively with 4 ml of salt-saturated 1*N* sodium hydroxide and 4 ml of salt-saturated 1*N* hydrochloric acid (*8*). Each organic extract was then evaporated to dryness under a nitrogen stream. The resulting dry salt deposit was dissolved in 3 ml of water and extracted with an equal volume of petroleum ether, which was aspirated and discarded. The aqueous phase was then extracted with 3 ml of chloroform, which was further washed with deionized water, filtered through anhydrous sodium sulfate, and evaporated to dryness under nitrogen. To eliminate the last traces of chloroform and other volatile contaminants, the walls of each tube were washed down with 1 ml of ethanol which was, in turn, evaporated under nitrogen (*9*). Finally, the residue in each tube was dissolved in 2 ml of water and its melatonin content was determined by bioassay.

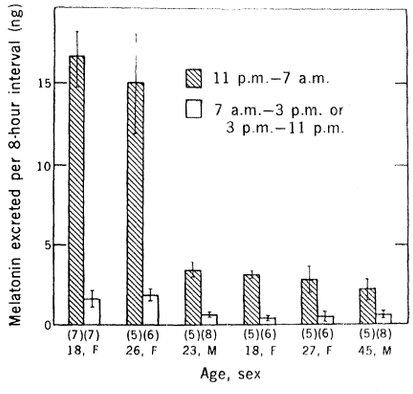

Fig. 1. Urinary melatonin content per 8-hour interval in numerous nonconsecutive samples obtained from six healthy adult subjects. The data are means for the numbers of samples shown in parentheses; error bars show standard errors.

A modification of the quantitative bioassay technique reported earlier [the dermal melanophore response of larval anurans to melatonin in their bathing medium (*10*)] was used to estimate the melatonin contents of urine extracts (*11*). The extent to which melatonin could be recovered from urine by this isolation procedure was determined by adding approximately 30 ng of [^{3}H]-melatonin (*12*) to 50- to 300-ml samples of urine and monitoring the decrement in radioactivity with each step of the process. Virtually all of the melatonin was retained by the column and eluated by the organic solvent mixture. Thereafter, a 20 to 25 percent loss was incurred in the course of the various washes and extraction steps. Melatonin recoveries were also determined by adding known amounts (2 to 16 ng) of authentic, unlabeled melatonin to 100-ml samples of urine, which were then run through the extraction procedure and bioassayed. Overall recoveries of 75 to 80 percent were obtained. Thin-layer chromatography was used to confirm that the biologically active component of the urine extract was melatonin. In each of five solvent systems, the melanophorotropic constituent of the urine extract was found to exhibit the same chromatographic mobility on silica gel–coated sheets as authentic melatonin chromatographed concurrently (*13*).

Urine was collected during seven consecutive 8-hour periods from six subjects. In all cases, the melatonin contents of the samples collected between 11 p.m. and 7 a.m. were several-fold greater than those observed in any of the daytime samples (7 a.m. to 3 p.m. or 3 p.m. to 11 p.m.). While the total amount of melatonin excreted varied markedly among individual subjects, the pattern of melatonin excretion tended to be consistent; the amplitude of the daily variation appeared to be characteristic for each individual (Table 1). In some daytime 8-hour specimens, no melanophorotropic activity could be detected. The

limit of sensitivity of the bioassay, as applied in this study, was estimated to be 0.6 ng of melatonin per 8-hour urine sample.

In several, nonconsecutive 8-hour urine specimens collected over a month from the same six subjects, the mean amount of melatonin in the nighttime samples for each subject significantly exceeded the mean amounts observed in daytime samples (Fig. 1).

These observations confirm that at least a portion of the melatonin secreted from the human pineal finds its way chemically unchanged into the urine (*4*). They also demonstrate that (i) the rate of melatonin excretion, and possibly secretion, is greatest during nighttime (that is, at the time of day when melatonin synthesis is greatest in rats (*3*) and (ii) the total amount of melatonin excreted in a 24-hour period varies considerably among individuals. Further studies will be needed to identify the sources of this variation (such as age- or sex-related differences in the secretion of melatonin, or in its rate of metabolism and clearance) and to determine the extent to which the human melatonin rhythm is entrained by light-dark cycles, sleep, rhythms in motor activity, or food consumption (*14*).

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7. Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa.) was washed and hydrated by stirring successively with four bed volumes of acetone four times, three bed volumes of methanol three times, and three bed volumes of deionized water three times. The washed resin was then suspended in degassed water, stirred under a vacuum for 30 minutes, and refrigerated in water for at least 1 week before it was used. General-purpose chromatographic columns measuring 6 by 200 mm with a 50-ml reservoir were plugged with glass wool, and hydrated Amberlite was slurried into each column to a depth of 15 cm. Each column was then washed with 50 ml of degassed water, and a small plug of glass wool was placed on top of the resin bed. An aliquot of urine was applied to a column by measuring the urine into a 125-ml Florence flask, which was then inverted over the column. In this way, a low constant hydrostatic head and a suitable flow rate were maintained.
8. Salt saturation prevented the partition of isopropanol into the aqueous phase and allowed the alkaline and acid washes to be accomplished with minimal loss of melatonin.
9. The evaporation under nitrogen of multiple small samples of organic solvent was facilitated by the use of a plastic manifold equipped with interchangeable glass capillary tubes. The construction of such an assembly will be described elsewhere (H. J. Lynch, R. J. Wurtman, M. A. Moskowitz, M. C. Archer, in preparation).
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11. Light-adapted larval *Rana pipiens* were placed in the urine extract. Test animals were also placed in solutions containing known concentrations of authentic melatonin that had been put through a similar extraction procedure. The melanophore response of each animal was then quantitatively evaluated by microscopic examination, and a calibration curve was plotted relating melanophore response to melatonin concentration. From this curve the melanophore response of test animals exposed to urine extracts could be interpreted in terms of the melatonin contents of the extracts.
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13. In each of the five solvent systems, the R_F of authentic melatonin, and the R_F range within which the melanophorotropic constituent of chromatographically separated urine extract was located, were as follows: (i) methyl acetate, isopropanol, and 25 percent ammonia (45 : 35 : 20), 0.92 and 0.82–1.00; (ii) ethanol, 0.75 and 0.65–0.85; (iii) *n*-butanol, 0.63 and 0.53–0.73; (iv) chloroform, methanol, and acetic acid (93 : 7 : 1), 0.58 and 0.48–0.68; and (v) ethyl acetate, 0.25 and 0–0.35.
14. Some of the studies have previously been described; H. J. Lynch and R. J. Wurtman, paper presented at the symposium, "Advances in fertility regulation through basic research," held at Rockefeller University, New York, 15 and 16 July, sponsored by the Population Council and the Center for Population Research of the National Institute of Child Health and Human Development.
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5-Methyltetrahydrofolic Acid Is Not a Methyl Donor for Biogenic Amines: Enzymatic Formation of Formaldehyde

Abstract. *Contrary to previous reports, 5-methyltetrahydrofolic acid does not mediate the methylation of dopamine to epinine. Instead, this methyl donor is degraded enzymatically to formaldehyde, which condenses with dopamine to form a tetrahydoisoquinoline derivative. The latter has chromatographic characteristics very similar to those of epinine, which likely accounts for the original misidentification of the product.*

It has recently been reported that 5-methyltetrahydrofolate (5-MTHF) is an active methyl donor for biogenic amines such as dopamine, serotonin, and tryptamine (*1, 2*). The reported production of methylated metabolites such as dimethyltryptamine, a highly active psychotomimetic (*3*), has led to speculation regarding the possible implication of 5-MTHF in psychotic disorders such as schizophrenia (*4*).

Because of our interest in biogenic amine methylation reactions (*5*), we attempted to duplicate these findings. In doing this, we found that the compounds formed were chromatographically very similar to, but not identical with, the expected methylated products.

We have shown that in human blood *S*-adenosylmethionine, under certain conditions, can generate formaldehyde by an enzymatic process. Formaldehyde then reacts nonenzymically with indoleamine substrates present in the incubation medium to yield condensation products that are chromatographically very similar to the expected methylated compounds (*6*). The apparent identity, on the basis of our criteria (*6*), of the products formed in 5-MTHF–mediated incubations with those formed in the *S*-adenosylmethionine–mediated blood studies prompted us to investigate the possibility that formaldehyde could be generated from 5-MTHF. Results of our studies on the reactions of indoleamine substrates with 5-MTHF have been described (*7*). We now present evidence that incubation of dopamine with 5-MTHF results not in the production of *N*-methyldopamine (epinine) but rather in the enzymatic formation of formaldehyde which condenses nonenzymatically with dopamine to form 6,7-dihydroxytetrahydroisoquinoline (TIQ) (*8*). We have also found that formaldehyde is generated from 5-MTHF in blood platelet preparations (*9*).

Rat brain enzyme was prepared and incubated essentially by the method of Laduron *et al.* (*10*). Incubation mix-