nection with the optic lobes. This pathway might or might not be part of the visual system. As suggested for the pineal of sparrows (*23*), the optic lobes of the cricket could house a self-sustaining oscillator, driving a damped oscillator in another part of the brain which directly controls singing activity. Entrainment, then, of bilobectomized crickets would be due to direct driving of the damped oscillator by the temperature cycle. Previous studies have argued against multiple oscillators for different behaviors in this cricket species (*24*). It seems even more likely that one behavior is not controlled by as many oscillators as there are Zeitgebers. Therefore, a more parsimonious explanation demands a single timing device which receives afferent input of hierarchically ordered environmental stimuli which act as Zeitgebers.

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8. Hereafter any use of the words singing, calling, or stridulation refers to the calling song of male *Teleogryllus commodus*.
9. Housing, recording, rearing, and surgical techniques are described in detail elsewhere (*6*). Forty unoperated and 20 bilobectomized crickets were recorded. The light intensity in all LL and LD experiments was 30 to 35 lux. All LD cycles had the lights on from 0800 to 2000 and the lights off from 2000 to 0800 P.S.T., and all HTLT cycles were at 35°C from 0800 to 2000 and 25°C from 2000 to 0800 P.S.T. Parallel experiments were run in DD (constant dark) with results similar to those obtained in LL conditions. Results of exposure of bilobectomized crickets to LD have also been described (*6*). The daily temperature cycle (inset, Fig. 3) was effected by combining a high-wattage heater with a forced air uptake and removal system. The temperature regime was recorded in each individual cricket chamber by copper-constantan thermocouples attached to a Honeywell recording potentiometer at 6-second intervals for the period around the transitions and 5-minute intervals for the rest of the 24-hour period. The total time for an experiment in *T. commodus* was restricted to between 40 and 60 days, which is the average lifespan (from imaginal molt to death) of a normal male.
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15. "Zeitgeber" is used here in its strictest sense as that forcing oscillation which entrains a biological rhythm [J. Aschoff *et al.*, in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), p. xiv]. We mean to make a sharp distinction between those driving oscillations that simply trigger or release the behavior and those that actually reset the biological clock with each cycle.
16. *T. commodus* housed in constant light (30 lux) and constant temperature (25° ± 1°C) were placed for 12 hours (0800 to 2000 P.S.T.) at 5°C, LL (30 lux), and then returned to the constant light and temperature conditions. Statistical analysis of the period beginning 24 hours after the initiation of the cold pulse in normal ($N = 2$) and bilobectomized ($N = 5$) animals shows a significantly (Student's t, $P < .01$) greater singing activity during the 12-hour period corresponding to the cold pulse (0800 to 2000) as compared to the subsequent 12-hour period (2000 to 0800). This difference lasts for approximately 2 days after the cold pulse and then begins to damp out.
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18. Best fit projection of the free-running rhythm in Fig. 1 reveals that if the thermoperiod was merely masking the expression of the free run we would find the singing on day 34 beginning at 0800 rather than at 2000. The 3.5-hour bout of singing that does begin at 0800 on day 34 is a transient extension of the previous day's singing activity due to the novel absence of the step up in temperature.
19. Normal ($N = 5$) and bilobectomized ($N = 3$) animals were allowed to entrain to HTLT 12 : 12, LL (30 lux) and then subjected to HTLT 15 : 15, LL (30 lux). Normal animals returned to a free-running singing rhythm and lobectomized ones to an arrhythmic pattern during exposure to the 30-hour cycle. Fourier analysis of the lobectomized animals showed no significant (± 2 standard errors) periodicities over the range from 1 hour to infinity. Both groups entrained again to HTLT 12 : 12 after termination of the 30-hour cycle.
20. Arrhythmia here means a total desynchronization with no apparent periodicities. For instance, Fourier analysis of the activity depicted in Fig. 2 for days 30 to 42 (LL, 25°C constant) shows no significant periodicities within 95 percent confidence limits. The similarity in the total amount of singing in lobectomized and normal crickets (*6*) indicates that the operation is not quantitatively releasing or suppressing the singing.
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3 June 1975

Heteropolypeptides from Poly-α-Cyanoglycine and Hydrogen Cyanide: A Model for the Origin of Proteins

Abstract. *Poly-α-cyanoglycine, a homopolymer synthesized from the* N-*carboxyanhydride of α-cyanoglycine, is converted by cumulative reactions of hydrogen cyanide to heteropolypeptides that can be hydrolyzed to protein amino acids, including glycine, alanine, valine, aspartic acid, and glutamic acid. These results are consistent with the hypothesis that the original heteropolypeptides on the earth arose spontaneously from hydrogen cyanide and water without the intervening formation of α-amino acids.*

During the past two decades, extensive research (*1*) on chemical evolution has led to the widespread belief that the formation of primitive proteins occurred in two stages: α-amino acid synthesis initiated by the action of natural sources of high energy on the components of a reducing atmosphere followed by polycondensation of the accumulated monomers in the oceans or on land. A more critical examination of the evidence for the second step, however, suggests that the inherent thermodynamic barrier to spontaneous polymerization of α-amino acids has only been overcome by invoking specific environments—anhydrous locales, high-temperature milieus, or acidic bodies of water, for example—that may not be characteristic of a young, developing planet. We now present experimental results consistent with an alternative route proposed for the origin of proteins—the direct synthesis of heteropolypeptides from hydrogen cyanide and water without the intervening formation of α-amino acids.

According to this hypothesis (*2*), a low-energy pathway exists (Fig. 1) that allows hydrogen cyanide (**1**) to polymerize readily to polyaminomalononitrile (**4**) via dimeric hydrogen cyanide (**2**) and its polymer (**3**). Semiempirical quantum-mechanical calculations (INDO) suggest that **2** is probably azacyclopropenylidenimine (*3*) rather than iminoacetonitrile or aminocyanocarbene (*4, 5*), other possible structures that could also lead to **3**. Successive reactions of hydrogen cyanide with the reactive nitrile groups of **4** then yield heteropolyamidines (**5**) which on contact with water are converted to heteropolypeptides (**6** and **7**) after a series of hydrolysis and decarboxylation steps (*6, 7*).

To demonstrate the feasibility of this postulated conversion of a homopolymer to a heteropolymer it would be desirable to synthesize **4** and transform it to **7** by treat-

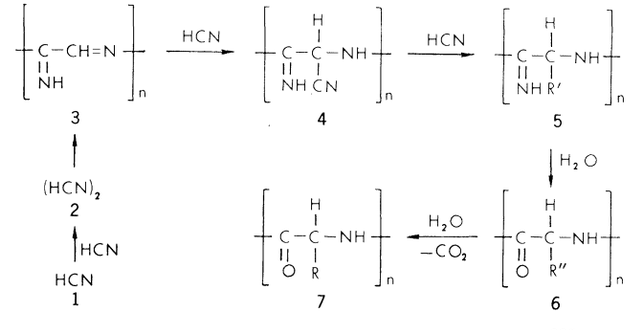
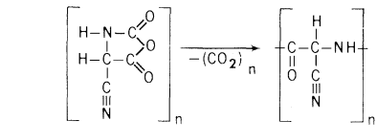


Fig. 1. Proposed route for heteropolypeptide synthesis from hydrogen cyanide and water (**1** → **7**). R″ and R′ are precursors of protein side chains R.

Table 1. Distribution of ^{13}C-labeled species for amino acids derived from poly-α-cyanoglycine and labeled HCN (90 percent H^{13}CN).

Amino acid	C atoms/ molecule	Mole percent of amino acid with the following number of ^{13}C atoms:					
		Zero	One	Two	Three	Four	Five
Glycine	2	57	12	31			
Alanine	3	61	21	11	7		
β-Alanine	3	62	23	9	6		
Aspartic acid	4	41	29	18	12	0	
Diaminosuccinic acid	4	49	30	13	8	0	
α-Aminobutyric acid	4	46	18	29	7	0	
Glutamic acid	5	78	13	5	2	2	0
Valine	5	67	33	0	0	0	0

ment first with hydrogen cyanide and then with water. A more accessible approach, however, is to use poly-α-cyanoglycine (**8**),



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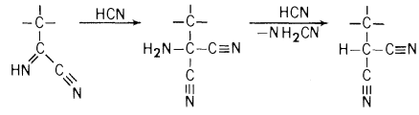

10 11 12

since this polyamide analog of the polyamidine (**4**) can now be obtained from α-cyanoglycine *N*-carboxyanhydride (**9**) (*8*) by well-established procedures (*9*). We report here the first synthesis of poly-α-cyanoglycine (*10*) and its modification by hydrogen cyanide and water to heteropolypeptides possessing at least five of the side chains of proteins today (*11*).

Ideally, such an investigation would involve simply the treatment of **8** with hydrogen cyanide followed by total hydrolysis and amino acid analysis. According to this model, hydrolysis of pure **8** would give glycine quantitatively since polyglycine would be formed by spontaneous decarboxylation of the α-carboxyl intermediate, polyaminomalonic acid (*12*), whereas after reaction of **8** with hydrogen cyanide and water, other amino acids would also be expected (*13*). A complication immediately arises, however, because of the concurrent synthesis of heteropolypeptides that might also take place via the polymerization of HCN. We therefore used labeled hydrogen cyanide (90 percent H^{13}CN) so that the relative contributions of the two possible processes for heteropolypeptide formation could be estimated.

In a typical experiment, labeled HCN generated from sulfuric acid (concentrated) and potassium cyanide (90 percent K^{13}CN, 1.0 g, 0.015 mole) was transferred in vacuo to a reaction chamber immersed in liquid nitrogen, containing a solution of **8** (0.0138 g) in freshly distilled acetonitrile (6 ml); any water from the generating flask was removed in transit by a cold trap cooled to −50°C by an acetone–water–Dry Ice bath. Ammonia (25 ml at standard pressure and temperature, 0.001 mole) was introduced as a catalyst, and the liquid nitrogen bath was replaced by a magnetic stirrer. After 40 days the reaction mixture had changed from yellow to orange to dark brown in color. Unreacted hydrogen cyanide and ammonia, together with the solvent, were removed in vacuo (*14*), leaving a brown residue, which was hydrolyzed (6*N* HCl) and lyophilized, and then converted to *N*-trifluoroacetyl *n*-butyl esters (TAB derivatives) for analysis by combined gas chromatography–mass spectrometry (*15, 16*). Protein amino acids detected (in micromoles per gram) included glycine (10,600), alanine (1,550), valine (20), aspartic acid (250), and glutamic acid (70), all with considerable ^{13}C incorporation. Also present were about 30 other compounds including the nonprotein amino acids β-alanine (100), diaminosuccinic acid (200), and α-aminobutyric acid (400). The distribution of labeled carbon for each amino acid was calculated (*17*) from the peak ratios of relevant fragment ions obtained from their mass spectra. A representative mass spectrum for the aspartic acid product is shown in Fig. 2, compared with a standard spectrum. Results for the eight observed amino acids are summarized in Table 1.

Under the conditions used, polymerization of hydrogen cyanide was not a predominant process since little or no fully labeled amino acids with side chains could be detected. Instead, the overall distribution of label (*13, 14*) suggests that extensive modification of the nitrile groups of **8** had taken place, probably by way of the intermediates **10**, **11**, and **12** (*18*). We conclude that hydrogen cyanide and water can convert poly-α-cyanoglycine **8** (and therefore polyaminomalononitrile **4**) to heteropolypeptides possessing side chains of today's proteins (*19*), in accord with the hypothesis that primitive proteins on the earth originated directly from hydrogen cyanide polymers rather than by the condensation of α-amino acids.

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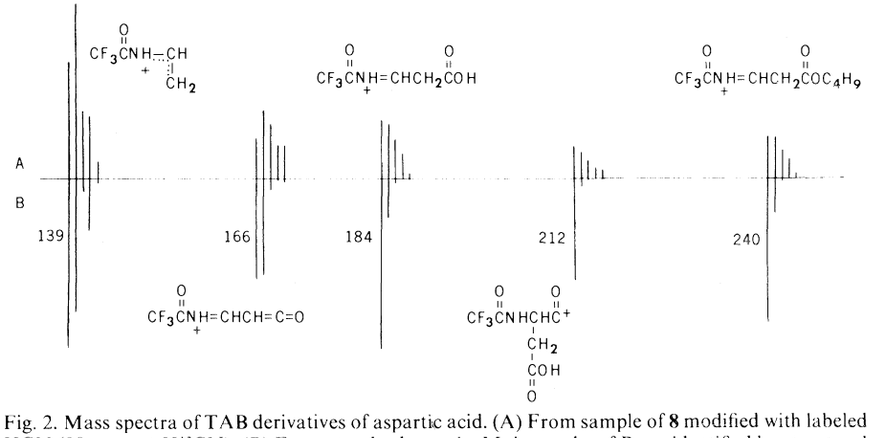

Fig. 2. Mass spectra of TAB derivatives of aspartic acid. (A) From sample of **8** modified with labeled HCN (90 percent H^{13}CN). (B) From standard sample. Major peaks of B are identified by structural formulas and numbers (amu).

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10. Poly-α-cyanoglycine (**8**) was synthesized by the base-catalyzed polymerization of α-cyanoglycine *N*-carboxyanhydride (**9**) (*8*). In a representative procedure carried out in a nitrogen dry box, a solution of **9** (4.5 g) in acetonitrile (50 ml) was stirred for 30 days at room temperature with two drops of a 1 percent solution of water in acetonitrile (*9*). A light brown precipitate formed that was filtered through fritted glass and dried in vacuo to yield a pale yellow powder (3.0 g). The weight average molecular weight determined from gel permeation chromatography was estimated at about 1300, corresponding to some 16 monomer units. Characteristic spectra show absorptions at 2245 cm^{-1} and 2200 cm^{-1} (infrared, $-C \equiv N$) and at 197 to 200 nm (ultraviolet).
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13. Hydrolysis of **8** yielded not only glycine but traces of alanine and aspartic acid, presumably formed from HCN eliminated during the synthesis of **8** from **9**. This suggests that in our experiment with $H^{13}CN$ and **8** exchange of nitrile groups might take place and could be one source of label in the amino acid products.
14. The material that was removed in vacuo was allowed to stand for 5 weeks when a tan polymer of hydrogen cyanide formed. On hydrolysis, this yielded glycine, alanine, valine, aspartic acid, and other compounds rich in ^{13}C (80 to 90 percent), showing that polymerization of this enriched HCN (90 percent $H^{13}CN$, 10 percent $H^{13}CN$) could be a source of label in the reaction with **8**.
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16. All mass spectral data were obtained on a model 3200 Finnigan gas chromatograph–mass spectrometer coupled with a model 6000 data system. The range m/e 35 to 300 was scanned approximately once per second and all data were stored on magnetic disc. Peak intensities were derived from single spectra after background subtraction, or in the case of low intensity peaks from the area of the reconstructed mass chromatogram for a particular ion. Gas chromatography conditions are described in (*15*).
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18. Structures **8** and **10** are precursors of glycine; **11**, of diaminosuccinic acid; and **12**, of aspartic acid and, perhaps alanine. Detailed mechanisms now being elucidated for side chain modification of **8**, particularly the steps involving the use of HCN as a reducing agent, are consistent with these labeling results as well as with the results obtained by deuterolysis of **8** with DCl-D_2O before and after HCN modification. We have found that for the amino acids derived from **8** by deuterolysis all hydrogen atoms attached to carbon atoms can be replaced by deuterium.
19. S. Akabori and M. Yamamoto [in *Molecular Evolution, Prebiological and Biological*, D. L. Rohlfing and A. I. Oparin, Eds. (Plenum, New York, 1972), p. 189] restate Akabori's 1953 proposal that the original heteropolypeptides on the earth were formed by polymerization of aminoacetonitrile to a polyamidine "fore-protein" which was hydrolyzed to polyglycine and then modified by aldehydes, and the like, to heteropolypeptides. We consider this model improbable because of the instability of aminoacetonitrile and the inertness of polyglycine. Diglycine and triglycine reported from attempts to polymerize aminoacetonitrile most probably were formed instead from eliminated hydrogen cyanide (*6*). We have shown that deuterolysis of **8** with DCl-D_2O yields glycine containing two atoms of deuterium per molecule more than glycine obtained by deuterolysis of polyglycine. This striking demonstration of the reactivity of the α-hydrogen and α-cyano groups of **8** suggests that its polyamidine analog **4**, derived solely from HCN, is a far more likely precursor of proteins than polyglycine or the fore-protein of Akabori.
20. We thank S. Kammeyer, R. Widing, and Dr. C. Warren for experimental help.

8 July 1975

Solubilization of a Stereospecific Opiate-Macromolecular Complex from Rat Brain

Abstract. *A [^{3}H]etorphine-macromolecular complex has been solubilized from rat brain synaptosomal fraction by extraction with the nonionic detergent Brij 36T. Stereospecificity of binding to this solubilized complex was demonstrated by the finding that radioactivity in the complex was virtually eliminated when binding had occurred in the presence of excess levorphanol, an active narcotic analgesic, while it was unaffected by its inactive enantiomorph dextrorphan. Bound radioactivity was dissociated by proteolytic enzymes, sulfhydryl reagents, and heat, suggesting the presence of protein. The bound solubilized macromolecular moiety may be the opiate receptor.*

The existence of stereospecific opiate binding sites in animal brain was discovered independently about 2 years ago in three laboratories, including our own (*1, 2*). Since then much has been learned about the properties of these binding sites by studying opiate binding in membrane preparations derived from brain homogenate. The observed properties are consistent with the hypothesis that these sites represent pharmacological opiate receptors. The exact chemical structure of these receptors and many aspects of receptor-drug interaction can only be learned when receptor molecules are available in soluble and highly purified form. As a first step toward this end we report the solubilization of etorphine (*3*) bound stereospecifically to a macromolecular moiety and present data suggesting that this moiety may be the opiate receptor.

The brains of Sprague-Dawley rats, after removal of the cerebella, were homogenized and used to prepare mitochondrial-synaptosomal (P_2) fractions (*4*). The P_2 fraction, resuspended in 0.32*M* sucrose, was diluted five-fold with 0.05*M* tris buffer, *p*H 7.4, to give the appropriate tissue concentration of an osmotically lysed membrane preparation. Prior to extraction with detergent, the P_2 membranes were incubated with [^{3}H]etorphine at 37°C for 20 minutes. As described earlier (*2, 4*), incubations were carried out in the presence of the active narcotic analgesic levorphanol (10^{-6}*M*) or in an equal concentration of its inactive enantiomorph dextrorphan, in order to establish the degree of stereospecificity of the etorphine binding (*5*). The membrane fraction was then centrifuged at 20,000*g* for 15 minutes at 4°C. The resulting pellet was resuspended in one-tenth the original volume of an ice-cold 1 percent solution of the nonionic detergent Brij 36T (Emulsion Engineering, Elk Grove Village, Ill.) in 0.01*M* tris buffer, *p*H 7.4, containing 0.2 m*M* dithiothreitol and 1 m*M* EDTA (*6*). This suspension was mixed briefly on a Vortex vibrator and immediately centrifuged in a Beckman preparative ultracentrifuge at 100,000*g* for 90 minutes. Free and bound etorphine in the supernatant were separated by passage through a column of XAD-4 Amberlite and elution with cold 0.05*M* tris, *p*H 7.4. Fractions (1 ml) were collected, and radioactivity was determined by counting portions in Aquasol in a liquid scintillation spectrometer. Protein concentration was determined by the method of Lowry *et al.* (*7*).

Free [^{3}H]etorphine adheres firmly and quantitatively (> 99 percent) to the XAD-4 column and can only be eluted with methanol or ethanol. Figure 1 shows another control in which [^{3}H]etorphine was added to a Brij extract of P_2 membranes (we previously ascertained that no binding occurs in the presence of 1 percent Brij). When the supernatant from ultracentrifugation of the extract was passed through a column of XAD-4, the bulk of protein (> 85 percent) appeared in the void volume, while the amount of radioactivity eluted was less than 1 percent.

When [^{3}H]etorphine was first bound to P_2 membranes in the presence of a 1000-