Table 2. Average genetic variability for all populations kept in constant environments, in which one factor varied, and in environments in which three factors varied.

Variables	Heterozygosity per individual (% ± S.E.)	Alleles per locus (No.)
0	7.81 ± 0.31	1.67 ± 0.02
1	9.62 ± 0.84	1.92 ± 0.13
3	13.36 ± 0.76	2.12 ± 0.17

drosophila medium) and f_2 [Spassky's medium (*13*)] indicate the type of medium each cup contained. Cages were kept at 25°C, 19°C, or on alternate weeks at 25°C and 19°C (symbolized 19/25). Thus five cages, 1, 2, 3, 6, and 9, had constant environments; six cages, 4, 5, 7, 8, 10, and 11, had one condition varied; two cages, 12 and 13, had all three conditions varied.

After the populations were maintained for 45 weeks, or about 15 generations, samples of adult individuals were assayed for genetic variability by starch-gel electrophoresis, with the use of techniques already described (*2*). Twenty-two enzyme loci were studied in each population (*14*). Fifty individuals from each population were assayed for each enzyme. Therefore, except for two sex-linked loci for which males were assayed, 100 genomes were studied at each locus in each population.

Two measures of genetic diversity are given in Table 1. The first is the percentage of average heterozygosity per individual, as calculated by Lewontin and Hubby (*1*) except that actually observed heterozygotes are used instead of assuming Hardy-Weinberg equilibria. (The Hardy-Weinberg assumption had to be used for the two sex-linked loci for which males were assayed.) The standard deviations for these figures were calculated by averaging the binomial variance of the frequency of observed heterozygotes at each locus (*15*). The second measure of diversity is the average number of alleles maintained at a locus. Since the sample size was the same in all cages, this meant that an allele had to be present at a frequency of 1 percent or greater to be included in this measurement.

As is shown in Table 1, populations in more variable environments have maintained more genetic variability. This is true for both those factors that are available to the population at the same time (spatial heterogeneity) and for the factor that varies with respect to time (temporal heterogeneity). Combining all three factors—yeast, medium, and temperature—increases genetic variability more than any single factor alone.

Table 2 gives the average genetic variability for all populations kept in constant, one-variable, and three-variable environments. Again, more variable environments maintain more genetic heterogeneity by either measure.

Using the technique of gel electrophoresis to determine total genetic variability in a population is subject to several biases (*1*). However, in my study, it is not so important to know the total genetic variability of the population, but it is sufficient to have shown that the type of variability detected by electrophoresis can be affected by varying the environment of the population.

The results show that at least some of the protein polymorphisms in these experimental populations are maintained by environmental heterogeneity and are not neutral to natural selection. In the natural population from which these experimental populations were begun, the average heterozygosity per individual was about 19 percent (*2*). In the experimental populations the average heterozygosity per individual ranged from about 8 percent in the most constant environments to about 13 percent in the most varied. As *D. willistoni* is native to Neotropical rain forests, it undoubtedly meets a more complex and diversified environment than can be easily created in the laboratory.

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15. Whenever the frequency of observed heterozygotes was less than .05, the variance was calculated as if its frequency were .05. This would cause overestimation of the variance. The variances were averaged over the 22 loci and the square root was taken as the standard deviation.
16. I thank Natalie Feng for technical assistance and Drs. Th. Dobzhansky, F. J. Ayala, and W. W. Anderson for advice and encouragement. This work was done at the Rockefeller University and was supported by NSF grant GB-20694 and AEC contract AT(30-1) 3096.

10 August 1971; revised 7 September 1971

Barbiturates: Radioimmunoassay

Abstract. *The development of a radioimmunoassay for barbiturate is described. The barbiturate is made antigenic by coupling it to a protein, bovine gamma globulin. The radioimmunoassay can measure as little as 5 nanograms of barbiturate.*

For study of the metabolism of barbiturates, quantitative assays that are rapid, sensitive, specific, and reliable even for small amounts of barbiturates would be most advantageous. Methods now available are suited for qualitative and quantitative analysis only if an adequate sample of biological tissue, 1 to 5 ml, is available (*1*). In addition, these methods require solvent extraction (*1*) and, in some instances, filtration and evaporation (*2*). Gas chromatography and spectrophotometry are sensitive to 10 μg/ml but require solvent extraction (*3*). Immunologic methods for assaying polypeptides, hormones, and drugs have been reported (*4*). We have conjugated a barbiturate to bovine

gamma globulin (BGG), produced antibodies against the barbituate hapten, and developed a radioimmunoassay capable of measuring nanogram levels of barbiturates.

Antibodies were induced by immunization of rabbits with a barbiturate-protein conjugate. The barbiturate, 5-allyl-5-(1-carboxyisopropyl) barbituric acid, was converted to 5-allyl-5-(1-*p*-nitrophenyloxycarbonylisopropyl) barbituric acid by reacting the free base (10 mg) with *p*-nitrophenol (12 mg) in *N*,*N*-dimethylformamide for 24 hours at 4°C. The 5-allyl-5-(1-*p*-nitrophenyloxycarbonylisopropyl) barbituric acid was coupled to BGG (10 mg) in a glycerin-water solution (1 : 1, by volume) in the presence of dicyclohexylcarbodiimide (5 mg) (*5*). The mixture was incubated overnight at 4°C, and the protein-hapten complex was dialyzed against distilled water. Conjugation of the barbiturate to the protein carrier was confirmed by the increase in absorbance at 202 nm of the barbiturate-BGG conjugate as compared to control BGG solutions. From the molar extinction coefficient of the barbiturate (E_m = 19,500), the degree of substitution was estimated to be 2 to 3 moles of barbiturate per mole of protein. New Zealand albino rabbits were immunized with 1 mg of barbiturate-BGG (Fig. 1). The immunogen (100 μg) in phosphate-buffered saline, *p*H 7.2, was emulsified with an equal volume of complete Freund's adjuvant. The initial dose was 1.6 ml, 0.4 ml injected into each footpad. A booster injection of 100 μg of antigen in adjuvant was given every 6 to 8 weeks, 25 μg in each of the footpads. Blood was collected 5 to 7 days after booster injections and the serum was examined for antibodies to barbiturates.

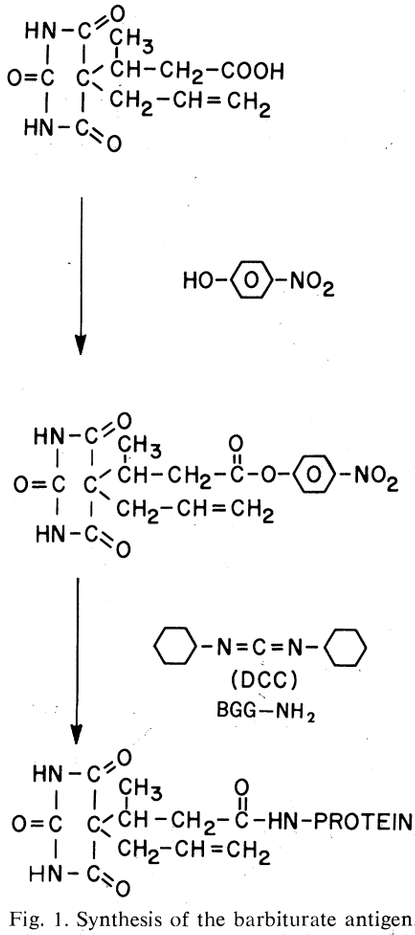

Fig. 1. Synthesis of the barbiturate antigen. Abbreviations used are as follows: DCC, dicyclohexylcarbodiimide; BGG, bovine gamma globulin.

Various dilutions of antiserums were incubated with 8×10^{-4} μc of [^{14}C]-pentobarbital sodium (New England Nuclear, 4.13 mc/mmole), approximately 1000 count/min, at 4°C overnight. After incubation, a neutral saturated ammonium sulfate solution (volume equal to incubation medium) was added to all tubes. The precipitate, containing pentobarbital bound to antibody, was washed two times with an equal volume of 50 percent saturated ammonium sulfate and then dissolved in 0.5 ml of Nuclear-Chicago Solubilizer (*6*), and the radioactivity was counted in a liquid scintillation spectrometer (Packard Tri-Carb). While normal rabbit serum failed to bind labeled pentobarbital, the serum from immunized rabbits bound 75 to 80 percent of the added labeled pentobarbital, and there was a linear relationship between bound [^{14}C]pentobarbital and the concentration of added antibody (Fig. 2A). When variable amounts of [^{14}C]pentobarbital were added to a constant amount of antibody, there was a linear relationship between added and bound [^{14}C]pentobarbital (Fig. 2B).

The radioimmunoassay depends on competition between unlabeled pentobarbital and a standard of [^{14}C]pentobarbital for combination with barbiturate antibodies in rabbit antisera. A tube that contained radioactive pentobarbital and antiserum, but no unlabeled pentobarbital, measured maximum radioactivity bound to antibody. The addition of increasing amounts of unlabeled pentobarbital to fixed amounts of [^{14}C]pentobarbital and antiserum resulted in competitive inhibition of binding of labeled pentobarbital by antibody (Fig. 3). The similarity of the standard curves obtained when pentobarbital was added in plasma, urine, or buffered saline indicates that there are no interfering substances in the two body fluids. In addition, the data demonstrate the sensitivity of the method. Pentobarbital (5 ng) in a sample volume of 10 μl caused a 20 percent inhibition of binding of the labeled compound. The same amount of pentobarbital can be assayed in a larger sample volume (200 μl), which increases the sensitivity 20-fold.

The antibody bound barbital, pentobarbital, and phenobarbital equally well. These three compounds differ only by the substituents on the C-5 position. Since BGG was conjugated to the barbituric acid moiety at C-5 it is understandable that the antibody fails to differentiate between these barbituric acids. In contrast, at equimolar concentration of hexobarbital or thiopental the antibody bound these compounds to a lesser degree. These compounds have

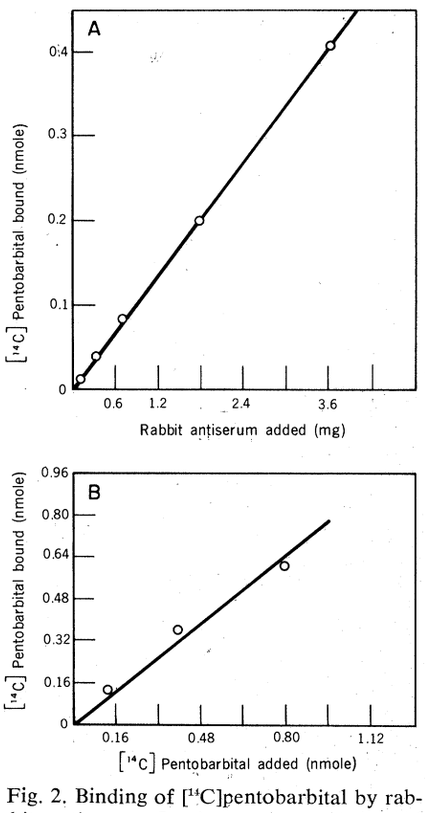

Fig. 2. Binding of [^{14}C]pentobarbital by rabbit antiserum. In A, varying amounts of antiserum (milligrams of protein per reaction tube) were added to a constant amount of [^{14}C]pentobarbital (0.8 nmole). In B, varying amounts of [^{14}C]pentobarbital were added to a constant amount of rabbit antiserum (3.5 mg of protein per reaction tube).

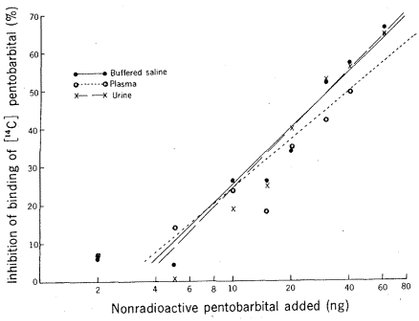

Fig. 3. Inhibition of binding of [^{14}C]pentobarbital to rabbit antiserum by nonradioactive pentobarbital in buffered saline (●——●), plasma (○——○), or urine (×——×). Incubation medium consisted of 0.10 ml of normal rabbit serum, 0.10 ml of rabbit antiserum (0.4 mg of protein), 0.01 ml of [^{14}C]pentobarbital (0.1 nmole), and 0.01 ml of either standard unlabeled pentobarbital (1 to 100 ng) or unknown sample and sufficient phosphate-buffered saline (0.01*M* phosphate, *p*H 7.4) to make a final volume of 0.50 ml. Lines of regression were calculated by the method of least squares.

different substituents at either position 2 or 3 in the barbituric acid ring. Thus, the urea portion of the ring may be critical in determining antibody specificity.

The barbiturate-BGG antigen was effective in eliciting antibodies against barbituric acid derivatives. We believe that this is the first report of the experimental production of antibodies capable of recognizing barbiturates. The radioimmunoassay technique reported here is rapid and extremely sensitive, and should be useful for the determination of barbiturate concentrations in biological tissues and fluids. Theoretically, since metabolic products with changes at the C-5 position may also be detected by the antibody, our procedure, coupled with a solvent extraction, could measure both total concentration of barbiturate and its hydroxylated metabolites. Antibodies directed against steroid haptens (*7*) and digitalis (*8*) have been reported to modify the physiological actions of these agents, and it would be interesting to determine whether antibodies against barbiturates interfere with the pharmacological effects of barbiturates.

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1 June 1971; revised 9 August 1971

Formaldehyde and Ammonia as Precursors to Prebiotic Amino Acids

Fox and Windsor (*1*) reported synthesis of a number of amino acids when mixtures of formaldehyde and ammonia were heated at 185°C for 8 hours. These reactants were chosen because they have been identified in contemporary interstellar matter, and thus "reaction of the two is more easily visualized as a consequence of this coexistence."

A number of molecules have been found in interstellar clouds, including several diatomic and several polyatomic species (*2*). These discoveries have naturally led to speculation on the relation between the existence of such molecules and the origin of life on earth. However these speculations usually disregard the extremely low volume densities (from 10^{-2} to 10^{-5} molecules per cubic centimeter) and column densities (from 10^{13} to 10^{16} molecules per square centimeter) in the clouds.

For example, consider the experiments cited above. Fox and Windsor used formaldehyde and ammonia concentrations in the moles per liter range. This is some 10^{24} times as great as the maximum expected in interstellar clouds. Mild extended thermal treatment of the type used in the experiments causes the system to approach thermodynamic equilibrium. Rough thermodynamic calculations show, for example, that for the reaction

$$2H_2CO + NH_3 \rightleftarrows \text{glycine} + H_2$$

the equilibrium concentration of glycine is quite high under the experimental conditions, but very low (of the order of one molecule per 1000 km^3) under the conditions existing in interstellar clouds. Equilibrium concentrations of other more complex amino acids would be less. Approach to even the low equilibrium concentrations would be very slow because of the extremely low concentrations of reactants.

The concentrations of formaldehyde and ammonia at the earth's surface could have been increased over those found in the clouds if the solar system, including the earth, formed from condensation of such a cloud. However, in this case most of the molecules would have been destroyed by the high temperatures in the condensing system. Whether or not such destruction occurred, any uncombined formaldehyde or ammonia present early in earth's history would have been lost in the primeval degassing episode that removed the rare gases.

Alternatively it might be supposed that the earth swept through a cloud containing the molecules. In this case most of them would have been destroyed by the ultraviolet radiation from the sun before reaching the earth (*3*). However, even if they were protected from photodissociation from ultraviolet light (as they seem to be, to some extent in outer space) and if all the molecules intercepted by the earth remained in its atmosphere, 10^{16} molecules of formaldehyde or ammonia per square centimeter in the cloud would give a partial pressure at the earth's surface less than 10^{-9} atmosphere—about 10^{-12} of the concentration used in the experiment. On the other hand, the molecules could have been generated easily on the primitive earth (NH_3 from outgassing, HCHO from various nonequilibrium energy sources acting on water and methane or other carbon-containing compounds). Ammonia concentrations were probably around 10^{-5} atm (*4*), orders of magnitude greater than would be obtained from a cloud, while formaldehyde gen-