Real Science Review: DNA Molecules

Student, Sally Gutierrez: Ms. Miller, we are told that DNA codes our genes with specific sequences of molecule pairs like A-T and C-G. How did scientists study the molecules that make up DNA before Watson and Crick figured out how they were bonded together in 1953?

Ms. Miller. That's a really good question, Sally. Many people don't think to ask it. The key to the Watson-Crick discovery was knowing what molecules were present in DNA and their relative amounts. It turns out that this information was discovered many years earlier, in the 1940s.

One important early paper is the one you and your teammates are going to use for a simulated peer review. That study had two key features: 1) They isolated the molecules that make up DNA by using acid to break its weakest chemical bonds. 2) Then they developed a way to separate, see, and measure the resulting molecules.

They also stumbled on a discovery that today we realize is more important than they realized. See if you can find it.

Original Report: Hotchkiss R. D. (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J. Biol. Chem. 175(1), 315–332 (1948).

Revising author: W. R. Klemm

Vocabulary Used in the Original Report

Hydrolysis: a chemical reaction in which a strong acid is used to break chemical bonds by adding the elements of water (hydrogen and oxygen). In the case of DNA, the reaction splits sugars from their bonding with nucleosides (defined below).

Hydrolysates (/hīˈdrälə¸sāt/): From the Greek words hydro and lysis, or "water break." Hydrolysates are the compounds that form when their parent molecules are split by hydrolysis.

Nucleic Acids: DNA and RNA. Though the nucleosides of DNA and RNA are bases, all the many phosphate groups (PO4) in DNA and RNA make them net acidic.

Nucleoside (n(y)o͞oklēə¸sīd)**:** a chemical derivative of purines and pyrimidines, such as DNA's bases A, T, C, G. In terms acidity, they are bases. They are bonded to the sugar (deoxyribose) in DNA.

Nucleotide (n(y)o͞oklēə¸tīd): a compound consisting of a nucleoside linked to the phosphate group (PO4) that is bonded to DNA sugar.

Optical Density: the intensity of light that passes through a target sample or solution. The light that does not pass through is absorbed by materials in the sample. Absorption differs depending on the wavelength of the light and the chemical nature of the target.

Paper Chromatography: a method used to separate different chemicals, which are put in a solvent that is dropped on a sheet of paper. As the solvent wicks along the paper, the various chemicals deposit at different points of the paper. Staining the paper and measuring the absorption of light passing through the paper, show where each chemical is located and how well it is isolated from the other chemicals in the sample.

Purine base: (pyo͝orēn): a chemical derivative of purine, such as DNA's adenine (A) and guanine (G).

Pyrimidine base (pəˈriməd͡ēn,pīˈrimə ˌd͡ēn): a chemical derivative of pyrimidine, such as DNA's thymine (T) and cytosine (C).

Ultraviolet Spectrometry (spĕk-trŏmʹĭ-trē): The measurement of light that passes through a target. In this case, it refers to the absorption of certain ultraviolet frequencies as the light passes through the nucleosides as they separate from the wicking of solvent on paper (paper chromatography).

The Quantitative Separation of Purines, Pyrimidines, and Nucleosides by Paper Chromatography.

Introduction

The separation of amino acid mixtures by migration with organic solvents in filter paper has been accomplished by many workers since it was first described in 1944 (ref. cited). The key to success is that each amino acid travels as a mostly well-defined spot as the solvent migrates through the paper. The differing properties of amino acids cause them to deposit at different points along the paper. Staining the dried paper allows one to see the location of spots. The amount is measured by the absorption of certain frequencies of ultraviolet light.

Introduction: Questions to Answer

1. If there was a hypothesis, either stated or implied, what was it?
2. How well did the authors justify doing this study?
3. What are some other related ideas that they did not test?

We separated purines and pyrimidines contained in nucleic acids and several related compounds. The solvent was n-butyl alcohol.

We present evidence that we believe indicates multiple advantages of this analysis method:

- The five bases, cytosine, thymine, uracil, adenine, and guanine, even in microgram quantities be completely separated from one another.
- The separated substances are detected by ultraviolet absorption.
- The method used an organic solvent that does not interfere with ultraviolet light measurement.
- The measurement error was less than 10%.
- Comparison of pure known standards of each base confirmed the identity of each spot.

- The method is sensitive enough to detect 1 mg. or less of a nucleic acid after it has been liberated by enzymatic or acid hydrolysis.

We identified several limitations of the method:

1. Guanine does not migrate well in butyl alcohol and lags behind at the initial point of sample application. Guanine can be migrated, by application of a second, different, solvent.
2. Two amino acids, if present, will migrate to the same point on the paper as cytosine does.
3. Large sample sizes are harder to separate, as with other kinds of paper chromatography.
4. The water content of the paper and other variables reduce consistency in the absolute location of spots in repeated trials. However, the relative separation remains reproducible.

Material and methods

Apparatus. Most chromatograms were obtained in a Pyrex glass cylinder with tight seals. Brass arms held the cylinder horizontally. An aluminum ring formed a tight seal and also served to hold horizontally placed glass troughs and rod separators, about 140 mm long. The metal used did not affect the results.

Methods: Questions to Answer

1. What acts as a control group by receiving no treatment? What is the purpose for having this group? How well does it serve that purpose?

2. What factors (variables) that might affect the results are not considered?

3. What are the advantages and disadvantages of the procedures and equipment used?

Volumes of solution larger than 0.017 cc. were placed upon a small area of the paper, locally heated by curving the strip over a horizontal glass tube,10 mm. in diameter, joined between a flask of boiling water and a reflux condenser. The fluid samples are delivered from a calibrated capillary pipette by touching it to the paper from time to time as drying occurs. There has been no indication that the presumed local drying of the paper has influenced the resolution of either pyrimidines, purines, or amino acids. Solutions were examined in 3 cc. quantities in the model DU Beckman photoelectric quartz spectrophotometer.

The paper used was Whatman No. 1. Aqueous extracts of this paper have only very low absorption in the ultraviolet, as discussed below. Butano1 was the organic solvent. The bottom of the chamber was covered with equal portions of butanol and water and the aqueous phase was brought to about 2.5 per cent concentration of gaseous ammonia by addition of concentrated ammonia. The troughs contained butanol saturated with water at, the prevailing room temperature, without added ammonia.

Two lots of each of the principal bases were used. Hoffmann-La Roche, Inc. (Basel). provided adenine, guanine, and uracil. The Schwarz Laboratories (New York) provided adenine sulfate, guanine, thymine, and yeast nucleic acid. Eastman Kodak Company (Rochester) provided uracil and yeast nucleic acid. The Dougherty Chemicals (Richmond Hill, New York) provided cytosine. In addition, we used crystalline samples of thymine, cytosine, xanthine,

hypoxanthine, adenosine, cytidine, guanosine, and thymidine, prepared in the laboratory of the late Dr. P. A. Levene. Desoxyribonucleic acid was prepared from calf thymus according to the method of Mirsky (ref).

Weighed amounts of the pure bases were dissolved in water, neutralized to about pH 7 if necessary. The concentration of nitrogen was accurately determined by Kjeldahl method. The nucleosides were not available in sufficient amount for nitrogen analysis. From these solutions the standard curves for pure bases were obtained and the mixtures submitted to separation were prepared. All calculations based upon absolute weight throughout this paper refer to the anhydrous free bases.

Nucleic acids were hydrolyzed in aqueous hydrochloric acid for 2 hours at 120 degrees. The acid was later removed by evaporation to dryness. Then the hydrolysate, dissolved in a small volume of water, was neutralized with sodium or ammonium hydroxide.

Preparation of Paper Chromatogram. Solutions to be investigated are deposited upon spots or bands distributed along a penciled "starting line" drawn transversely about 50 mm. from one end of the paper strip. The spots are narrow (10 mm. or less) in the direction of flow. Along the starting line, each mm. of length carries about 1 gamma (and preferably not more than 5 gamma) of the individual bases. We usually used paper strips 125 by 460 mm., bearing three or four initial spots approximately 10 by 20 to 25 mm. at least 10 mm. apart. Such spots can carry 20 to 100 gamma of individual bases or the degradation products of 1 mg. of nucleic acid.

The end of the paper strip is inserted into a glass trough. The end is weighted by a large glass rod so that the starting line is just at the point where the paper curves over the glass rod separators and projects downward. After the strips are in place, the bottom of the chamber is loaded with room temperature butanol-water. Then the chamber is closed. A jacket of corrugated paper is placed around the cylinder to protect it from air drafts and exposure to radiators or windows.

After 16 to 24 hours at about 25 degrees, the position of the butyl alcohol front is marked, and the strip is removed and hung upside down to dry. The individual "lanes" can be cut apart at this time for treatment with ninhydrin or other stains or for separate cutting of strip segments.

Transverse segments are now cut from each lane on a trimming board or with scissors at successive chosen intervals, such as every 10 mm. The precision of the method may often justify making segments as narrow as 5 mm., and in vacant areas segments of 20 or 30 mm. width is convenient.

Each cut piece is placed in a clean test-tube and then soaked 1 hour or more in 3.5 cc. of distilled water to dissolve the chemicals in that piece. The absorption of the solutions at 260 millimicrons is determined in the spectrophotometer. A graph or table of this absorption correlated with the distance travelled from the starting line (to the mid-point of the segment) reveals a series of absorption peaks corresponding to the separated components of the original mixture.

Results

The separation of components is illustrated in a known mixture in Figure 1 and in a tissue sample in Figure 2.

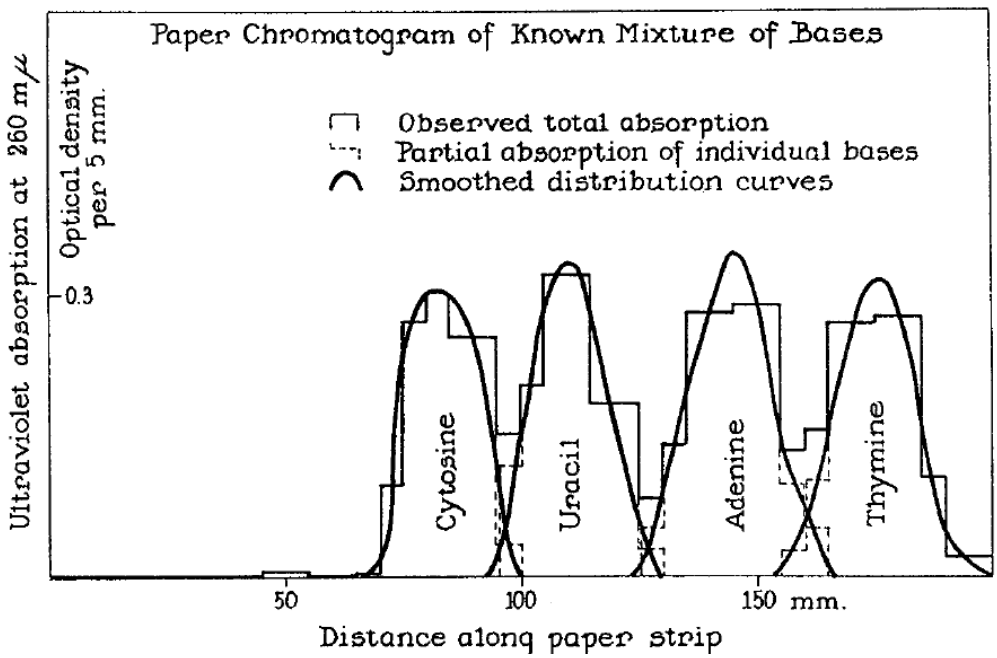

Figure 1. Ultraviolet absorption showing the separation of in known mixture of four nucleosides.

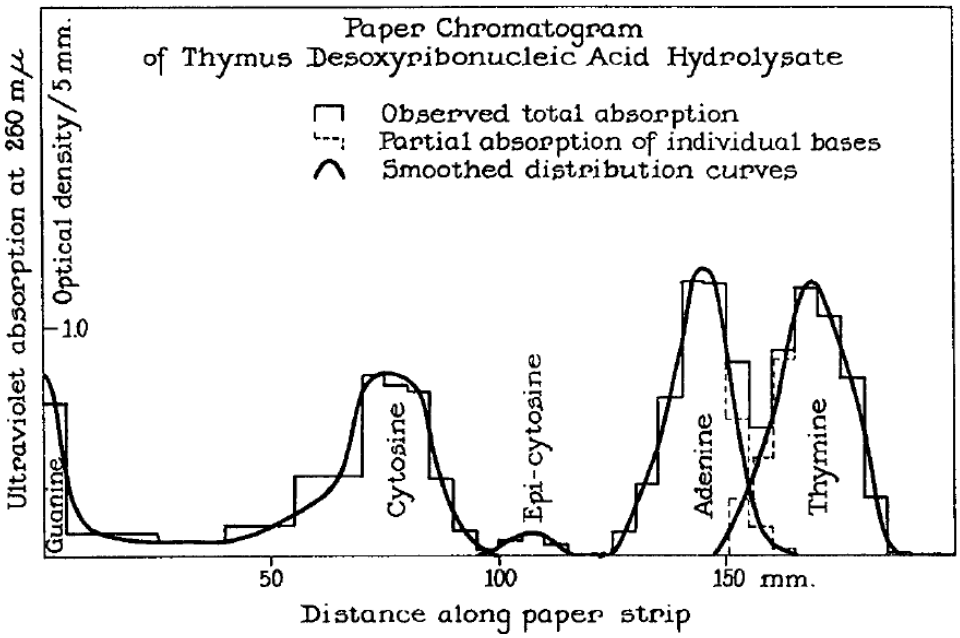

Fig. 2. Ultraviolet absorption of a mixture of nucleosides from thymus tissue.

The small “epicytosine” peak may be an artifact, but it occurs consistently in thymus samples. Perhaps it is cytosine that has been modified by adding a methyl group. A recent study has shown that there is a methyl cytosine in tubercle bacilli (ref. cited).

We also occasionally saw another abnormality, an “epiguanine,” traveling about 0.7 as fast as cytosine and having a double band spectrum somewhat like guanine. This substance is believed to be a derivative of guanine, probably an artifact, formed in the hydrolysis of certain DNA preparations.

The range of migration of separated components along the paper is shown for mixtures and for yeast and thymus biological samples are reported in Table 1 (data not shown here).

More precise information was obtained when the spots at different points along the paper strip were cut out and dissolved for individual spectrophotometer analysis. Results of pure known nucleosides recovered from paper strips are shown in Figures. 3 and 4 (not shown). These data were then used to determine the identity of unknown nucleosides in the biological samples. Somewhat atypical results for guanine are shown in Figure 5.

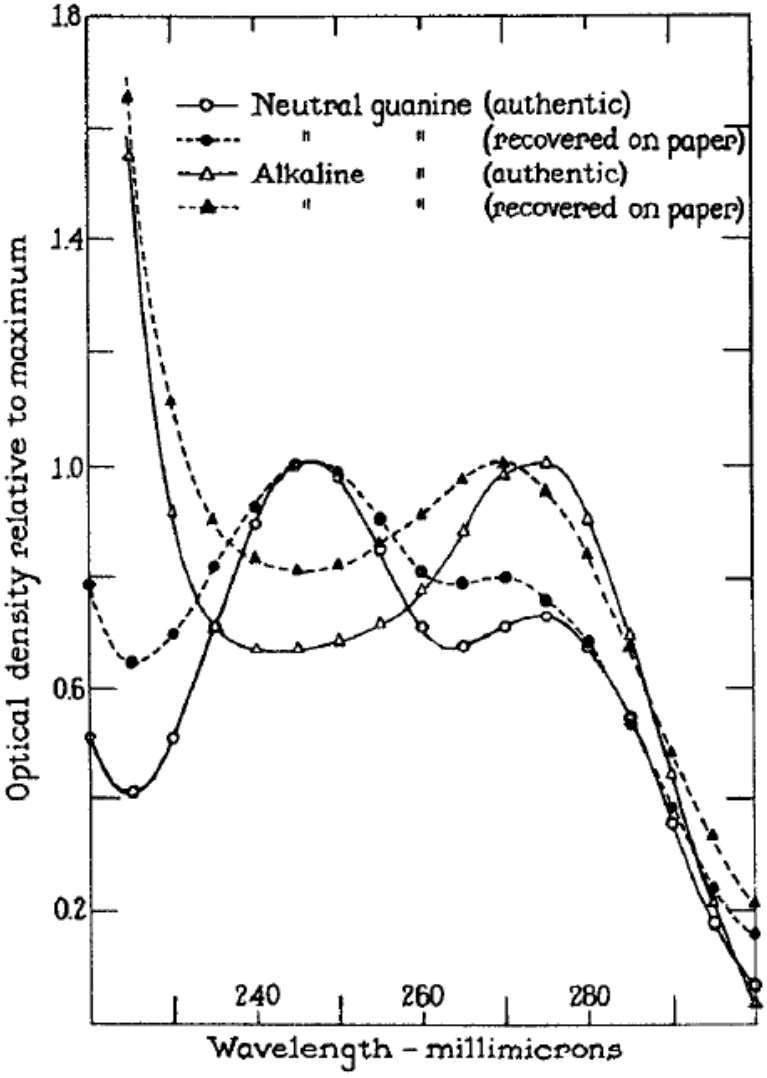

Figure 5. Comparison of optical density of known guanine and the biological samples.

Results: Questions to Answer

1. Do the results support the hypothesis or not? How convincing is that support?

2. Do you notice anything of possible importance in the data that authors failed to mention?

3. Is the variation in data large enough to suggest that some unknown variables interfere with reliable results? What might these be?

4. How big is the 'treatment' effect? Is it large enough to be of much practical importance?

The close correspondence of the data for authentic and recovered bases indicated that the substances are isolated essentially free of other material. Known and unknown solutions of different concentrations were most conveniently expressed in terms of the ratios of peak optical density.

The lack of correspondence seen in the range 220 to 240 millimicrons (Fig. 5), even with authentic samples indicates that this portion of the ultraviolet spectrum is not useful. The relative absorption data for the five bases in neutral, alkaline, and acid solutions are collected in Table II (data not shown here).

For less rigorous identification of the isolated fractions, use is made of certain characteristic features of the absorption at a few chosen points in the absorption spectra. The distinguishing wavelength features that are most effective in distinguishing the bases are indicated in Table III (data not shown here).

Quantitative Recovery of Bases. Since only small amounts of any base are present outside the region surrounding its peak absorption, the recovered quantity can be immediately determined by totaling the absorption found in this region. This is the accumulated sum of the area under the curve of an isolated peak. Table V illustrates the microgram recovery.

TABLE V

Quantitative Recovery of Purines and Pyrimidines

Preparation analyzed		Thymine	Adenine	Uracil	Cytosine	Guanine
		γ	γ	γ	γ	γ
Pure substances	Theory	90	89	94	91	
	Found	79	93	92	86	
Mixture I	Theory	84	52	66	84	
	Found	81	50	65	79	
Mixture II	Theory	72	71	75	72	
	Found	67	69	73	69	
2 mg. yeast nucleic acid hydrolyzed 2 hrs. at 120° with hydrochloric acid						
normality of acid						
0.4		0	123	7	9	125
0.7		0	112	11	9	125
2.4		0	112	54	18	140
6		0	132	117	52	125

Discussion

The method outlined above appears able to estimate with some accuracy purine and pyrimidine bases, and probably the nucleosides, in hydrolysates of nucleic acids. Sugar constituents contribute very little to ultraviolet absorption either before or after hydrolysis. This method is much more effective than paper chromatography attempts to separate amino acids.

Since this present study was completed, another group (ref. cited) has extended the paper chromatography method to isolating thymine and uracil. Their procedure uses solvents that do not absorb ultraviolet light. Their hydrolysis method converts cytosine to uracil. We have not observed this in our method.

Limitations of this method are that it is can be somewhat tedious. The careful quantitative study of twenty or thirty strip segments for one single analysis may require several hours. It is also not a simple matter to hydrolyze quantitatively a nucleic acid preparation without destroying some of the bases themselves. Further, two unexpected bases were found, and one is believed to be an artifact, a degradation product of guanine.

Discussion: Questions to Answer

1. Summarize how the authors discussed the results in terms of their original hypothesis.

2. Did the authors point out ideas that go beyond the hypothesis?

3. What ideas for future research did the authors generate?

4. What ideas for future research do you generate?

5. How would you state the "so what" or take-home lesson?

Summary (Note: the format of this journal did not call for an Abstract at the top of the report)

1. By paper strip chromatography in a butyl alcohol system, the DNA components, cytosine, uracil, adenine, and thymine, may be isolated from mixtures and from hydrolysates of nucleic acids. Guanine can also be isolated under favorable circumstances.

2. The nucleosides that have been examined (cytidine, guanosine, adenosine, and thymidine), can also be separated from each other and from most of the free bases in the same system.

3. The quantity of substances is determined by ultraviolet spectrophotometry.

4. Mixtures of any two nucleosides can be separately measured and their amounts measured.

References

Identification of the references can be found in the original report and are not necessary for our purposes here.