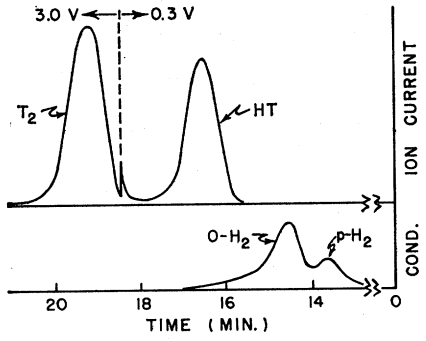


Fig. 1. Separation of ortho- and para- $H_2$, HT, and $T_2$ by gas-solid partition chromatography.

sparking mixtures of about 1 percent $T_2$ in $H_2$ with a Tesla coil. They were passed through a 6.1-m molecular sieve column with helium carrier gas. The column was kept at −160°C with a bath of liquid methane containing about 10 percent ethane. The gas chromatograph was modified by the addition of a 10-cm³ ionization chamber in series with the thermal conductivity cell (*2*). Both detectors were kept at 30°C. The chemical peaks (thermal conductivity) and radiochemical peaks (ion current) were recorded on synchronized recording potentiometers. The results are depicted in Fig. 1. Hydrogen showed two peaks with relative heights of 3 to 1, corresponding to ortho- and para-hydrogen (*3*). A sample of pure para-hydrogen gave only one peak at retention time of 13.5 minutes. Prolonged treatment of a sample with a Tesla coil increased the HT peak and decreased the $T_2$ peak. When the sample was placed on activated uranium (*4*), then taken out, the peak identified as $T_2$ disappeared.

One of the easily controlled variables in the analysis was the flow rate of the carrier gas. In Fig. 2, the heights equivalent to a theoretical plate (H.E.T.P.) for HT and $T_2$ are plotted against flow rate of carrier gas (*5*). From this figure, it is concluded that a flow rate of about 10 cm/sec gives maximum separation. The number of theoretical plates for HT and $T_2$ were then 4060 and 4580, respectively.

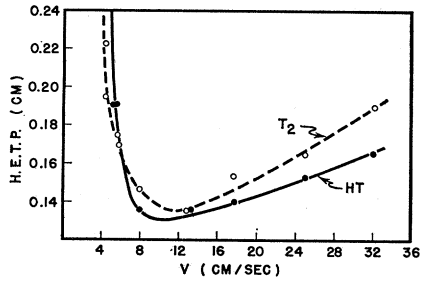


Fig. 2. H.E.T.P. (cm) for HT and $T_2$ versus flow rate of helium carrier gas (cm/sec).

The results reported here can be utilized for investigating the kinetics of some interesting systems: for example, the radiolysis of $T_2 + H_2$ mixtures and the formation of HT in Wilzbach labeling (*6*).

PRESTON L. GANT
KANG YANG
*Research and Development Department, Continental Oil Company, Ponca City, Oklahoma*

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7. We gratefully acknowledge the help of L. D. Lively in the design of chromatographic apparatus. L. O. Morgan and F. H. Dickey offered valuable discussions in the preparation of this manuscript. D. H. Weitzel of the National Bureau of Standards furnished us the sample of pure para-hydrogen.

12 January 1959

## Electrophoretic Method for Desalting Amino Acids

*Abstract.* A solution to be desalted is placed on a paper strip along which an ammonium formate buffer gradient has been established. Application of a potential brings about migration of amino acids to their isoelectric *p*H's and removal of salt ions. The strip is eluted with water, and the eluate is freed of ammonium formate by vacuum sublimation at 40°C.

Salts in amino acid preparations (for example, protein hydrolyzates or tissue extracts, to be analyzed chromatographically) are objectionable (*1, 2*). Several desalting procedures have been developed but, according to Block *et al.* (*1*), not one is completely satisfactory.

The need for a simple general method of desalting micro quantities of amino acids and peptides from protein hydrolyzates led to the development of an electrophoretic procedure that takes advantage of the volatility of ammonium formate [previously exploited in ion-exchange chromatography of amino acids by Hirs *et al.* (*3*)]. The sample to be desalted is subjected to electrophoresis on a paper strip along which a *p*H gradient is established by use of ammonium formate buffer. The amino acids migrate to the region of their isoelectric *p*H at or near the midpoint of the strip, the unwanted salt ions move to the solutions around the electrodes, and ammonium formate is left as the only electrolyte on the strip. After electrophoresis, the strip is eluted with water, and the eluate is vacuum dried at 40°C. The ammonium formate sublimes, and the desalted hydrolyzate remains as a residue.

The method was tested as follows with single-strip Durrum-type paper electrophoresis cells (*1*, p. 511; *4*): A 1- by 10-in. strip of Whatman No. 1 paper, held vertically, was washed by allowing 200 ml of water to flow down through it (as in descending chromatography). The ends of the dried strip were placed in the electrode compartments with ammonium formate buffers of 0.1 ionic strength at *p*H's above and below the isoelectric points of any amino acids present [for example, above 10.8 and below 2.3 if both arginine (*pI* 10.8) and aspartic acid (*pI* 2.3) are present]. The paper was supported at its midpoint by a horizontal glass rod (3 mm in diameter) rubbed with silicone stopcock grease (*1*, p. 512). The sample of solution to be desalted (usually 10 to 30 μl) was applied at the midpoint of the strip, a glass cover was placed over the strip, and the buffer solutions were allowed to migrate up the strip to complete the liquid junction. A potential of about 50 v per cell was applied overnight, the electrode in the low *p*H buffer being positive. After electrophoresis, the ends of the strip were cut off just above the buffer vessels, the strip was allowed to dry at room temperature, and the amino acids were eluted by allowing 20 ml of water to flow down through the vertically suspended strip. The eluate was vacuum-dried for at least 16 hours at 40°C (*5*). The possibility of scaling up the quantity of sample is indicated by the recovery of 0.98 g of glycine from a solution of 1.00 g of glycine in 5 ml of 0.1*M* KCl on a single large sheet of Whatman No. 1 paper in the Spinco model R apparatus.

Figure 1 shows two-dimensional chromatograms of 20-μl samples of amino acid solutions in 1*N* NaCl containing

Table 1. Data on recovery and salt removal for 80 μl of 1 percent DL-phenylalanine in 1*N* NaCl. The values shown in parentheses were obtained before desalting.

| Absorbance (at 280 mμ) | Recovery of amino acid (%)* | NaCl (mg) | NaCl remaining (%)* |
|---|---|---|---|
| | *Experiment 1* | | |
| 0.161 (0.137) | 95 | 0.12 (4.68) | 1.1 |
| | *Experiment 2* | | |
| 0.170 (0.137) | 102 | 0.08 (4.68) | 0.2 |
| | *Experiment 3*† | | |
| 0.031 (0) | | 0.07 (0) | |

* After correction for blank.
† Blank (no sample added to filter paper strip).

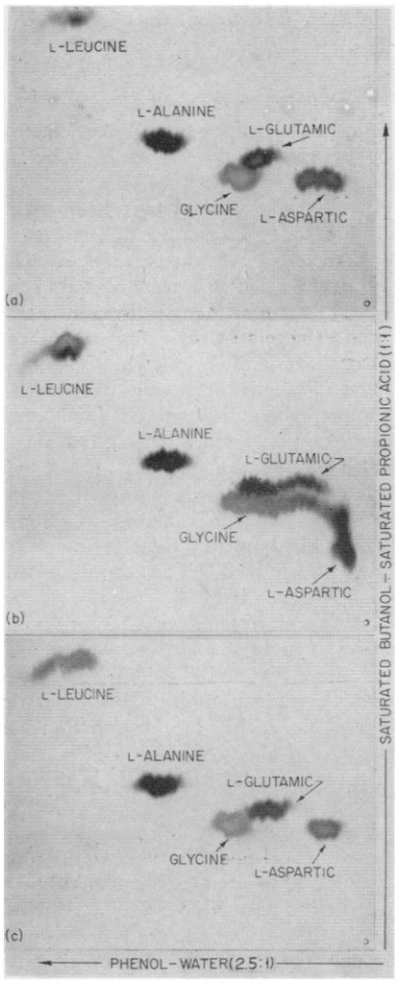


Fig. 1. Chromatograms of an amino acid mixture: (a) water solution, (b) 1*N* NaCl solution, (c) 1*N* NaCl solution followed by desalting.

1.5 mg/ml of each amino acid present. These are typical of results obtained when a variety of amino acids as well as other salts were used, including $CaCl_2$, $MgCl_2$, and KCl. The tailing and overlapping in the presence of NaCl and the improvement effected by the desalting procedure are evident.

To determine the extent of migration on the filter paper and to check the recovery of the amino acids, strips were sprayed with Ninhydrin after electrophoresis and both before and after elution with 20 ml of water. Sprayings before elution (*6*) revealed that the amino acids concentrated rapidly in a band 1 to 2 cm wide and 0 to 3 cm from the point of application, depending on their isoelectric points and the *p*H gradient in the strip. Sprayings after elution did not result in any color development, even on prolonged heating at 105°C, indicating fairly complete removal of both the amino acids and the ammonium formate. In the foregoing, solutions of glycine, glycylglycine, L-alanine, L-glutamic acid, L-cystine, L-methionine, DL-phenylalanine, L-arginine, L-lysine, L-tyrosine, L-tryptophan, and L-valine, were used.

Recovery of DL-phenylalanine and the extent of desalting were also investigated by light absorption and electrical conductance measurements. A typical set of results is shown in Table 1 for experiments in which Whatman No. 1 paper treated with LiOH was used. Because of soluble light-absorbing and conducting material in the paper, corrections with a blank (*7*) were necessary. All absorbances and conductances are for samples diluted to 5 ml with water. The conductances (corrected for solvent) are expressed as milligrams of NaCl. The data show that both the recovery of the amino acid and the removal of salt are essentially complete.

Preliminary experiments with 2′- and 3′-uridylic and 2′- and 3′-cytidylic acids indicate that the procedure can be applied to nucleotides. A strongly acid buffer (for example, 0.1*M* ammonium formate in 50 percent formic acid for uridylic acid) was necessary on the low *p*H side because of the low isoelectric points of these substances (*8*).

JAMES C. NICHOL*
*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee*

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4. A few runs, which proved quite satisfactory, were made with the Spinco model R eight-strip Durrum-type apparatus with Whatman No. 1 paper. With heavier papers, such as 3MM, desalting was not so effective.
5. A laboratory Glass and Instruments Corporation Evapo-Mix evaporator accommodating ten sample tubes was used. The small quantity of ammonium formate present either was completely removed to the cold trap or was condensed as a few hard crystals in the lead-off line between the sample tubes and the trap.
6. If the strip is heated after spraying, color caused by the presence of ammonium formate develops rapidly and obscures the amino acid color. Therefore, the strips were allowed to develop at room temperature.
7. The paper could be temporarily freed of absorbing and conducting material by thorough washing with water or with dilute acid or alkali followed by water. However, the offending material would again appear in reduced amounts in eluates obtained a few hours later. Finally, this effect was eliminated by heating the strips in 1 percent LiOH, at just below boiling temperature overnight, and washing thoroughly with water, although this treatment did not reduce the blank correction to zero (Table 1). Since it was found that the chromatograms (Fig. 1) were not significantly improved by the LiOH treatment, the matter was not pursued further. Evidently a much more thorough treatment, possibly one such as that developed by G. E. Connell, G. H. Dixon, and C. S. Hanes [*Can. J. Biochem. and Physiol.* 33, 416 (1955)], which included a 22-day elution with LiOH, would be necessary to remove all soluble conducting and light absorbing matter.
8. The work described in this report originated in discussions with Dr. N. G. Anderson of the Biology Division, Oak Ridge National Laboratory. I wish to thank Mr. R. E. Canning for running the chromatograms.
* Present address: University of Minnesota, Duluth Branch, Duluth, Minn.

26 January 1959

## Incorporation of Unnatural Pyrimidine Bases into Deoxyribonucleic Acid of Mammalian Cells

*Abstract.* When a mammalian cell strain was incubated with 5-iododeoxyuridine and 5-bromodeoxyuridine, DNA thymine was partially replaced by the halogen-containing pyrimidines. The extent of incorporation of the unnatural bases increased when amethopterin and hypoxanthine were added to the medium. It is thus evident that the replacement of DNA thymine by selected structural analogs, a phenomenon previously reported for bacterial systems, is applicable to cells of higher organisms.

Following preliminary observations of Weygand *et al.* (*1*), Zamenhof *et al.* and Dunn and Smith (*2–5*) have demonstrated the introduction of 5-chloro, 5-bromo, and 5-iodouracil into the deoxyribonucleic acid (DNA) of several bacterial strains. These pyrimidines are considered structural analogs of thymine for which the Van der Waals' radii of the halogen atoms are approximately equal to the radius of the methyl group (*5, 6*). The extensive incorporation (reported in this paper) of 5-bromouracil and 5-iodouracil into the DNA of a mammalian cell strain demonstrates that this phenomenon can be extended to cells of higher organisms.

In vitro experiments in our laboratory had shown that 5-bromodeoxyuridine severely depressed the incorporation of labeled thymidine into the DNA of H.S. No. 1 human tumor transplant slices (*7*). However, a search for incorporation of bromouracil into the DNA of this tissue slice system had yielded negative results.

Cells (H.Ep. No. 1) derived from a human cervical carcinoma (*8*) were grown in large Blake bottles with Eagle's medium (*9*) plus 20 percent horse serum. After cell growth on glass had been established, 5-iododeoxyuridine (*10*), 5-bromodeoxyuridine, hypoxanthine, and amethopterin were added to the culture medium as indicated in Table 1. After approximately 3 days the cells were washed with saline and harvested with 0.05 percent trypsin (*11*). The DNA bases were obtained by a procedure previously described (*12*) and separated from each other by two-dimensional paper chromatography. In agreement with the observations of Dunn and Smith, the Marshak-Vogel hydrolytic procedure for DNA containing iodouracil yielded a compound identified by chromatography and ultraviolet spectrum as uracil (*3*). In the case of DNA containing bromouracil, the hydrolytic procedure yielded a mixture of bromouracil and uracil. Consequently, the data for iodouracil and bromouracil shown in Table 1 are based on the yield of these hydrolysis products.