Substituting equation 5 in equation 6, recalling that $V_A^1 + V_B^1 = 1$, and subtracting 1 from both sides for simplification

$$\alpha - 1 = \frac{(V_A^1 - V_A^1)X}{V_A^1[V_B^1 Z - (V_A^1 - V_A^1)X]} \qquad (7)$$

Equation 7 is an expression of the separation factor in terms of readily determined quantities.

Error due to any non-additivity in volume can be reduced by employing an experimentally-determined density–composition curve to find the value for X used in equation 7.

Expressions similar to equation 7 may be derived using mole fraction or weight fraction, but both involve the density of a liquid mixture having the composition of the adsorbed phase. Assuming density to be linear with composition, or experimentally determining a density–composition curve, it is possible to calculate the separation factor using either mole fraction or weight fraction, but the equations are more complex. From the nature of the separation factor expression, any non-additivity in volume will lead to the same error, independent of the method of expressing the compositions.

A comparison of separation factors determined by the column method and the static method can be made for *n*-hexane/benzene mixtures on silica gel. Mair, Westhaver and Rossini[8] have determined separation factors for this hydrocarbon pair by the use of the column method. These values are shown graphically in Fig. 1. Equilibria data for the same hydrocarbon pair and the same type adsorbent (Davison Chemical Corporation, No. 22-08, "through 200 mesh") in a static system have been reported by Lombardo.[8] From these data we have calculated separation factors, also plotted in Fig. 1.

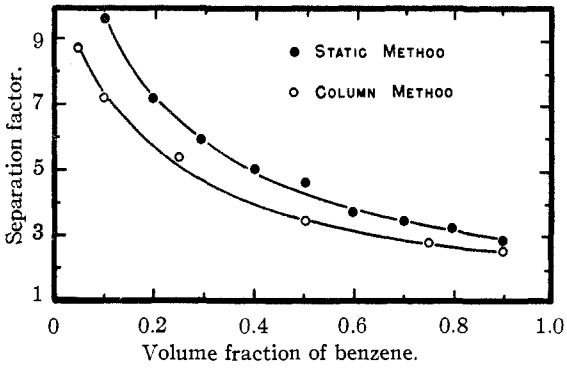

Fig. 1.—Separation factors for benzene/*n*-hexane mixtures.

The results indicate that the separation factors determined by the static method are higher than those determined by the column method. Since both methods assume additivity in volumes, and since both methods define the adsorbed phase in terms of the adsorbent capacity as determined by equilibration through the vapor phase, any deviations from these two assumptions could not account for the differences between the two curves. Due to the broad composition range covered in the column method, involving a broad range of separation factors, it is believed that the separation factors determined by the static method are more accurate. Furthermore, the attainment of equilibrium was determined experimentally in the static method, and must be assumed in the column method.

(8) R. J. Lombardo, Ph.D. Thesis, The Pennsylvania State College (1951). An equation similar to 5 was employed to determine the composition of the adsorbed phase.

Experimental

The experimental work consisted of determining the adsorbent capacities listed in Table I.

Adsorbents.—Activated alumina (Alcoa, F-20 grade, 80–200 mesh) was pretreated by heating to 200° for 16 hours in a 10 mm. i.d. column with passage of a slow stream of nitrogen gas through the column. Silica gel (Davison Chemical Corporation, No. 11-08-08-01, 28–200 mesh) was heated to 175° for 20 hours in the same apparatus.

Test Liquids.—Table II lists the liquids, their sources, refractive indices and vapor pressures at room temperature. Methylcyclohexane was purified by distillation in a helix packed column of 35–40 plates at a reflux ratio of 15/1 and passed over silica gel. Cyclohexane was passed over silica gel twice before use. The distilled water was boiled to remove any dissolved gases.

TABLE II

TEST LIQUIDS

Liquid	Source	$n^{25}D$	Approx. v.p. at 25°, mm.
Methylcyclohexane	Phillips, Tech. Grade, 95 mole % pure	1.4213[a]	48.0
Cyclohexane	Eastman, Practical	1.4236[a]	96.0
n-Heptane	Pure, Westvaco	1.3851	49.0
Benzene	Phillips, 99.97% pure	1.4976	95.2
Distilled water	Laboratory	1.3321	23.8
5-*n*-Butylnonane	API Project 42 Penn. State	1.4246	0.2

[a] Refractive index following purification.

Procedure for Determining Adsorbent Capacity.—The pretreated adsorbent was placed in a weighed covered Petri dish, and then dish and contents were weighed to the nearest ±0.0001 g. to give the weight of the adsorbent. The Petri dish and adsorbent were placed in a desiccator along with pure liquid in another open Petri dish. The desiccator was evacuated until the vapor pressure of the liquid was approached. The desiccator was then sealed and equilibration allowed to proceed. At 24-hour intervals, the adsorbent plus adsorbate was weighed to the nearest ±0.0001 g. until no increase in weight was observed. The weight of the adsorbate divided by the density of the liquid at room temperature gave the volume of the liquid adsorbed.

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WHITMORE LABORATORY
SCHOOL OF CHEMISTRY AND PHYSICS
THE PENNSYLVANIA STATE COLLEGE
STATE COLLEGE, PENNA.

A Study of the Quantitative Dinitrophenylation of Amino Acids and Peptides

BY W. A. SCHROEDER AND JOANN LEGETTE

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During an investigation of peptides in partial hydrolysates of gelatin,[1] the method of Sanger[2] has been used not only for the identification of the N-terminal amino acids but also for the identification and estimation of the other amino acids of the peptides. According to this method, the dinitrophenyl (DNP) peptide is completely hy-

(1) W. A. Schroeder, L. Hounen and F. C. Green, *Proc. Nat. Acad. Sci.*, **39**, 23 (1953).

(2) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

drolyzed and the N-terminal DNP-amino acid so released is extracted and identified. The remaining amino acids of the peptide which are present as the free amino acids in the hydrolysate are identified and estimated after conversion to the DNP-amino acids. When known amounts of DNP-peptides are hydrolyzed, one would not expect to recover an equimolar quantity of N-terminal DNP-amino acid because of the known partial destruction during hydrolysis. However, because there is no evidence that the other amino acids are destroyed during hydrolysis of a DNP-peptide, one would expect to be able to isolate them in amounts equimolar to the starting amount of DNP-peptide if their conversion to the DNP-derivative were quantitative. In most of our experiments this anticipated result was not realized and the DNP-amino acids, both terminal and other, failed to correspond to the amount of DNP-peptide. The present investigation was made in order to ascertain what conditions were required to produce quantitative dinitrophenylation of amino acids and peptides: this aim has to some extent been achieved. A correlation of the present results with some previously recorded in the literature will be presented in the discussion.

Experimental

Reagents.—The sources of amino acids were the same as those previously used.[3] Gly-DL-ala[4] and DL-ala-gly were obtained from Amino Acid Manufactures (University of California at Los Angeles), L-leu-gly-gly from Delta Chemical Works, and ala-ala-ala-ala (4 L) from Dr. Erwin Brand. Sodium bicarbonate was J. T. Baker analyzed C.P. grade and sodium carbonate was Baker and Adamson reagent grade. 2,4-Dinitrofluorobenzene (DNFB) was prepared by dinitration of fluorobenzene and purified both by distillation at reduced pressure and by chromatography on silicic acid–Celite. Absolute alcohol was obtained from U.S. Industrial Chemicals Co. Anhydrous ether from General Chemical Co. was distilled before use.

Dinitrophenylation Procedures.—All procedures for dinitrophenylation had certain common features despite variations which were introduced from time to time. One ml. of a standard solution which contained one micromole of amino acid or peptide per ml. was pipetted into a 100-ml. glass-stoppered flask, and sodium carbonate or bicarbonate was added followed by a solution of DNFB in 2 ml. of undistilled absolute alcohol. (Distilled alcohol gives extraneous zones on chromatograms of a blank dinitrophenylation.) The mixture was then mechanically shaken for 3 hr.

Three distinct procedures of dinitrophenylation were used. These differed in the quantities of reagents as shown in the following tabulation:

Reagent	Dinitrophenylation procedure First	Second	Third
$NaHCO_3$, mg.	100	....	20
Na_2CO_3, mg.	....	100	...
DNFB, ml.	0.1	0.1	0.02

Other variants on these three procedures will be given when the results are presented.

Extractive Procedures.—After any of the above procedures had been used, the reaction mixture was transferred to a separatory funnel with 10 ml. of water. In those experiments in which the amount of water and alcohol in the reaction mixture was increased, 20 ml. of water was used for the transfer. When sodium carbonate was used, the transfer of the large amount of dinitrophenol was expedited by using, in addition to the water, the 25 ml. of ether which was used for the first extraction (see below).

The **original extractive procedure** was as follows. The transferred reaction mixture was extracted with 4 × 25 ml. of ether to remove unreacted DNFB, acidified with one ml. of 6 *N* hydrochloric acid, and further extracted with 2 × 25 and 5 × 10 ml. of ether to remove the DNP-amino acid or DNP-peptide. The latter ether extracts were then washed with 3 × 10 ml. of water to each portion of which two drops of 6 *N* hydrochloric acid was added. Unless acid is added to the wash waters, some DNP-amino acids and DNP-peptides tend to re-enter the aqueous phase during washing.

The **revised extractive procedure** differed from the above only in that the transferred reaction mixture was acidified directly and the preliminary extraction of the basic solution to remove DNFB was omitted.

For the extraction of DNP-peptides, ethyl acetate replaced ether in the extraction of the acidified solutions.

Chromatographic Procedures.—During a dinitrophenylation, 2,4-dinitrophenol is formed to a greater or less extent. One cannot therefore determine directly the quantity of DNP-compound which has been extracted from the dinitrophenylation mixture, for example, by spectrophotometry. This interfering substance has been removed chromatographically.

The extracts of the dinitrophenylation mixtures were prepared for chromatography as previously described,[5] and each DNP-amino acid and DNP-peptide was then chromatographed in all particulars by the method of Green and Kay.[6] Each compound was developed with 6 or 7 *V* ml. of a developer appropriate to the group to which it belonged. Dinitrophenol is removed by 1 or 2 *V* ml. of any developer which is used in the method. DNP-Gly-ala and DNP-ala-gly were developed with 3AA-15A-L, DNP-leu-gly-gly with 2AA-10A-B and DNP-ala-ala-ala-ala with 3AA-15A-B (abbreviations as in Green and Kay[6]).

Spectrophotometric Procedures.—After the compound had been chromatographed and eluted, the eluent was evaporated and the residue of DNP-compound was taken up in glacial acetic acid for spectrophotometric determination. For spectrophotometric calculations, it has been assumed that the molecular extinction coefficient of DNP-amino acids and DNP-peptides is 1.61×10^4 liters per mole cm. at 340 ± 2 mμ in glacial acetic acid, except in the case of α,ε-di-DNP-lysine in which the determined value is 3.22×10^4 at 342 mμ and of DNP-proline in which the determined value is 1.75×10^4 at 360 mμ. If these coefficients are recalculated in terms of the ratio: concn. in micromoles per 100 ml. of soln./optical density for a 1-cm. length of soln., the values of 6.2, 3.1 and 5.7, respectively, are obtained. These are more convenient for calculation of quantity.

The assumed constants were arrived at from a study of values which had been determined for several compounds.[5] DNP-Aspartic acid which has been much used in the present study was found by determination to have exactly the assumed value.

Results and Discussion

Table I records the percentage of the theoretical amount of various DNP-amino acids and DNP-peptides which was isolated after the use of the several procedures described above.

In these experiments, one micromole of compound has been used because this quantity forms nicely visible zones on the chromatographic column and also is sufficient to require dilution to about 20 ml. before the spectrophotometric reading is taken. At such dilution, the blank correction introduced by the chromatographic procedure is inconsequential: it would reduce the values in Table I by no more than one per cent. and has not been applied. One correction, however, has been applied to give the results in Table I. It has been found that when such diverse DNP-derivatives as α,ε-di-DNP-lysine, DNP-aspartic acid, DNP-valine and DNP-phenylalanine are chromatographed, there is a loss of about 7% per chromatogram. It has been assumed that all the compounds listed

(3) W. A. Schroeder, L. M. Kay and R. S. Mills, *Anal. Chem.*, **22**, 760 (1950).

(4) Abbreviations follow the suggestions of E. Brand (*Ann. N. Y. Acad. Sci.*, **47**, 222 (1948), and F. Sanger (*Adv. Prot. Chem.*, **7**, 5 (1952)).

(5) W. A. Schroeder, THIS JOURNAL, **74**, 5118 (1952).

(6) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952).

TABLE I

PERCENTAGE OF THE THEORETICAL AMOUNT RECOVERED AFTER VARIOUS METHODS OF DINITROPHENYLATION

Amino acid or peptide[a]	Dinitrophenylation procedure: First[b]	Second[b]	Third[c]
Ala	97, 87, 90[d]	97, 102, 96, 101	100, 100
Asp	61, 60, 66, 69	83, 80, 84	93, 94
Glu	70, 61, 69, 68	86, 80, 83	96, 98
Gly	85, 80	95, 95	94, 98
Leu			97, 99[e]
Lys	78, 78, 80, 81	92, 86, 89	99, 96
Pro	72, 75	77	82, 76
Thr			99, 98
Ala-gly			95, 95
Gly-ala	82	88	82
Leu-gly-gly			85, 90
Ala-ala-ala-ala			82, 82

[a] One micromole of each was taken. [b] Original extractive procedure. [c] Revised extractive procedure. [d] Individual replicate values in chronological order of determination. [e] Original extractive procedure was used because contamination, apparently by DNFB, invalidates the results.

in Table I are lost to this degree in the course of one chromatogram and, hence, the values given have been corrected to this extent. It has not been possible to ascertain the cause of the losses which are suffered during chromatography. Experiments have shown, however, that it is not due to the action of light or to the use of ether in the procedure.

The proportions of water, alcohol, sodium bicarbonate and DNFB which were used in the first dinitrophenylation procedure are those which Sanger[2] used for dinitrophenylating insulin and they are very similar to his conditions for dinitrophenylating phenylalanine. It is evident from Table I that the dinitrophenylation is not quantitative in any instance and that the conversion of aspartic acid is especially poor. Mills[7] has also noted the difficulty with which aspartic acid is dinitrophenylated but he achieved quantitative results by increasing the time of reaction. He claims quantitative conversions for the other amino acids. Middlebrook[8] found that increase in reaction time did not improve the yield of DNP-aspartic acid but that the substitution of sodium carbonate for sodium bicarbonate did give quantitative recovery.

The second dinitrophenylation procedure (in which this substitution was used) very definitely improved the recoveries. The yield of DNP-alanine and DNP-glycine is almost quantitative and that of DNP-aspartic acid, DNP-glutamic acid and di-DNP-lysine is much improved; on the other hand, that of DNP-proline is little altered. The use of sodium carbonate has the disadvantage that rather large amounts of dinitrophenol are formed in this more basic solution although the dinitrophenol does not interfere chromatographically. In addition, artefacts appear on the chromatograms.

Within the framework of these two procedures, a number of variations were introduced. Thus, with the second procedure as a basis and with aspartic acid as a test substance, the time of reaction, the amount of sodium carbonate, and the method of extracttion were varied. The results may be tabulated as follows:

Time of reaction, hr.	Yield of DNP-aspartic acid, %
0.5	79, 79
3	83, 80, 84
7.5	83, 73
Amount of sodium carbonate, mg.	
30	83, 83
100	83, 80, 84
Extractive procedure	
Original	83, 80, 84
Revised	83, 88, 89

The reaction clearly is very rapid and essentially the same results are obtained over the period from 0.5 to 7.5 hours. Variation in the amount of sodium carbonate has no effect while the revised extractive procedure increased the yield slightly. Thus, of the above variations only the revised extractive procedure showed even minor improvement.

One variant of the first dinitrophenylation procedure was also tried in order to test the effect of light. Mills,[7] for example, recommends that photochemical reactions be reduced by shielding the DNP-derivatives from light. When aspartic acid was dinitrophenylated and carried through the procedure with the exclusion of all but the dimmest light required to carry out the various operations, the yield of 61% was not improved over those of 61, 60, 66 and 69% which were obtained under normal conditions of laboratory lighting. Our laboratories are lighted by fluorescent fixtures and the amount of natural light is negligible.

It was, of course, possible that the scale of the experiments was so small that manipulative losses were responsible for the poor yields. Consequently when the first dinitrophenylation procedure was used but the quantity of aspartic acid was increased from one to 10 micromoles, the yield also increased from an average of 64% to 81 and 79%. The definite improvement warranted further experiments. When 10 micromoles of aspartic acid was dinitrophenylated in 5 ml. of water and 10 ml. of alcohol which contained the usual 100 mg. of sodium bicarbonate and 0.1 ml. of DNFB, the recovery was 92%: when, under these same conditions, only one micromole of aspartic acid was used, the recovery was 94%. These results lead to the conclusion that the concentration of the reagents is important in improving the yield of the dinitrophenylation. Consequently, the third dinitrophenylation procedure was devised and the revised extractive procedure was used because it had given slightly higher yields.

Before discussing those results which were obtained with the third procedure and which are recorded in Table I, let us mention the effect of variation from the third procedure since in actual practice it is not always possible to use exactly these conditions. Aspartic acid was again chosen as the test substance because it seems to be one of the most difficult to dinitrophenylate. The variables which were introduced into the third procedure were time of reaction, amount of amino acid, amount of sodium bicarbonate, and extractive procedure. The following results were obtained:

(7) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).

(8) W. R. Middlebrook, private communication.

Time of reaction, hr.	Yield of DNP-aspartic acid, %
0.5	86
1	91
3	93, 94
8	93
Amount of amino acid micromoles	
0.3	83, 84
1.0	93, 94
10.0	98, 97
Amount of sodium bicarbonate, mg.	
5	94
20	93, 94
Extractive procedure	
Original	85
Revised	93, 94

As previously noted, the reaction is rapid and the results are constant between 1 and 8 hr. Very probably the concentration of amino acid rather than incidental manipulative losses is responsible for the differences which appear when the amount of amino acid is altered. Thus, if one micromole is used but the concentration is reduced by using 5 ml. of water and 10 ml. of alcohol, the yield is only 85%. Further decrease in the amount of sodium bicarbonate is without effect but the revised extractive procedure apparently improves the recovery by a few per cent.

When the third procedure is used, the recoveries as shown in Table I are generally satisfactory. After this method was found to give improved results, the scope of the study was broadened to include other representative amino acids and some peptides. Of the amino acids, proline alone behaves much the same in each procedure and gives a rather low yield. Structural features may influence the result in this case as there is no reason to believe that the proline is impure. It is more difficult to assess the results from the peptides because studies of their purity have not been made.

The literature records only a few studies of the quantitativeness of the dinitrophenylation of amino acids. As already mentioned, the first procedure of the present study is essentially that of Sanger.[2] Porter and Sanger[9] studied the quantitative dinitrophenylation of valine but fail to describe experimental details. Mills[7] has obtained quantitative dinitrophenylation of all amino acids and Krol[10] has shown that glycine reacts quantitatively. In the light of the present results, Mills' description of procedure is significant. "Residual HCl (from a hydrolysate) was neutralized by the careful addition of a *slight excess* of sodium bicarbonate...." (Italics ours.) Likewise, Krol used only 30 or 40 mg. of sodium bicarbonate per 3 ml. of reaction mixture. Apparently, their quantitative results stemmed from their use of small amounts of sodium bicarbonate.

Several observations have been made which may throw some light on the reason why the reaction is more quantitative when the reagents are less concentrated. In the third procedure, the reaction mixture is homogeneous in contrast to the others. This may mean that the actual concentration of reagents is greater because the bicarbonate cannot have salted out the DNFB. Although, in both the first and third procedures, the DNFB is in excess at the end of the reaction, much less dinitrophenol is produced in the third procedure. The reduction of this competitive reaction may influence the course of the dinitrophenylation in the homogeneous reaction mixture. It may be noted that a homogeneous solution was present in that experiment in which an improved yield of DNP-aspartic acid was obtained simply by changing the amount of solvents in the original procedure from 1 to 5 ml. of water and from 2 to 10 ml. of alcohol. The change in conditions does not seem to involve the shifting of an equilibrium. Thus, when a known amount of DNP-aspartic acid was "dinitrophenylated" by both the first and the third procedures, the recovery was 94 and 95%, respectively. If an equilibrium were involved, the former value should be about 64%.

(9) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).
(10) S. Krol, *ibid.*, **52**, 227 (1952).

Applications

The third dinitrophenylation procedure has been used with success in the study of peptides from partial hydrolysates of gelatin.[1] The estimation of the amino acids other than the N-terminal amino acids of the peptides is generally better than 90% of the starting DNP-peptides. It is advisable, however, to use the original rather than the revised extractive procedure. When the original extractive procedure is used, dinitroaniline is removed during the extraction of the basic solution, but if the revised procedure is used, dinitroaniline is always present on the chromatograms and requires definite identification in order to distinguish it from DNP-phenylalanine. The source of the dinitroaniline is unknown and if the revised procedure is used, its presence on the chromatograms leads to unnecessary complications.

Conclusions

The investigation has shown that the quantitative dinitrophenylation of amino acids is best achieved by carrying out the reaction in a homogeneous solution of amino acid, sodium bicarbonate, DNFB, alcohol and water.

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GATES AND CRELLIN LABORATORIES OF CHEMISTRY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA 4, CALIFORNIA

Correlation between the Structure of Some Dinitrophenyl Peptides and their Chromatographic Behavior on Silicic Acid–Celite

BY W. A. SCHROEDER AND LEWIS R. HONNEN

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During the determination of the N-terminal amino acid of lysozyme,[1] dinitrophenyl (DNP) peptides were detected in certain hydrolysates and

(1) F. C. Green and W. A. Schroeder, THIS JOURNAL, **73**, 1385 (1951).