Ray, W. J., Jr., and Koshland, D. E., Jr. (1960), *Brookhaven Symposia in Biol. 13*, 135.
Sanger, F., and Tuppy, H. (1951), *Biochem. J. 49*, 463, 488.
Sela, M., Anfinsen, C. B., and Harrington, W. F. (1957), *Biochim. et Biophys. Acta 26*, 502.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spackman, D. H., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 648.
Stark, G. R., Stein, W. H., and Moore, S. (1961), *J. Biol. Chem. 236*, 436.
Stein, W. H., and Moore, S. (1949), *J. Biol. Chem. 178*, 79.
Taborsky, G. (1959), *J. Biol. Chem. 234*, 2652.
Toennies, G., and Callan, T. P. (1939), *J. Biol. Chem. 129*, 481.
Vithayathil, P. J., and Richards, F. M. (1960), *J. Biol. Chem. 235*, 2343.

Spectrometric Evaluation of the Approximate pK of the Carboxyl Group in 2,4-Dinitrophenyl-Amino Acids

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The absorption at 360 mμ shown by 2,4-dinitrophenyl-amino acids in aqueous solution is very sensitive to changes in hydrogen ion concentration in the pH range 1 to 5. Twenty-one 2,4-dinitrophenyl derivatives have been examined for changes in absorbancy at 360 mμ at various hydrogen ion concentrations, and the approximate pK of the carboxyl group in many of these compounds has been evaluated from a curve relating absorbancy to pH. The effect of ionization of the carboxyl on the contribution to absorbancy at 360 mμ by the chromophore is highly dependent on the distance of the carbon carrying the chromophore system from the carboxyl group. When this distance exceeds three carbons, carboxyl ionization has little effect on absorbancy. The observed changes in spectra would be consistent with resonance stabilization of the anion.

The determination of 2,4-dinitrophenyl(DNP)-amino acids is generally done by the measurement of their absorbancy in solution in glacial acetic acid or aqueous sodium bicarbonate at 340 and 360 mμ respectively (Fraenkel-Conrat *et al.*, 1954), or by measurement of the absorbancy in the visible region of the spectrum after reduction of the compounds with sodium borohydride in aqueous sodium bicarbonate (Ramachandran, 1961). Molar absorbancy values of DNP-amino acids in acetic acid or acid solutions are somewhat lower than the values obtained for solutions in aqueous bicarbonate, and the peak found at 360 mμ in alkaline solutions is shifted to a lower wave length of 340–350 mμ in acid solutions. We present in this communication data on the dependence of the absorbancy of the compounds on the hydrogen ion concentration of the medium, pointing to the possibility of evaluation of the approximate pK values of the carboxyl groups from a curve relating absorbancy to pH. Potentiometric determination of the pK of the carboxyls is difficult owing to the very low solubility of most DNP-amino acids in water; in such cases spectrometric methods are useful if the spectrum changes with changes in hydrogen ion concentration of the solution (Gillam and Stern, 1954).

EXPERIMENTAL AND RESULTS

2,4-Dinitrophenyl Derivatives.—DNP derivatives of glycine, DL-valine, L-phenylalanine, L-isoleucine, L-tryptophan, L-alanine, L-arginine, L-aspartic acid, L-asparagine, DL-glutamic acid, L-proline, β-alanine, DL-isoserine, L-lysine (ε-DNP), DL-ornithine (δ-DNP), aminoethanol, α-, β-, and γ-aminobutyric acids, and α- and β-aminoisobutyric acids were prepared with 1-fluoro-2,4-dinitrobenzene (Sanger, 1945) and had physical constants in agreement with those recorded in the literature (Fraenkel-Conrat *et al.*, 1954; Rao and Sober, 1954; Green and Kay, 1952). DNP-β-aminobutyric acid, DNP-DL-α-aminobutyric acid, DNP-β-aminoisobutyric acid, and DNP-DL-isoserine had melting points of 166–8°, 190°, 154°, and 145–8° respectively.

Spectrum of DNP-Arginine.—Figure 1 shows the spectrum of DNP-arginine in aqueous solution at pH 5.8 and 2.0. At the lower pH the peak at 360 mμ found in solutions at pH 5.8 is shifted to the lower wave length of 350 mμ, and the absorbancy is lower. The trough at 300 mμ is likewise shifted to the lower wave length of 297 mμ at the more acid pH. Gradual changes in pH from 5 to 2 resulted in a family of curves which also shifted gradually from A to B, and the common isobestic point was found close to 348 mμ.

Spectrometric Determinations of pK.—The DNP-amino acids were dissolved in a series of buffers (to give molar concentrations in the range 2×10^{-5} to 10^{-4}) whose pH decreased from the alkaline side down in steps to acid pH values where the change in spectrum ceased—namely, until the lowest absorbancy at 360 mμ had been reached. Buffer solutions of known pH were made by using hydrochloric acid, sodium acetate, acetic acid, sodium bicarbonate, and sodium carbonate. Buffering constituents were usually present in a concentration of 0.2

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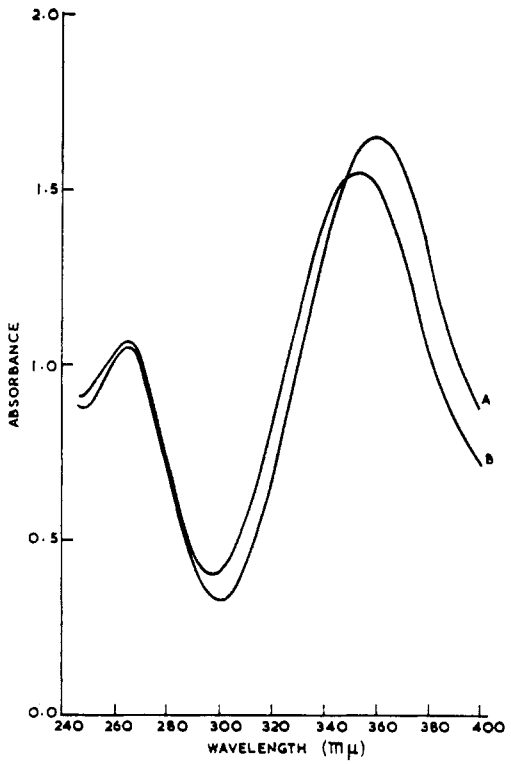


FIG. 1.—A, Spectral characteristics of DNP-arginine at pH 5.8; B, Spectrum at pH 2.0. Concentration 0.0966 μmole per ml.

M. Changes of ionic strength (in the range 0.04 to 1.3) alone had no effect on absorbancy due to the chromophore. The buffers themselves showed negligible absorbancy at 360 mμ. Readings around pH zero, when recorded, were simply taken on solutions in 1 N HCl. A Beckman DU spectrophotometer and cells of 1-cm path length were employed. Measurements of absorbancy were made at 360 mμ, since at this wave length marked differences in molar absorbancy existed between the ionized ($\epsilon_{M(COO^-)}$) and the non-ionized ($\epsilon_{M(COOH)}$) forms of the DNP-amino acids.

Curves were drawn for each compound, relating absorbancy of solution to pH. Points on the curves were picked corresponding to $^1/_2\Delta\epsilon_M$ [where $\Delta\epsilon_M = \epsilon_{M(COO^-)} - \epsilon_{M(COOH)}$], and the pH corresponding to this point was taken as the pK of the carboxyl group. In Table I are recorded values of $\epsilon_{M(COOH)}$, $\epsilon_{M(COO^-)}$, and $\Delta\epsilon_M$ characteristic of each compound studied, and Table II gives the pK value for each compound as evaluated above. Some typical titration curves are shown in Figure 2.

Potentiometric Titrations.—A Beckman G pH-meter, with glass electrode and reference AgCl-KCl electrode, was used for measuring pH. For the acid region of the pH scale, 0.05 M potassium hydrogen phthalate (pH 4.03) was used as a standard. Approximately 0.5 to 1.0 × 10^{-4} moles of the dried specimen (DNP-arginine) was dissolved in 10 to 20 ml water, the pH was adjusted to 7 to 8 with a dilute solution of sodium hydroxide, and the solution was titrated with standard HCl. The pH was recorded after each portion of acid was added. Corrected titration curves were constructed after

TABLE I

MOLAR ABSORBENCY VALUES (360 mμ) AND $\Delta\epsilon_M$ VALUES FOR DNP-AMINO ACIDS

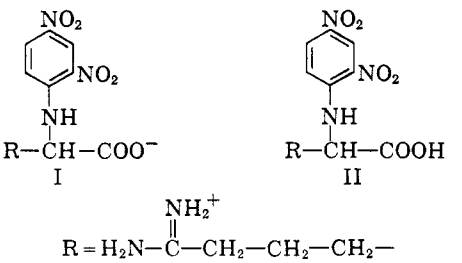
DNP-Amino Acid	$\epsilon_{M(COOH)}$	$\epsilon_{M(COO^-)}$	$\Delta\epsilon_M = \epsilon_{M(COO^-)} - \epsilon_{M(COOH)}$
Glycine	12,840	15,890	3,050
Alanine	14,430	16,720	2,290
Valine	15,550	17,480	1,930
Isoleucine	14,120	16,820	2,700
Asparagine	15,880	18,410	2,530
Glutamic acid	15,750	17,960	2,210
Arginine	15,630	17,200	1,570
Phenylalanine	13,740	16,670	2,930
α-Aminobutyric acid	15,590	17,570	1,980
α-Aminoisobutyric acid	15,700	17,700	2,000
β-Aminobutyric acid	18,470	19,000	530
β-Aminoisobutyric acid	16,480	16,960	480
γ-Aminobutyric acid[a]	16,970	17,200	230
Ornithine (δ-DNP; $HCl \cdot H_2O$)[a]	17,120	17,300	180
Lysine (ε-DNP; $HCl \cdot H_2O$)[a]	17,470	17,530	60
Tryptophan	16,230	16,960	730
β-Alanine	16,310	17,940	1,630
Proline[b]	15,640	18,970	3,330
Aspartic acid	13,340	18,310	4,970
Isoserine	5,410	18,580	13,170
Aminoethanol[c]	16,310	16,300	

[a] $\Delta\epsilon_M$ values represent the difference between the molar absorbency values at pH 8.3 and pH 1.0. [b] Measurements at 390 mμ. [c] No carboxyl group present; measurements made at pH 1.0 and pH 6.

subtraction of values obtained from an appropriate blank titration, and pK was calculated by the Henderson-Hasselbach equation. The titration of DNP-γ-aminobutyric acid, which is sparingly soluble in water at acid pH, was done in 40% acetone. Values obtained are recorded in Table II.

DISCUSSION

The sensitiveness of the structure of DNP-arginine to changes of hydrogen ion concentration in the pH range 2 to 5, at which carboxyl groups generally titrate, warrants the assumption that curves A and B of Figure 1 represent the spectra of species I and II, respectively:



The values of 3.1 ± 0.1 and 3.0 ± 0.05 for the pK of the carboxyl group of DNP-arginine obtained by spectrometric and potentiometric titration, respectively, also agree fairly with each other. DNP-aminoethanol, which bears no carboxyl group, exhibits the spectrum of the chromophore, but as expected the absorption at 360 mμ is not affected by changes in hydrogen ion concentration.

The influence of the proximity of the carboxyl on absorption by the chromophore at 360 mμ is

TABLE II

pK OF CARBOXYL OF 2,4-DINITROPHENYL-AMINO ACIDS

DNP-Amino Acid	pK from Spectrometric Titration[a]	pK from Potentiometric Titration	pK of Carboxyl of the Parent Amino Acid	Δ_{pK}[b]
DNP-α-aminobutyric acid	3.1 ± 0.1		2.55[e]	0.55
DNP-β-aminobutyric acid	3.5 ± 0.2			
DNP-γ-aminobutyric acid	...	4.7[c]	4.23[f]	
DNP-α-aminoisobutyric acid	3.2 ± 0.1		2.36[e] (calcd.)	0.84
DNP-β-aminoisobutyric acid	4.2 ± 0.2			
DNP-glycine	2.8 ± 0.1		2.34[g]	0.46
DNP-alanine	3.5 ± 0.1		2.34[g]	1.16
DNP-valine	3.1 ± 0.1		2.32[h]	0.78
DNP-isoleucine	3.5 ± 0.1		2.36[h]	1.14
DNP-phenylalanine	2.9 ± 0.1		1.83[i]	1.07
DNP-tryptophan	2.8 ± 0.2		2.38[j]	0.42
DNP-arginine	3.1 ± 0.1	3.0 ± 0.05[d]	2.17	0.93
DNP-β-alanine	3.9 ± 0.1		3.6[e]	0.30
DNP-aspartic acid	(3.3)[l]		pK_1 1.9; pK_2 3.7	
DNP-asparagine	3.2 ± 0.1		2.02[g]	1.18
DNP-isoserine	(3.9)[l]		2.76[k]	(1.14)
DNP-glutamic acid	(2.8)[l]		pK_1 2.2; pK_2 4.3[e]	(0.6)
DNP-proline	2.8 ± 0.1		1.99[e]	(0.8)

[a] Temperature 28–30°, except DNP-arginine at 25°. [b] ΔpK represents the difference: pK(COOH) of the DNP-amino acid minus pK(COOH) of the amino acid. [c] 31° in 40% (v/v) acetone in water. [d] 25°. [e] Cohn and Edsall (1943). [f] Neuberger (1937). [g] Czarnetsky and Schmidt (1931). [h] Miyamoto and Schmidt (1931). [i] Cohn (1931). [j] Schmidt *et al.* (1929). [k] Emerson *et al.* (1931). [l] Assignment of values, in parentheses, as pK of the α-carboxyl group is difficult.

well illustrated in Figure 2. The slope in the titration curve of DNP-α-aminobutyric acid becomes less pronounced in DNP-β-aminobutyric acid and has almost disappeared in DNP-γ-aminobutyric acid. The corresponding $\Delta\epsilon_M$ values are 2190, 530, and 230 respectively (Table II). The lack of pronounced inflections in the curve for DNP-γ-aminobutyric acid makes it impossible to evaluate the pK of the carboxyl, and it may be anticipated that the spectrometric method will be limited in application to DNP-α-amino carboxylic acids and if applied to DNP-β-amino acids would be subject to considerable error. Thus, for example, in ε-DNP-lysine, in which the chromophore is separated by five methylene groups from the carboxyl, carboxyl ionization has only an insignificant effect (Table I, $\Delta\epsilon_M$ = 60; value subject to considerable error) on absorption by the chromophore. The relation of $\Delta\epsilon_M$, exhibited by the chromophore in various derivatives, to the distance of the carbon bearing the chromophore from the carboxyl group is depicted in Figure 3 and reveals the pronounced dependence. The bathochromic shift and hyperchromicity that accompany ionization of the carboxyl group, and the disappearance of this effect when the dinitrophenylamino chromophore is separated from the carboxyl by several carbon atoms, would accord with resonance stabilization in the anion.

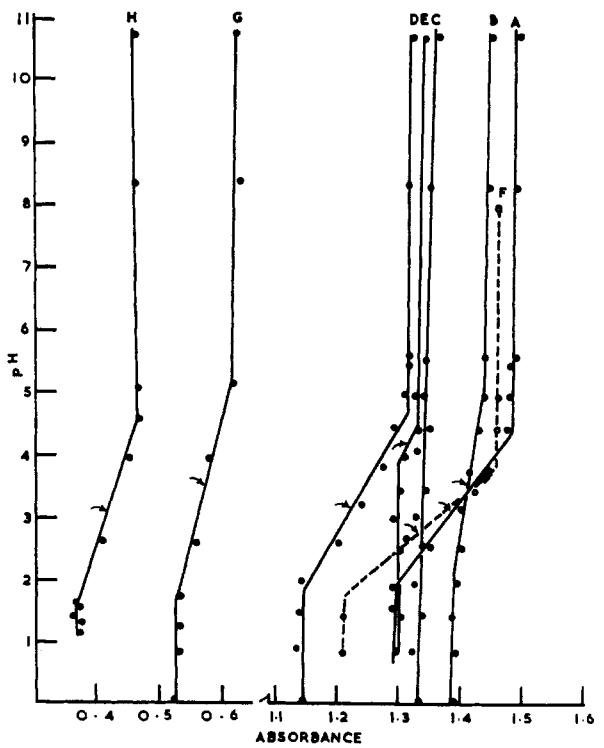


FIG. 2.—Spectrometric titration curves of DNP-amino acids. Arrows point to pK corresponding to pH at $^1/_2\Delta\epsilon_M$. A, DNP-α-aminobutyric acid; B, DNP-β-aminobutyric acid; C, DNP-γ-aminobutyric acid; D, DNP-α-aminoisobutyric acid; E, DNP-β-aminoisobutyric acid; F, DNP-proline; G, DNP-isoleucine; H, DNP-valine. All measurements at 360 mμ, except DNP-proline at 390 mμ.

Comparison of the pK of the carboxyl in the DNP-amino acid with the value for the carboxyl of the parent amino acid (Table II) reveals that dinitrophenyl-amino acids are invariably weaker acids, the difference in pK being +0.3 to +1.2 units. Substitution of the amino group of α-amino acids is known to have this effect (Zief and Edsall, 1937). In the substituted butyric acids, DNP-α-aminoisobutyric acid is found to be a stronger acid than DNP-β-aminoisobutyric acid, even as α-aminobutyric acid is a stronger acid than γ-aminobutyric acid. DNP-γ-aminobutyric acid (pK 4.7 by potentiometric titration in 40% acetone, and possibly close to 4 in aqueous solution) is only as strong an acid as γ-aminobutyric acid itself, which has a pK_1 of 4.23.

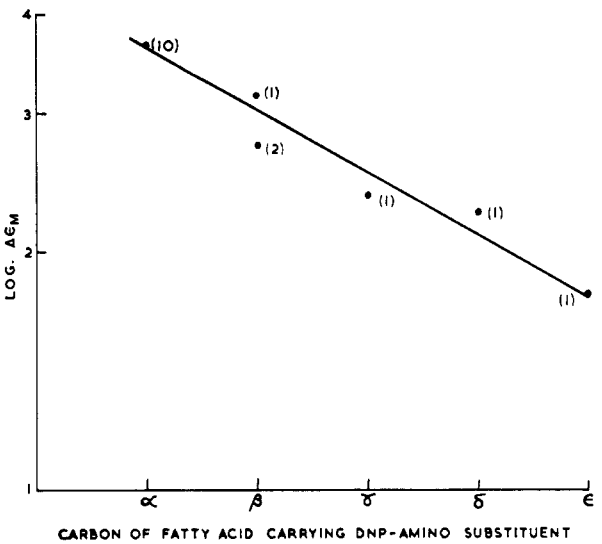
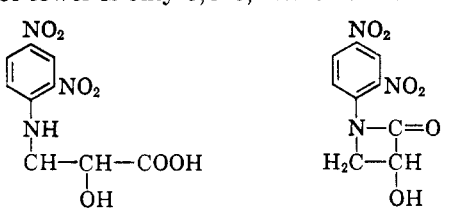


FIG. 3.—Relation of $\Delta\epsilon_M$ to distance of chromophore from the carboxyl group. α, Average of the $\Delta\epsilon_M$ found for the first 10 compounds listed in Table II. β, Average of the $\Delta\epsilon_M$ for DNP-β-amino-n- and β-amino-isobutyric acids, and one for DNP-β-alanine; γ-value for DNP-γ-aminobutyric acid; δ-value for δ-DNP-ornithine; ϵ-value for ϵ-DNP-lysine.

Some of the derivatives studied deserve special comment. That of β-alanine shows almost thrice the $\Delta\epsilon_M$ value observed in the other two β-amino acids studied. The $\Delta\epsilon_M$ for DNP-tryptophan is only about one seventh of that seen in ten of the DNP-α-amino acids studied. DNP-β-alanine and DNP-tryptophan show the least weakening effect of acid strength of the carboxyl compared to other DNP-α-amino acids. DNP-proline, the only imino acid studied, shows a slightly higher $\Delta\epsilon_M$ (3,330), compared to the average of 2,320 seen in the derivatives of ten α-amino acids. At all pH levels below 2, DNP-proline decomposes rapidly and shows decreasing absorption. Values have been recorded at acid pH as rapidly as possible. DNP-aspartic acid shows a $\Delta\epsilon_M$ of 4,970, and this is not unexpected since the ionization of the two carboxyls may each independently influence the extinction due to the dinitrophenyl group.

The same phenomenon could occur also in DNP-glutamic acid. In both these compounds there is the problem of spectrally overlapping pK values, and unambiguous assignment of the pH value corresponding to $^1/_2\Delta\epsilon_M$ of these compounds as the pK of the α-carboxyl group is not possible. Both compounds have such low solubility in water at low pH levels that direct titrations cannot be done. DNP-isoserine alone among the compounds studied shows a very unusual behavior, with a $\Delta\epsilon_M$ as high as 13,170, and it has an apparent pK corresponding to $^1/_2\Delta\epsilon_M$ of 3.9. Since the ϵ_M of the compound at pH 2 or lower is only 5,410, and this is of the same



order as the value of 3,000 for a dinitrophenyl substituent on an iminazole ring (Ramachandran, 1961), there is a possibility that a β-lactam-like structure has formed in acid solution. The phenomenon is being further studied. The spectra of DNP-isoserine at pH 10.7 and 1.36 are shown in Figure 4. The 360-mμ peak absent in the medium

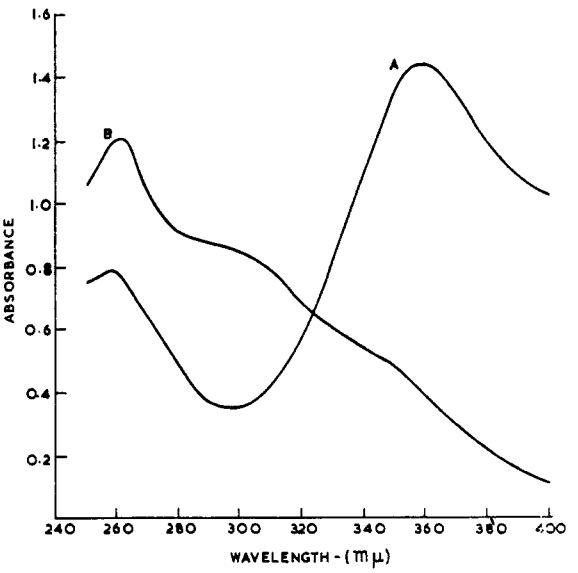


FIG. 4.—Spectrum of DNP-DL-isoserine at pH 10.7 (A) and at pH 1.36 (B).

at pH 1.36 reappears when the pH is raised. N → O acyl migration is ruled out, as the product formed at low pH has no free amino group.

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REFERENCES

Cohn, E. J. (1931), *Ergeb. Physiol. 33*, 781.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions. New York, Reinhold Publishing Co., pp. 80–84.
Czarnetsky, E. J., and Schmidt, C. L. A. (1931), *Z. physiol. Chem. 204*, 129.
Emerson, O. H., Kirk, P. L., and Schmidt, C. L. A. (1931), *J. Biol. Chem. 92*, 449.
Fraenkel-Conrat, H., Levy, A. L., and Harris, J. I. (1954), *Methods of Biochem. Anal. 2*, 359.
Gillam, A. E., and Stern, E. S. (1954), An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry. London, Edwin Arnold (Publishers) Ltd., p. 289.
Green, F. C., and Kay, L. M. (1952), *Anal. Chem. 24*, 726.
Miyamoto, S., and Schmidt, C. L. A. (1931), *J. Biol. Chem. 90*, 169.
Neuberger, A. (1937), *Proc. Roy. Soc. A 158*, 68.
Ramachandran, L. K. (1961), *Anal. Chem. 33*, 1074.
Rao, K. R., and Sober, H. A. (1954), *J. Am. Chem. Soc. 76*, 1328.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Schmidt, C. L. A., Appleman, W. K., and Kirk, P. L. (1929), *J. Biol. Chem. 81*, 723; *85*, 137.
Zief, M., and Edsall, J. T. (1937), *J. Am. Chem. Soc. 59*, 2245.