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THE EFFECTS OF HYDRAZINES ON PYRIDOXAL PHOSPHATE IN RAT BRAIN*

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Abstract—The effects have been examined of hydrazines, primarily unsymmetrical dimethylhydrazine and symmetrical dimethylhydrazine, upon pyridoxal phosphate in rat brain. No significant change was found. Injected pyridoxal phosphate was as effective as pyridoxine in raising brain pyridoxal phosphate levels, although only pyridoxine is therapeutic for dimethylhydrazine poisoning. It is concluded that the toxic action of these hydrazines is not due to a direct effect upon brain pyridoxal phosphate.

HYDRAZINE and several of its derivatives interfere *in vivo* and *in vitro* with systems involving pyridoxal phosphate (PALP). Thus glutamic decarboxylase and γ-aminobutyric (GABA) transaminase, for which PALP is a cofactor, are inhibited[1–3] perhaps because of reduction of PALP levels by formation of a Schiff base with the hydrazines; and pyridoxal kinase is inhibited *in vitro* and *in vivo* by hydrazine, perhaps because the hydrazone of pyridoxal is a potent inhibitor of the kinase.[4, 5]

The toxic action of some hydrazines has been attributed to depletion of GABA, after direct or indirect interference with glutamic decarboxylase, which produces it. The idea is supported by the fact that pyridoxine is therapeutic for poisoning by unsymmetrical dimethylhydrazine (UDMH) or monomethylhydrazine, but there are numerous anomalies, such as the failure of pyridoxine to restore the brain glutamic decarboxylase in rats poisoned by hydrazine or monomethylhydrazine, although restoration occurred with UDMH;[6] and the fact that GABA levels are modified similarly by symmetrical dimethylhydrazine (SDMH) which has no acute toxicity to rats, and by hydrazine, UDMH, and monomethylhydrazine.[5–8]

MATERIALS AND METHODS

Female albino rats weighing 180–200 g were obtained from the Holtzman Co., Madison, Wis. UDMH was obtained from Eastman Organic Chemicals, Rochester, N.Y.; SDMH dihydrochloride from Chemicals Procurement Laboratories; pyridoxal hydrochloride from Sigma Chemicals Co., St. Louis, Mo.; PALP and pyridoxine hydrochloride from Eastman Organic Chemicals; tyrosine decarboxylase apoenzyme from Worthington Biochemical Corp., Freehold, N.J.; and *l*-tyrosine from California Corp. for Biochemical Research, Los Angeles, Calif.

UDMH and SDMH were dissoved in saline and the pH was adjusted to neutral with 10 N HCl or 10 N NaOH and the volumes adjusted appropriately. UDMH at

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102 mg/kg (LD_{50} dose),[8] and SDMH at 500 mg/kg, and 25 mg of pyridoxal, PALP, and pyridoxine per kg were injected intraperitoneally. All values are calculated as the free base form. The rats were decapitated 75 min after the injection, which has been determined as the minimal time for onset of convulsions with hydrazines in our previous work,[7] and the brains were removed as quickly as possible. Rats convulsing before this time were discarded.

Determination of PALP in brain homogenate

The method of Bain and Williams[9] is sensitive and gives values for a variety of B_6-vitamers. However, each sample requires column chromatography yielding 100 fractions, and these fractions are assayed by a cumbersome yeast bioassay. As the authors wished only to examine PALP effects (since this is the actual cofactor for decarboxylases) and needed to perform a large number of determinations, the Bain and Williams method was unsuitable.

A whole brain, weighing 1·8–2·0 g, was homogenized in 20 ml of water in a glass Potter-Elvehjem homogenizer. The homogenate was poured into a 50-ml volumentric flask which contained 12·5 ml of 2 N NaOH, made up to volume with water and mixed well. Ten ml of this homogenate was heated in a boiling water bath for 5 min and cooled. Boxer *et al.*[10] state that hot alkaline treatment is necessary to release bound PALP from tissue. Samples were stored in the dark until assay. Just prior to assay the pH was adjusted to 6·5 with 2 N HCl and the sample centrifuged. The supernatant was diluted with water to give 3 to 6 mg of brain per ml. PALP was then determined manometrically by the tyrosine decarboxylase method.[11, 12]

Recovery values were obtained by adding a known amount of PALP to whole brain homogenates and treating them as above. The recovery was 88·3% ± 1·6 (standard error). The PALP values reported herein have been corrected appropriately. Values for PALP in whole rat brain of 7·8 mμmoles/g were obtained (Table 1), as compared with 4·3 for whole mouse brain (Bain and Williams[9]).

TABLE 1. DISTRIBUTION OF PALP IN RAT BRAINS*

	PALP (mμmoles/g brain)
Cerebellum	6·0 ± 0·6
Medulla oblongata	12·0 ± 0·2
Mesencephalon–diencephalon	11·3 ± 0·1
Cerebral cortex	7·5 ± 0·7

* Figures are means ± S.E. The mean values were obtained from triplicate determinations on three runs of experiments each with three pooled brains.

Preparation of the hydrazones

Thirty mg of UDMH aqueous solution was mixed with 62 mg of PALP, a few drops of acetic acid added, and boiled for 20 min. The yellow precipitate was recrystallized twice from hot water. The yield was 25%. The elemental analysis found C was 37·95%, calc. 41·56%; H was 5·65%, calc. 5·58%. Pyridoxal–UDMH hydrazone was prepared the same way with 30 mg of UDMH and 49 mg of pyridoxal. The yield was 30%. The elemental analysis found C was 58·52%, calc. 57·42%; H was 6·36%, calc. 7·18%.

The purity of the compounds was tested by paper chromatography in a *n*-butanol–acetic acid–water (2:1:1) system. Phosphorus was detected by the Hanes-Isherwood spray reagent and UDMH by trisodium pentacyanoaminoferroate (TPF) reagent. PALP–UDMH hydrazone gave positive test for both reagents. PALP could be detected by ammonia vapor. With these reagents, the hydrazones were the only materials present.

Measurement of the ultraviolet spectrum

A 25% brain homogenate from rats injected with UDMH and hydrazine was prepared in 5% trichloracetic acid solution and centrifuged. The u.v. spectrum of the supernatant was measured with distilled water as standard. Five per cent trichloracetic acid solution showed no absorption above 260 mμ but absorbed u.v. strongly below that wavelength. The u.v. spectra of pyridoxal, pyridoxal–UDMH hydrazone, PALP, and PALP–UDMH hydrazone in aqueous solution were measured with the Beckmann DB-type spectrophotometer.

RESULTS

Table 1 shows the distribution of PALP in brain. Because PALP was not markedly localized in one area, subsequent experiments used whole brain.

Table 2 shows that, contrary to expectation, neither UDMH nor SDMH had any significant effect on brain PALP.

TABLE 2. PALP LEVELS IN BRAIN OF RATS INJECTED WITH HYDRAZINES AND B_6 COMPOUNDS

	PALP (mμmoles/g brain)*			
	None	PALP	Pyridoxal	Pyridoxine
None	7·8 ± 0·5	14·2† ± 1·8	8·5 ± 0·7	11·0† ± 0·9
UDMH	8·3 ± 0·5			9·4† ± 0·3
SDMH	9·1 ± 0·7			11·1† ± 0·6

* Figures are means ± S.E. The mean values were obtained from triplicate determinations on five sets of experiments each with three pooled brains.

† These values are significantly different from controls as judged by the 't' test at the 5% level. For data in columns 2, 3, and 4 the controls are the corresponding values in column 1. For the values with UDMH alone and SDMH alone, the control is the 7·8 value.

It is known that pyridoxine protects rats from convulsive seizures induced by some hydrazines;[6, 8, 13] pyridoxal and PALP, however, have a weak synergistic effect.[6, 8] Table 2 shows that injection of PALP and pyridoxine caused substantial increases in brain PALP. Pyridoxal had little effect. Pyridoxine injected after UDMH caused a significant increase in brain PALP over the effect of UDMH alone. SDMH did not influence the increase in brain PALP caused by injected pyridoxine, in harmony with the belief that SDMH cannot form hydrazones.

Possible interferences

The brain extract used for PALP assay contained all the heat-stable constituents. The method is known to be sensitive to PALP but not to its analogues such as pyridoxine phosphate and pyridoxamine.[14] The possibility of interference by hydrazines

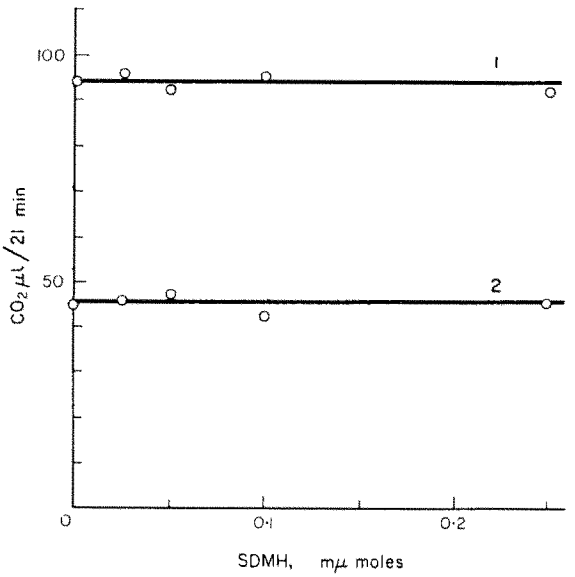

FIG. 1. Effect of SDMH upon CO_2 release in PALP determination. Each point is the mean of three determinations.

1. SDMH with $5{\cdot}0 \times 10^{-2}$ mμmoles PALP. 2. SDMH with $2{\cdot}5 \times 10^{-2}$ mμmoles PALP. Main compartment of Warburg flasks contained 0·5 ml of SDMH aqueous solution, 0·5 ml of PALP aqueous solution, 0·5 ml of tyrosine suspension (2·5 g/100 ml 0·2 M acetate buffer solution, pH 5·5) and 1·0 ml of 0·2 M acetate buffer solution, pH 5·5. Side arm of flasks contained 0·5 ml of tyrosine decarboxylase apoenzyme suspension.

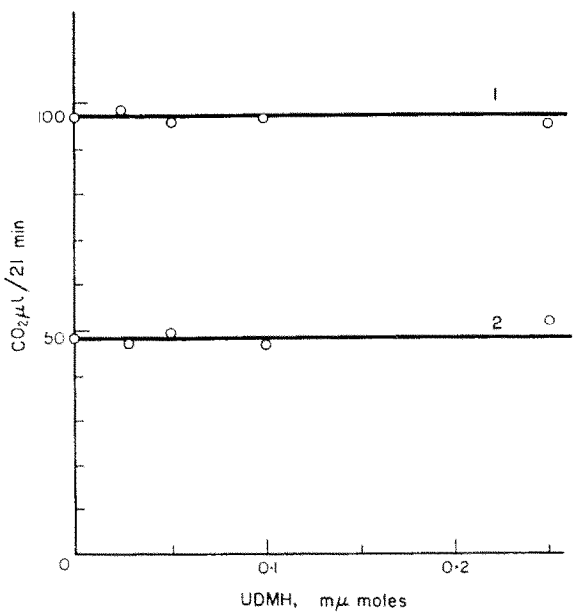

FIG. 2. Effect of UDMH upon CO_2 release in PALP determination. Each point is the mean of three determinations.

1. UDMH with $5{\cdot}0 \times 10^{-2}$ mμmoles PALP. 2. UDMH with $2{\cdot}5 \times 10^{-2}$ mμmoles PALP. Experimental conditions are as in Fig. 1.

and hydrazones had to be considered, and it was decided to study them in detail for SDMH and UDMH. Figures 1 and 2 show that concentrations of these hydrazines, even at 10 times molar excess over PALP, had no effect when added *in vitro*. It has been shown[15] in the rat that about 0·5% of the radioactivity is present in brain between 30 min and 4 hr after injection of 40 mg ^{14}C-UDMH/kg. If this figure can be used for the dose of 102 mg/kg used herein, it corresponds to 10^{-5} M in brain. This includes all derivatives; if 25% of this radioactivity was as free UDMH, then the UDMH levels in the brain would equal the highest level shown in Fig. 2. It may be concluded that, under these conditions, no interference is anticipated from residual free UDMH.

The possibility that the hydrazone formed with PALP would give a positive test in the enzyme assay was examined. UDMH–PALP hydrazone was prepared, and shown on the basis of chromatography and elemental analysis to be substantially pure (see Methods). Its activity in the enzyme assay was examined; Medina *et al.*[3] have shown that no hydrolysis to free PALP occurs under buffered conditions comparable to those used here. Figure 3 shows that the hydrazone gave a small positive response,

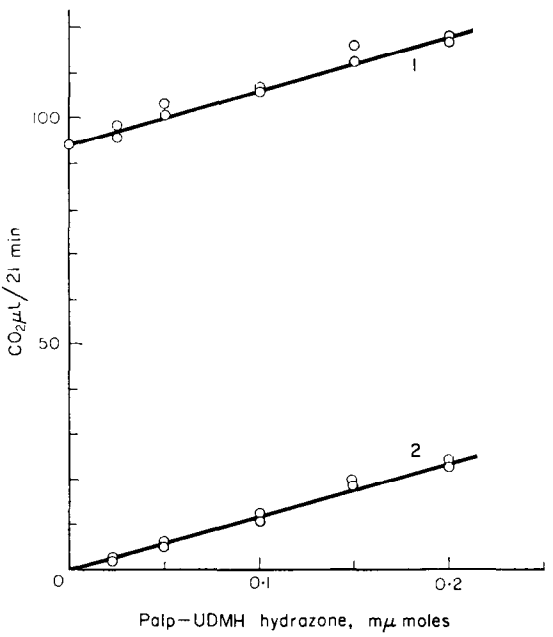

FIG. 3. Effect of UDMH–PALP hydrazone upon CO_2 release in PALP determination. Each point is the mean of three determinations.
1. The hydrazone with $5{\cdot}0 \times 10^{-2}$ mμmoles PALP. 2. The hydrazone alone. Experimental conditions are as in Fig. 1.

which was strictly additive with that due to added PALP. The response was 6·6% of that expected if all the hydrazone acted like equimolar PALP. If this effect was due to contamination with about 6·6% of PALP, that PALP would have been readily detected chromatographically. In fact it was not. It is concluded that the hydrazone is weakly active in the enzyme assay for PALP, but the additivity of its effect with that due to pure PALP permits corrections as calculated below.

Influence of hydrazines on PALP levels

Table 2 shows that, contrary to expectation, treatment of rats with UDMH or SDMH had little or no effect on PALP levels. Briefer studies with monomethylhydrazine and hydrazine also showed no decrease in PALP; mean values of 9·4 and 14·5, respectively, were found at the LD_{50} dose injection. In consequence of the small interference by PALP–UDMH hydrazone described above, the values in Table 2 would have to be somewhat reduced if hydrazones were present. An attempt was made to calculate this reduction by measuring the hydrazone levels in brains of poisoned rats. Medina[16] used an absorption maximum of 365 mμ to measure UDMH–pyridoxal and UDMH–PALP hydrazones in rat brain, after injection of UDMH plus pyridoxal or UDMH plus PALP. In the present study, extracts of brains from rats treated with UMDH alone or hydrazine alone at the LD_{50} dose showed no absorption in the 330–400 mμ range. Calculations from Fig. 4 show that the detectable limit for

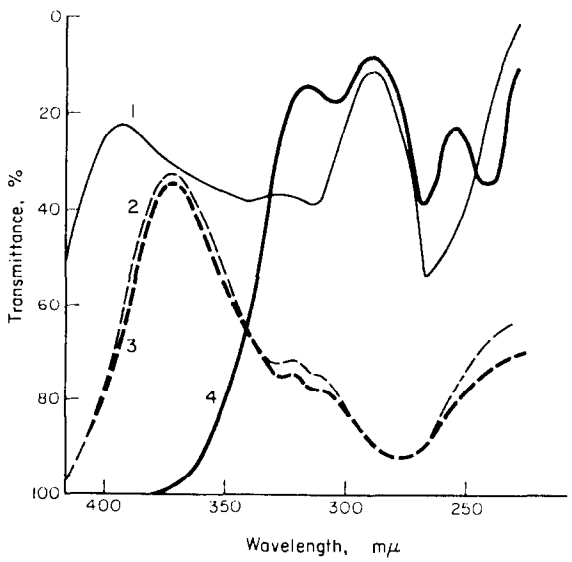

FIG. 4. Ultraviolet spectrum of pyridoxal analogues and their hydrazones. All are in aqueous solution. 1. PALP, 200 μM. 2. PALP–UDMH hydrazone, 20 μM. 3. PAL–UDMH hydrazone, 20 μM. 4. PAL, 200 μM.

PALP–UDMH hydrazone is about 3·03 μM in the extract, corresponding to 12·6 mμmoles/g of brain. Since the normal level of PALP in brain is only 7·8 mμmoles/g, one would not be able to detect formation of hydrazone by ultraviolet absorption, even if all the PALP was converted to hydrazone.

In considering the correction downward of the values in Table 2, since PALP–UDMH hydrazone is only 6·6% as active as PALP in the enzymic assay, the only way in which the figures of Table 2 could be compatible with a reduction of free PALP would be if a huge quantity of PALP–UDMH hydrazone occurred in brains of UDMH-treated rats. Thus, in principle, the value of 8·3 mμmoles/g found after UDMH treatment could be due to 3·9 mμmoles/g of free PALP (a 50% reduction compared with controls) plus 67 mμmoles/g of UDMH–PALP hydrazone. Such a

possibility would imply a massive synthesis of PALP for subsequent hydrazone formation. It is nevertheless a possibility, if in fact PALP levels are controlled by a feedback inhibition mechanism analogous to that demonstrated in purine biosynthesis,[17] so that PALP normally inhibits an enzyme earlier in the pathway of PALP synthesis; it would also be required that PALP hydrazones could not inherit this enzyme. The possibility of this explanation is reduced by the observation (Table 2) that injection of PALP into normal rats almost doubles the PALP level in brain. A feedback mechanism should prevent such an increase.

DISCUSSION

The results suggest that the short-term effects of these hydrazines upon brain metabolism are not due to a reduction of the prevailing levels of PALP. This finding very greatly reduces the number of possible biochemical lesions in brain during hydrazine poisoning, for B_6 is a cofactor in a great variety of critical reactions, including transaminases, decarboxylases, and glutathione metabolism.[18] Some other explanation must be sought for the findings that hydrazine *in vivo* inhibits rat brain glutamic decarboxylase and γ-aminobutyric transaminase. The findings are in harmony with the observation of Medina[6] that SDMH, which cannot form a Schiff base, inhibits the above enzymes substantially. The findings seem to conflict with the ability of hydrazines to inhibit rat brain pyridoxal kinase by 90% at doses of 100 mg/kg,[4] for one would expect a consequent fall in PALP, the product of pyridoxal kinase. However, there is precedent for enzymes existing in a large excess over their essential levels; about 96% of cholinesterase has to be inhibited in order to kill rabbits.[19] Consequently pyridoxal kinase might be substantially inhibited with little resultant change in PALP level.

Bain and Williams[9] gave isonicotinic acid hydrazide, 250 mg/kg i.p., to mice and found that during subsequent seizures the PALP level of brain dropped to 26% of normal but that, amazingly enough, the pyridoxal was raised four-fold. The drop in total B_6-vitamers was small (10%), but the totals differ widely from the sum of the individual compounds. They also report the unexpected observation that injected pyridoxal lowered brain PALP to 44% (in spite of raising brain pyridoxal 89-fold), quite unlike our finding in rat that a similar dose of injected pyridoxal had no effect on brain PALP. Again, they report that pyridoxine had no effect on mouse brain PALP (in spite of raising brain pyridoxal 28-fold), quite unlike our finding that injected pyridoxine increased rat brain PALP by 40%. Additional evidence of a fundamental difference between the actions of isonicotinic acid hydrazide in mice and various hydrazides in rats is that Medina[16] has been unable to detect hydrazone formation with endogenous PALP or pyridoxal after injection of hydrazines (in harmony with our observations above) and that Furst[20] has been unable to detect carbazone formation after injection of thiosemicarbazide. By contrast, Bain and Williams[9] found PALP–isonicotinoyl hydrazine (0·43 μg/g) in mouse brain after injecting isonicotinic acid hydrazide.

In considering species variation, an important variable may be the relative B_6 levels in different tissues. Levels of B_6-vitamers per unit weight of tissue in rat liver and kidney,[21] dog plasma, beef serum, and mouse liver[12] are all far greater than typical values for brain, and all these tissues together far exceed brain in total weight per animal. Variations in the ratio of non-brain to brain B_6 may account for differences

in mouse and rat effects. Certainly hydrazines can behave very differently in these species: 70 mg of SDMH/kg is fatal to mice but harmless to rats.[16]

Some alternative hypotheses which would not conflict with the above findings but would retain the view that in fact hydrazines do inhibit glutamate decarboxylase via formation of inactive PALP hydrazones, are as follows. (a) Possibly the hydrazones are formed with enzyme-bound PALP, but not with free PALP. However, Greenberg *et al.*[5] have data suggesting that in brain *homogenates* the glutamate decarboxylase is not saturated with PALP, although the applicability of this finding to intact brain is uncertain. It rather suggests the absence of much free PALP. (b) There may be metabolic compartmentalization of PALP, so that the PALP available as coenzyme for glutamate decarboxylase is bound by hydrazines, without much effect on the total PALP levels. (c) The data may represent a decrease of PALP, along with a massive accumulation of PALP–hydrazone, as discussed above. But if this combination permits approximately normal functioning of the tyrosine decarboxylase apoenzyme in the bioassay, one might suspect that it would allow approximately normal functioning of glutamic decarboxylase in intact brain.

In conclusion, the data presented, taken with the findings of Medina[6] and Uchida and O'Brien[7] that SDMH affects brain glutamate decarboxylase and γ-aminobutyric acid levels in a way similar to that of UDMH, hydrazine, and monomethylhydrazine, suggest that the simplest hypothesis is that these four hydrazines act directly upon glutamate decarboxylase and not by lowering the PALP levels of brain.

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REFERENCES

1. E. F. ROBERTS and S. FRANKEL, *J. biol. Chem.* **188**, 789 (1951).
2. K. E. KILLAM and J. A. BAIN, *J. Pharmacol.* **119**, 255 (1957).
3. M. A. MEDINA, H. D. BRAYMER and J. L. REEVES, *J. Neurochem.* **9**, 307 (1962).
4. D. B. MCCORMICK, B. M. GUIRARD and E. E. SNELL, *Proc. Soc. exp. Biol. (N. Y.)* **104**, 554 (1960).
5. S. M. GREENBERG, T. MASURAT, E. G. RICE, J. F. HERNDON and E. F. VAN LOON, *Biochem. Pharmacol.* **2**, 308 (1959).
6. M. A. MEDINA, *J. Pharmacol.* **140**, 133 (1963).
7. T. UCHIDA and R. D. O'BRIEN, *Biochem. Pharmacol.* In press.
8. R. D. O'BRIEN, M. KIRKPATRICK and P. S. MILLER, *Toxicol. appl. Pharmacol.* In press.
9. J. A. BAIN and H. L. WILLIAMS, in *Inhibition in the Nervous System and Gamma-Aminobutyric Acid*, E. ROBERTS, Ed., p. 275. Pergamon Press, New York (1960).
10. G. E. BOXER, M. P. PRUSS and R. S. GOODHART, *J. Nutr.* **63**, 623 (1957).
11. M. WACHSTEIN, J. D. KELLNER and J. M. ORTIZ, *Proc. Soc. exp. Biol. (N. Y.)* **103**, 350 (1960).
12. W. W. UMBREIT, in *The Vitamins; Chemistry, Physiology, and Pathology*, W. H. SEBRELL, JR. and R. S. HARRIS, Eds., vol. 3, p. 239. Academic Press, New York (1954).
13. K. C. BACK, M. K. PINKERTON and A. A. THOMAS, Technical Documentary Report No. AMRL-TDR 63-44. Aerospace Medical Research Laboratories (1963).
14. W. W. UMBREIT, W. D. BELLAMY and L. C. GUNSALUS, *Arch. Biochem.* **7**, 185 (1945).
15. M. A. MITZ, F. L. ALDRICH and B. M. VASTA. Private communication (1962).
16. M. A. MEDINA. Private communication (1963).
17. A. B. PARDEE, in *The Enzymes*, P. D. BOYER, H. LARDY and K. MYRBACK, Eds., vol. 1, p. 681. Academic Press, New York (1959).
18. J. M. HSU, E. BUDDEMEYER and B. F. CHOW, *Biochem. J.* **90**, 60 (1962).
19. D. NACHMANSOHN and E. A. FELD, *J. biol. Chem.* **171**, 715 (1947).
20. A. FURST. Private communication to M. A. Medina (1963).
21. J. C. RABINOWITZ and E. E. SNELL, *J. biol. Chem.* **176**, 1157 (1948).