Biochemical Pharmacology, Vol. 19, pp. 275–283. Pergamon Press. 1970. Printed in Great Britain

EFFECT OF HYDRAZINE TREATMENT ON HEPATIC PROTEIN BIOSYNTHESIS *IN VIVO*

W. L. BANKS, JR.

Department of Biochemistry, Medical College of Virginia, Richmond, Va. 23219, U.S.A.

(*Received* 27 *January* 1969; *accepted* 30 *May* 1969)

Abstract—The effect of a single subconvulsive dose of hydrazine (40 mg/kg body weight) on several parameters associated with hepatic protein biosynthesis was studied in adult, male rats. The results suggested that hydrazine treatment might have stimulated hepatic protein biosynthesis *in vivo*. This conclusion was based upon increased levels of liver $\frac{\text{RNA}}{\text{DNA}}$ and $\frac{\text{Protein}}{\text{DNA}}$ as well as increased incorporation of radioactive leucine into liver protein of the hydrazine-treated compared to the control rats. No alterations in these parameters due to hydrazine treatment were observed in the brain.

The free amino acid patterns of the liver and skeletal muscle implied that the amino acids required to obtain the increased liver protein content in the hydrazine-treated rat might have been supplied via mobilization of skeletal muscle "protein reserves".

INCREASED uptakes of ^{14}C-labeled amino acids into protein of liver slices from hydrazine-treated compared to control rats were reported by Amenta and Johnston.[1] They attributed the enhanced protein labeling to an enlarged free amino acid pool size in the liver due to a hydrazine-specific inhibition of transamination. However, they did not measure the free amino acid pool size. An increased liver protein content which correlated with an increased liver RNA content was demonstrated 24 hr after a single subconvulsive dose of hydrazine to rats.[2] These changes were associated with an enlarged liver and could not be ascribed to nutritional factors. The increased liver RNA and protein contents were preceded by increased levels of liver DNA.[3] These results indicated a possible sequence of changes in nucleic acid metabolism which brought about enhanced protein biosynthesis in the liver after hydrazine treatment.

In a preliminary report,[4] the increase in liver protein and RNA contents due to hydrazine treatment was correlated with an enhanced uptake of radioactive leucine into protein. The present study presents data that support the contention that hydrazine treatment might stimulate liver protein biosynthesis *in vivo*. In addition, the free amino acid patterns indicate that the skeletal muscle could serve as the source of the amino acid supply to the liver after hydrazine treatment.

EXPERIMENTAL

Experiment A. Twenty adult, male Holtzman rats were separated into two groups and injected i.p. with either hydrazine (40 mg/kg; brought to pH 7·4 with 100% CO_2) or isotonic saline (1·0 ml/kg). All of the animals were fasted for 24 hr and given water *ad lib.* At 22 hr after the initial injections, each rat was injected i.p. with 5·0 μc of L-leucine (uniformly labeled).

After 24 hr, the rats were anesthetized with sodium pentobarbital (40 mg/kg) and exsanguinated via the renal vein and artery. The brains and livers were removed, weighed and frozen. Homogenates were prepared from a sample of the liver or the whole brain.

The concentrations of protein, RNA and DNA were determined from aliquots of the tissue homogenates according to the method described by Wannemacher *et al.*[5] with the modifications suggested by Munro and Fleck.[6] Radioactivity measurements were made by a liquid scintillation system using Bray's solution[7] as the scintillator. The uptakes of ^{14}C-leucine into protein were determined by precipitating the protein with trichloracetic acid, digesting the precipitate with "Nuclear Chicago Solubilizer" (NCS) and counting an aliquot of this digest. The nonprotein radioactivity was determined by counting an aliquot of the TCA soluble material. In addition, the total ^{14}C-leucine radioactivity was determined by counting an aliquot from an NCS digest of the tissue homogenate. All of the radioactivity measurements were corrected for quenching using an external standard.

Experiment B. Twenty adult, male Sprague–Dawley rats were separated into two groups. The animals were treated with hydrazine or saline as in experiment A. After 24 hr, the rats were killed and the liver and a sample of gastrocnemius muscle were removed, weighed and frozen. The concentrations of protein, RNA and DNA were determined in homogenates of liver and muscle by the methods described above.[5, 6] Samples of the homogenates of each group were pooled and the amino acid patterns of the protein free filtrates were determined by elution chromatography.[8]

Statistical comparisons were made between the means of the control and experimental groups by the Student's *t*-test. The levels of significance for the various comparisons are indicated in the Tables.

RESULTS

In experiment A, hydrazine treatment produced a marked enlargement of the liver after 24 hr when expressed as either the actual liver weight or the relative liver weight (Table 1). The increase in liver size was accompanied by increased quantities of total liver protein and total liver RNA. The ratio, protein/RNA, was the same in both groups of rats. The proportional increase in liver protein was less than the percentage

TABLE 1. EFFECT OF HYDRAZINE ON BODY WEIGHT, LIVER SIZE, LIVER PROTEIN AND RNA CONTENTS

Group	Initial body weight (g)	Actual liver weight (g)	Relative liver weight (g/100 g body wt.)	Total liver protein (mg/liver)	Total liver RNA (mg/liver)	Protein / RNA (mg/mg)
Control	334 ± 7*	8·721 ± 0·218	2·84 ± 0·04	1470 ± 55	64·6 ± 2·2	22·7 ± 0·8
Hydrazine	315 ± 9	11·301 ± 0·375	3·93 ± 0·04	1660 ± 69	77·8 ± 3·7	21·5 ± 2·7
Significance	NSD†	$P < 0·001$	$P < 0·001$	$P < 0·05$	$P < 0·01$	NSD

* Standard error (S.E.).
† No significant difference.

increase in the liver wet weight due to hydrazine. Thus, the increase in liver protein due to hydrazine was not an exact reflection of the increased liver size.

The total liver DNA content (mg DNA/liver) was unaffected, whereas the DNA concentration (mg DNA/g liver) was significantly lowered by hydrazine treatment. Expression of the protein and RNA on a DNA basis revealed that the protein/DNA and RNA/DNA were significantly elevated in the liver by hydrazine treatment (Table 2).

The total incorporation of ^{14}C-leucine into the liver tissue was increased 53 per cent by hydrazine treatment (Table 2). Moreover, the data indicate that the increased incorporation of ^{14}C-leucine into the liver was associated with the protein rather than the

TABLE 2. EFFECT OF HYDRAZINE ON LIVER PROTEIN/DNA, RNA/DNA AND ^{14}C-LEUCINE UPTAKES

Group	Protein / DNA (mg/mg)	RNA / DNA (mg/mg)	Total radioactivity (cpm/ mg DNA)	Nonprotein radioactivity (cpm/ mg DNA)	Protein radioactivity (cpm/ mg DNA)	Specific activity (cpm/ mg Protein)
Control	43·6 ± 1·0*	1·92 ± 0·04	28,600 ± 1420	3010 ± 515	23,900 ± 1360	551 ± 32
Hydrazine	51·6 ± 1·8	2·41 ± 0·07	43,600 ± 2580	3940 ± 529	34,760 ± 2580	677 ± 49
Significance	$P < 0·001$	$P < 0·001$	$P < 0·001$	NSD	$P < 0·01$	$P < 0·05$

* S.E.

TABLE 3. EFFECT OF HYDRAZINE ON BRAIN PROTEIN/DNA, RNA/DNA AND ^{14}C-LEUCINE UPTAKES IN BRAIN

Group	Protein / DNA (mg/mg)	RNA / DNA (mg/mg)	Total radioactivity (cpm/ mg DNA)	Nonprotein radioactivity (cpm/ mg DNA)	Protein radioactivity (cpm/ mg DNA)	Specific activity (cpm/ mg protein)
Control	376 ± 13*	8·02 ± 0·25	95·5 ± 5·3	27·7 ± 5·3	62·1 ± 2·6	1·66 ± 0·048
Hydrazine	388 ± 10	7·90 ± 0·18	96·1 ± 4·6	31·9 ± 2·4	58·5 ± 2·9	1·51 ± 0·054
Significance	NSD	NSD	NSD	NSD	NSD	NSD

* S.E.

nonprotein compartment of the cell. The radioactivity in the protein compartment was increased 45 per cent by hydrazine treatment, whereas there was no significant difference in the nonprotein radioactivity between the hydrazine-treated and control groups. The specific activity of the liver protein was elevated 23 per cent by hydrazine treatment (Table 3). Hence, the proportion of the total liver protein that was labeled was enhanced significantly after a relatively short period of exposure to the radioactive precursor. These results demonstrate a marked increase in the incorporation of ^{14}C-leucine into protein due to hydrazine treatment.

In the brains of the same rats, there were no significant differences between the control and experimental groups in the protein/DNA, RNA/DNA and the uptake of ^{14}C-leucine into protein (Table 3). In this tissue, neither the uptake of C^{14}-leucine into the tissue, the protein compartment nor the nonprotein compartment was altered by hydrazine treatment.

In experiment B, the liver weight, total liver protein and liver protein/DNA were increased by hydrazine treatment after 24 hr (Table 4). In addition, the total liver RNA and the liver RNA/DNA were elevated significantly by hydrazine treatment. These results were consistent with those obtained in experiment A (Tables 1 and 2). Since two different strains of rats were used in these experiments, it would appear that the elevation in these parameters by hydrazine treatment was not a single strain anomaly. In this experiment, no significant differences were noted in the skeletal muscle protein when this data was expressed as the ratio, protein/DNA, or as the skeletal muscle protein concentration (mg protein/g muscle), (Table 4).

TABLE 4. EFFECT OF HYDRAZINE ON LIVER SIZE, LIVER PROTEIN AND SKELETAL MUSCLE PROTEIN CONTENT

Group	Liver weight (g)	Total liver protein (mg/liver)	Liver protein / DNA (mg/mg)	Skeletal muscle protein / DNA (mg/mg)	Skeletal muscle protein concn (mg/g muscle)
Control	8·200 ± 0·233*	925 ± 31	51·8 ± 1·8	244 ± 8	95·7 ± 2·2
Hydrazine	11·383 ± 0·240	1215 ± 29	61·8 ± 1·7	243 ± 4	94·3 ± 2·2
Significance	$P < 0·001$	$P < 0·001$	$P < 0·001$	NSD	NSD

* S.E.

TABLE 5. SELECTED FREE AMINO ACID LEVELS IN THE LIVER AND SKELETAL MUSCLE OF HYDRAZINE-TREATED AND CONTROL RATS

Amino acid	Control liver	Hydrazine liver	Control muscle	Hydrazine muscle
	(μmoles/mg DNA)			
ASP	3·87	6·63	4·45	9·42
GLU	14·1	15·7	14·5	14·4
ALA	11·2	15·6	34·0	56·4
TYR	1·56	5·19	3·80	9·77
VAL	4·05	3·78	6·55	14·0
LEU	3·11	4·02	7·07	16·3
MET	2·21	1·44	2·85	6·95
THR	3·65	5·25	9·08	14·5
ANB	0·20	2·78	nil	2·83
ORN	7·11	11·8	nil	4·36

The data presented in this table are taken from a single representative experiment.

The levels of selected amino acids from this representative experiment are illustrated in Table 5. In the liver, the most pronounced increases resulting from hydrazine treatment were in tyrosine, α-amino-*n*-butyric acid (ANB) and ornithine. Relatively small increases were noted in the levels of glutamate, aspartate, alanine and leucine, whereas somewhat decreased levels of valine and methionine were found in the liver as the result of hydrazine treatment. In comparison to the liver, the muscle levels exhibited marked increases in all of the free amino acids with the exception of glutamate as the consequence of hydrazine treatment.

DISCUSSION

On the basis of previous results from this laboratory[2–4] and others,[1] it might be postulated that a single, low dose of hydrazine would stimulate hepatic protein biosynthesis in the rat. Experiment A was designed to test this hypothesis.

Hydrazine treatment produced a marked enlargement of the liver size which correlated with increased quantities of total liver RNA and total liver protein. On a percentage basis, the increases in liver weight, protein and RNA in both experiments were similar to those reported previously.[2, 3] Since the ratio, protein/RNA, was the same for both groups of animals (Table 1), it appears that the quantity of liver protein was a direct function of the quantity of liver RNA. When expressed on a DNA, or cellular basis,[9] the liver protein (Tables 2 and 4) and RNA (Table 2) were elevated by hydrazine treatment.

Since the nonprotein radioactivity was the same for the control and experimental animals, it might be assumed that the radioactive pools of ^{14}C-leucine were similar in size. In experiment B, the content of free leucine in the liver did not differ substantially between the control and experimental group. If the nonprotein radioactivity in experiment A were corrected to a constant pool size by the free leucine levels determined in experiment B, values of 968 and 980 cpm per μmole leucine were obtained for the control and experimental groups respectively. Although the data from the two experiments cannot be rigidly interrelated, both experiments indicate that the pools of leucine in the livers of the control and experimental groups were similar in size.

The increased ratios of protein/DNA, RNA/DNA and the uptake of ^{14}C-leucine into protein due to hydrazine treatment strongly support the contention that a single, low dose of hydrazine stimulated liver protein biosynthesis *in vivo*. Since the time for uptake of the labeled amino acid into liver protein was relatively short, the predominant factor in the increased radioactive uptakes and specific activities of the protein was the rate of protein biosynthesis. However, a part of the increased labeling of liver protein in the hydrazine-treated animals might have been the result of decreased protein catabolism. The absolute magnitude of any alteration in liver protein catabolic pathways was not evaluated in this study. A stimulation in liver protein biosynthesis would be consistent with the ultrastructural results reported by Ganote and Rosenthal.[10] These investigators observed a substantial enlargement of both nuclear and nucleolar area as well as a moderate polysomal aggregation in rat liver as the result of hydrazine sulfate treatment. Assuming that the liver DNA content was related to the number of cells in these animals, then expression of the various parameters studied on a DNA basis would be tantamount to describing the cellular changes in those constituents due to hydrazine. Thus, the nuclear enlargements noted ultrastructurally might be one aspect of an overall cellular enlargment which in turn may account for the increased organ size due to hydrazine treatment.

Since no alterations in these parameters were observed in the brain and no alterations in the content of protein and RNA in the kidneys and gonads were reported after hydrazine treatment,[2] the results thus far indicate that the hydrazine-produced stimulation of protein biosynthesis *in vivo* might be confined to the liver.

The magnitude of the increase in liver protein content due to hydrazine treatment would require a substantial endogenous source of amino acids since the animals were fasted for 24 hr. Both groups of animals were fasted because the hydrazine-treated animals will refuse to consume food for several days after treatment with the com-

pound.[11] Thus, a valid comparison of protein biosynthesis *in vivo* can be made only against similarly fasted control animals. In this regard, Allison *et al.*[12] have shown that the liver protein/DNA of rats declined by fasting to a level less than 60 per cent of the fed value at 24 hr and remained essentially constant during the succeeding 48-hr period. Moreover, these investigators reported that the skeletal muscle protein/DNA was reduced by 20 per cent during the initial 24-hr period of fasting and continued to fall to a level of 75 per cent the fed value after 3 days. These workers indicated the skeletal muscle as a source of the "labile protein reserve" that could supply amino acids to other tissues of the body when they were scarce. In particular, the essential amino acids cannot be synthesized metabolically and must be supplied either by the diet or through protein catabolic pathways. Hence the hydrazine-produced increase in liver protein content in the 24-hr fasted rat would stress greatly the supply of all amino acids and, particularly, the essential amino acids. On the basis of the above discussion, catabolism of skeletal muscle protein might be expected to supply the

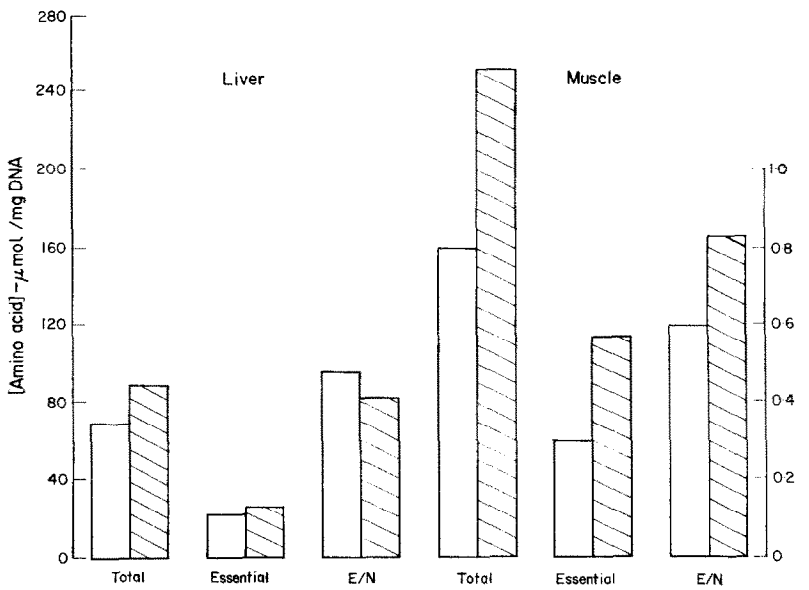

FIG. 1. The free amino acid composition of liver and muscle.
The bar-graphs illustrate the total and essential free amino acid levels per DNA and the ratios of essential to nonessential free amino acids (E/N) in liver and skeletal muscle of hydrazine-treated (cross-hatched bars) and control rats (open bars). The left ordinate denotes the free amino acid levels and the right ordinate, E/N.

additional quantity of amino acids required by the liver in the hydrazine-treated rat. Experiment B was designed to determine whether this was the case.

The levels of the total free amino acids and the levels of the free essential amino acids as well as the ratio of the essential to nonessential amino acids (E/N) for the liver and skeletal muscles from the control and hydrazine-treated animals from this representative experiment are illustrated in Fig. 1. In the liver, the total free amino acid level on a DNA basis was increased about 30 per cent by hydrazine treatment.

This increase in liver free amino acid level appears to be due principally to the nonessential rather than the essential amino acids. In the muscle, the total free amino acid level of the hydrazine-treated group was approximately 37 per cent greater than the control value. This increase in free amino acid content of the muscle due to hydrazine treatment was approximately 60 per cent with respect to essential amino acids and 40 per cent with respect to nonessential amino acids.

The absence of an alteration in protein content (Table 4) would indicate that the skeletal muscle was not the supplier of the additional amino acids required by the liver in the hydrazine-treated rat. However, examination of the data presented in Fig. 1 would tend to contradict this conclusion. This disproportionate rise of skeletal muscle essential amino acids in the hydrazine-treated compared to the control animals would be consistent with increased skeletal muscle protein catabolism in the treated group. Thus, a very small mobilization of skeletal muscle amino acids through protein catabolic pathways summed over the large muscle mass could account for the additional amino acid requirement of the liver in the hydrazine-treated rat. Since the fasted rat represents a one-way system with respect to essential amino acids, a hepatic stimulation of protein biosynthesis would tend to preferentially reduce their levels in the liver. However, the absolute level of free essential amino acids in the liver and the hepatic ratio, E/N, do not appear to be reduced by hydrazine treatment. The levels of free amino acids in liver of the hydrazine-treated rats could be maintained by increased mobilization of skeletal muscle amino acids or by enhanced metabolic formation of amino acid precursors within the liver. Mobilization of skeletal muscle amino acids would be consistent with the increased levels of serum amino acids that have been reported in hydrazine-treated dogs.[13] Since nonessential amino acid precursors could arise from carbohydrate and citric-acid cycle intermediates, a large reduction in liver glycogen after hydrazine treatment, as demonstrated by Amenta and Johnston,[11] could provide sufficient precursors to maintain the observed free nonessential amino acid levels even if hepatic protein biosynthesis was stimulated.

Hydrazine and hydrazine derivatives have been shown to inhibit transamination *in vitro*; presumably by formation of pyridoxal hydrazones.[14, 15] Inhibition of transamination *in vivo* would tend to reduce the reversible interconversions of alanine, glutamate and aspartate to pyruvate, α-ketoglutarate and oxalacetate respectively. Whether this inhibition would augment or reduce the levels of these amino acids would be a complex function of the relative rates of transamination in either direction, the rate of transport of amino acids into the organ and the relative rates of protein biosynthesis and catabolism in the liver cells.

An overall reduction in the levels of the free amino acids would be expected in the liver from the hydrazine-treated rats if stimulation of protein biosynthesis were the single factor regulating the amino acid pool size. Yet, the level of the free amino acids in the liver was elevated by hydrazine treatment (Fig. 1). Furthermore, there was no marked alteration in the levels of the principal participants in liver transamination, that is, aspartate, glutamate and alanine (Table 5). Thus, neither the stimulation in liver protein biosynthesis nor inhibition of transamination can be implicated as the sole determinant of the liver amino acid pool size in our study. However, a much larger dose of hydrazine to mice produced a substantial elevation in the levels of alanine, aspartate, and glutamate[16] which might imply a predominant effect of transaminase inhibition on the amino acid pool size.

The levels of free tyrosine were markedly elevated in both the liver and the muscle by hydrazine treatment (Table 5). This elevation represented the largest proportional change in any single protein precursor after hydrazine treatment. The increase in free tyrosine could be accounted for by inhibition of transamination. A large elevation in the level of tyrosine has been found in serum of hydrazine-treated dogs.[13, 17]

The patterns of valine and leucine in the liver and muscle are typical of most of the essential amino acids (Table 5). Essentially no differences were noted in the liver between hydrazine-treated and control animals, whereas a pronounced mobilization of these amino acids was seen in the skeletal muscle after hydrazine treatment. Since transamination is the first catabolic step for these amino acids, their levels in the liver would not be consistent with a general inhibition in transamination.

In the muscle, all of the free amino acids were elevated with the exception of glutamate. This would indicate a general mobilization of amino acids in this tissue that most likely occurred as a consequence of accelerated protein catabolism.

The most pronounced change of amino acid levels was found in α-amino-*n*-butyric acid (ANB) in both of the tissues studied. This amino acid, which is not a constituent of protein, was increased from negligible values many-fold by hydrazine treatment (Table 5). ANB is the product of transamination of α-ketobutyrate which in turn could arise metabolically via minor pathways from methionine and threonine in animal tissues.[18] Blockage of transamination would not result in the appearance of appreciable levels of ANB since the major catabolic pathway of α-ketobutyrate involves oxidative decarboxylation to propionyl CoA. Accumulation of ANB would be expected only when oxidative decarboxylation, rather than transamination, was blocked. From the levels of free amino acids found in the liver, it might be concluded that the ANB arose from methionine catabolism to a greater extent than from the threonine pathway. However, in the muscle the levels of these two amino acids do not reveal any direct relationship to the marked rise in ANB. These data taken together would imply that the high levels of ANB following hydrazine treatment were due to a pathway that is yet to be defined.

The free amino acid patterns suggest that a general blockade of transamination would not account exclusively for the increase in hepatic protein content following hydrazine treatment. Nevertheless, in the case of specific amino acids, i.e. tyrosine, inhibition of transamination could account for the observed free amino acid levels.

Finally, it is interesting to note the build up of ornithine in the liver and muscle since this amino acid plays a central role in the metabolic mechanism for the catabolism of amino acid nitrogen. The increased level of ornithine could be the result of a reduction in the conversion of ornithine to citrulline due to a diminished production of carbamyl phosphate from amino acid catabolism. In this regard, hydrazine has been shown to be a competitive inhibitor of carbamyl phosphate synthetase from frog-liver particles.[19]

If it can be assumed that the liver DNA content is related to the number of cells in the case of hydrazine-treated animals as it is in normal liver,[9] then a qualitative comparison of the various parameters that were studied on a DNA basis would reveal the hepatic cellular changes resulting from hydrazine treatment. In comparison to the controls, the liver cell of the hydrazine-treated animal contains more RNA and protein and shows a greater uptake of radioactive leucine into protein. These facts taken together would support the hypothesis that hydrazine treatment stimulated

liver protein biosynthesis. In addition, it appears that the amino acids required to supply an accelerated liver protein biosynthesis might arise from skeletal muscle protein catabolism.

Acknowledgement—The study was supported in part by grants from the National Institutes of Health (MH 12602) and the A. D. Williams Fluid Fund for Research of the Medical College of Virginia.

REFERENCES

1. J. S. AMENTA and E. H. JOHNSTON, *Lab. Invest.* **12**, 921 (1963).
2. W. L. BANKS, JR. and E. R. STEIN, *Proc. Soc. exp. Biol. Med.* **120**, 1 (1965).
3. W. L. BANKS, JR., D. A. CLARK and E. R. STEIN, *Proc. Soc. exp. Biol. Med.* **124**, 595 (1967).
4. W. L. BANKS, JR., *Toxic. appl. Pharmac.* **11**, 71 (1968).
5. R. W. WANNEMACHER, JR., W. L. BANKS, JR. and W. H. WUNNER, *Analyt. Biochem.* **11**, 320 (1965).
6. H. N. MUNRO and A. FLECK, *Analyst* **91**, 78 (1966).
7. G. A. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
8. D. H. SPACKMAN, W. H. STEIN and S. MOORE, *Analyt. Chem.* **30**, 1190 (1958).
9. J. N. WILLIAMS, *J. Nutr.* **73**, 199 (1961).
10. C. E. GANOTE and A. S. ROSENTHAL, *Lab. Invest.* **19**, 382 (1968).
11. J. S. AMENTA and E. H. JOHNSTON, *Lab. Invest.* **11**, 956 (1962).
12. J. B. ALLISON, R. W. WANNEMACHER, JR. and W. L. BANKS, JR., *Fedn Proc.* **22**, 1126 (1963).
13. P. KORTY and F. L. COE, *J. Pharmac. exp. Ther.* **160**, 212 (1968).
14. J. W. SIZER and W. T. JENKINS, *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN) vol. V, p. 677. Academic Press, New York, 1962.
15. K. F. KILLAM and J. H. BAIN, *J. Pharmac. exp. Ther.* **119**, 255 (1957).
16. D. C. SIMMONSEN and E. R. ROBERTS, *Proc. Soc. exp. Biol. Med.* **124**, 806 (1967).
17. H. H. CORNISH and C. E. WILSON, *Toxic. appl. Pharmac.* **12**, 265 (1968).
18. A. MEISTER, *Biochemistry of the Amino Acids*, vol. II, pp. 680–685, 759. Academic Press, New York, 1965.
19. S. MCKINLEY, C. D. ANDERSON and M. E. JONES, *J. biol. Chem.* **242**, 3381 (1967).