Biochemical Pharmacology, 1961, Vol. 5, pp. 323–331. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE GABA PATHWAY—I

THE INHIBITION OF γ-AMINOBUTYRIC ACID–α-KETOGLUTARIC ACID TRANSAMINASE *IN VITRO* AND *IN VIVO* BY U-7524 (AMINO-OXYACETIC ACID)

D. P. WALLACH
with the technical assistance of N. J. CRITTENDEN

The Pharmacology Department, The Upjohn Co., Kalamazoo, Mich.

(*Received* 15 *July* 1960)

Abstract—(1) Amino-oxyacetic acid has been found to be a potent inhibitor of the enzyme γ-aminobutyric acid-α-ketoglutaric acid transaminase derived both from *E. coli* and mammalian brain.

(2) The nature of this inhibition has been demonstrated to be strictly competitive.

(3) Amino-oxyacetic acid has also been demonstrated to inhibit the transaminase in the brains of several species of animals causing marked elevations in the brain concentrations of γ-aminobutyric acid.

(4) An assay method for γ-aminobutyric acid was developed using naturally occurring γ-aminobutyric acid-α-ketoglutaric acid transaminase and succinic semialdehyde dehydrogenase derived from *E. coli*.

THERE is abundant evidence extant[1–3] that γ-aminobutyric acid (GABA) is found in remarkably high concentrations in the central nervous system and that it may have an important physiological role in the transmission of certain types of nervous impulses. Administration of GABA in massive intravenous doses does not cause an elevation in brain concentrations of this amino acid, presumably because GABA does not cross the blood–brain barrier in appreciable quantities. Thus, it has been difficult to elucidate the effects of increased concentrations of GABA in the brains of intact animals. Roberts and Frankel[4, 5] demonstrated that GABA is formed in brain by the decarboxylation of glutamic acid. Subsequently, as shown by Bessman *et al.*[6] and Roberts and Bregoff[7], GABA transaminates with α-ketoglutaric acid (KGA) to form glutamate and succinic semialdehyde. As this is the principal pathway for the degradation of this amino acid in brain, inhibition of this transamination step should result in increased brain concentrations of GABA. Baxter and Roberts[8] have reported that such an inhibition can be induced with suitable doses of hydroxylamine, approximately doubling the concentration of GABA in the brain of rats.

This report is concerned with the enzymic and some of the physiological properties of a much more potent inhibitor of this enzyme, namely amino-oxyacetic acid (AOAA) ($H_2N.O.CH_2.COOH$) and its effects on the levels of GABA in the brain in various species of animals.

EXPERIMENTAL

Material and methods

When this study was initiated it was realized that the GABA–KGA transaminase found in brain of most species is not a very active enzyme compared to the same transaminase from bacterial sources. It is also more labile to freezing and thawing. When the brain transaminase is isolated from acetone powders it is usually contaminated with free α-amino acids which chelate copper. These interfere with the copper chelation method of Baxter and Roberts[9], which is the most rapid method currently available for the estimation of glutamic acid. Thus, it was felt that the brain enzyme was a poor one to employ in testing potential inhibitors. Baxter and Roberts[9] reported that *E. coli*, strain E-26 had a very active GABA–KGA transaminase. An investigation of this and other strains of *E. coli* showed that a number of them had the required enzyme and one strain, ATCC-26, when grown in nutrient broth (Difco), was superior to other strains for this transaminase activity. This organism was also found to have a very active succinic semialdehyde dehydrogenase. Jakoby and Scott[10] described a coupled system in which these two enzymes, isolated as adaptive enzymes from *Pseudomonas fluorescens* ATCC 13,430, grown on a synthetic medium rich in pyrollidine, could be used in an assay for either GABA or KGA by following the reduction of TPN at 340 mμ. The naturally occurring enzymes in *E. coli* ATCC-26, when isolated in the manner to be described, can be used either as a coupled system for the assay of GABA, or for transaminase activity alone, when testing the activity *in vitro* of potential inhibitors.

The organism is grown on 10-ml slants of nutrient agar (Difco) and is transferred monthly to fresh slants. When a batch of cells is to be grown, 6 l. of nutrient broth (Difco) are divided into 150-ml aliquots in 500-ml shake flasks and autoclaved. When cool, the flasks are seeded with a suspension of organisms from a slant and are then shaken at 250 rev/min at from 28 to 29 °C for from 16 to 18 hr. Good growth should be evident at this time. The cells are harvested by centrifugation and washed twice in at least 10 vols. of cold saline, after which they are frozen. The usual yield of cells is from 17 to 18 g. When frozen, the cells may be stored indefinitely without loss of enzymic activity.

When desired, the cells are thawed and washed once in 10 vols. of 0·1 M potassium phosphate buffer, pH 7·35, containing 0·01 per cent 2-mercaptoethanol. The cells are then resuspended in 1 vol. of this buffer and are treated for 30 min at from 0 to 3 °C in a Raytheon sonic oscillator.* The preparation is centrifuged for 20 min at 12,000 *g* in order to remove cellular debris and unbroken cells. The supernatant fluid is then adjusted to a protein concentration of from 65 to 75 mg/ml and is designated E_0. This crude extract has contaminating enzymes which react with compounds in brain other than GABA to cause a reduction of TPN. This makes accurate assays impossible without further purification.

In the fractionation scheme to be described, all volumes refer to the initial volume of sonicate taken for fractionation. To 15 ml of the crude sonicate, 0·05 vols. of 1 M $MnCl_2$ are added dropwise and with good mixing. The precipitate of nucleic acid which forms is removed by centrifugation and discarded. To the supernatant solution are added dropwise and with rapid mixing 0·8 vol. of cold saturated ammonium sulfate

* The machine used is rated at 200 W and 10 kc. The power supply was set at a current output of 1·0 A.

solution adjusted to a pH of 7·4 (measured at a 1:10 dilution in water at room temperature). The precipitate is removed by centrifugation and discarded. To the supernatant fluid is added 1 vol. of the ammonium sulfate solution (pH 7·4). The precipitate is collected by centrifugation and dissolved in 1 vol. of 0·1 M potassium phosphate buffer, pH 7·35, containing 0·01 per cent 2-mercaptoethanol. This fraction contains the desired enzymes. The yield is from 60 to 70 per cent of the initial activity of the sonicate, with a 1·5- to 2-fold purification.

The components of the mixture used in the determination of enzyme activity which are added to a Beckman DU cuvette, are as follows: 6 μmoles of GABA; 5 μmoles of 2-mercaptoethanol; 6 μmoles of α-ketoglutaric acid; 600 μmoles of tris buffer, pH 8·35; 1 mg of TPN; enzyme; water to a final volume of 3 ml. (An enzyme unit is arbitrarily defined as that amount of enzyme which will induce an OD change of 0·001 unit per min. under specified conditions of assay.)

The contents of the cell are well mixed and a reading at 340 mμ is immediately taken against a blank containing all the components except GABA. Readings are taken at 1-min intervals for 5 min at room temperature. Over this period of time, with limiting amounts of enzyme, the reaction is linear and zero order. The reading taken at zero time is subtracted from the reading taken at 5 min, and the corrected reading divided by 5 indicates the units of OD change per minute. Calculations of specific activity of the enzyme, total units, and per cent recovery may then be made. As isolated, the purified preparation contains from 18 to 20 mg of protein and approximately from 2500 to 3000 units per ml. One-tenth of a milliliter of this preparation is more than adequate to assay samples under the specified conditions within a range of concentration of from 0·1 to 0·6 μmoles of GABA. On known GABA standards made up in ethanol–water (75:25, v/v), the error of the assay is less than 3 per cent. The method is sensitive to 0·05 μmoles of GABA.

This preparation has a pH optimum of 8·2 to 8·4. The specificity of the system has not been extensively studied, but there is no reduction of TPN when glycine, ornithine, β-alanine, butyronitrile, 4-methylaminobutyric acid, or 2-hydroxy-4-aminobutyric acid are added. Tissues other than brain do contain one or more compounds which are extractable with the aqueous ethanol and which cause a slight reduction of TPN in the system. Chromatographic examination of these extracts has not revealed the presence of GABA, and the concentrations of these compounds remain unaffected by doses of AOAA which cause from four- to five-fold increases in the amounts of GABA in the brain.

The preparation is stable when frozen and may be kept in this state for at least a month without appreciable loss of activity. With multiple freezing and thawing, however, there may be some loss of activity and it is well to run a calibration curve on known standards before attempting to use the preparation for tissue assays.

Brain samples are prepared for analysis essentially in the manner described by Roberts and Frankel[1]. Small animals are decapitated and large animals are sacrificed by intravenous injections of magnesium sulfate. The brains are rapidly removed and frozen in dry ice, after which they are blended in 10 vols. of ethanol–water (75:25) in a micro-head of a Waring blendor. One minute is usually sufficient for from 1 to 3 g of tissue. The homogenate is centrifuged at 5000 *g* and a 10-ml aliquot of the clear supernatant fluid is then dried *in vacuo*. The residues, when dissolved in 0·1 vol. of the aqeuous ethanol, are ready for assay. Frequently it is not possible to dissolve all of

the dry residue, but it appears that all the GABA present in the sample goes readily into solution. Of this solution, the usual aliquot which is added to the cuvettes for analysis is 0·05 ml.

It must be pointed out that the enzyme preparation is contaminated with a small amount of alcohol dehydrogenase. Also, the system is inhibited to some extent by ethanol. To compensate for this it is necessary to include in the blank the same volume of the aqueous ethanol as is added with the brain samples. The components added to the cuvettes for tissue determinations are the same as given for the estimation of enzyme activity, with the obvious omission of GABA.

While the system described could be used for screening compounds, the discovery of an inhibition would require the demonstration that the transaminase and not the dehydrogenase was the affected enzyme. For this reason the preparation was used for its transaminase activity alone when testing potential inhibitors. The test system which is the same as that used by Baxter and Roberts[9], is composed in the following manner. Into 15-ml conical glass centrifuge tubes are added: 40 μmoles of GABA; 40 μmoles of KGA; 50 μmoles of sodium borate buffer, pH 8·2; enzyme sufficient to form from 3 to 4 μmoles of glutamate per hour; water to a final volume of 1·5 ml.

The test compounds were made up in 0·2 M sodium borate buffer, pH 8·2, at a concentration of 0·1 M, and 0·1 ml was added to the tubes. Appropriate enzyme and substrate blanks were included, as well as blanks for each compound tested. The contents of the tubes were well mixed and placed in a water bath at 38 °C for 30 min, after which they were removed from the bath and chilled. The reaction was stopped by the addition of 0·5 ml of 25 per cent trichloroacetic acid. The analysis for glutamic acid was carried out in the manner described by Baxter and Roberts[9].

When an inhibitor of the transaminase was found it was titrated in decreasing concentrations to determine its potency. Confirmation of inhibition was obtained from chromatograms developed in a descending front with phenol–water (88:12) as the developing solvent. The strips were usually left in the tanks for a 24-hr period and then dried in an oven at 80° C for 1 hr. The strips were sprayed with a 1 per cent solution of ninhydrin in butanol and again heated for 30 min in the oven to develop the color.

When a promising inhibitor was disclosed with the *E. coli* transaminase, it was administered intravenously to rats at several different doses. The intravenous route was used to obviate any difficulties which might be encountered with absorption. The rats were then held for a minimum of 4 hr before they were sacrificed for determinations of GABA in the brain.

RESULTS

Amino-oxyacetic acid (AOAA) was found to inhibit the bacterial transaminase 100 per cent at a concentration of $3{\cdot}3 \times 10^{-4}$ M and 40 per cent at a concentration of $3{\cdot}3 \times 10^{-6}$ M. It has no effect on the bacterial succinic semialdehyde dehydrogenase at concentrations of $3{\cdot}3 \times 10^{-2}$ M.

When the brain transaminase was tested under similar conditions, it was inhibited 92 per cent at a concentration of AOAA of 1×10^{-5} M.

When AOAA was tested in rats, in the manner described, it was apparent that the compound, in spite of being a strong acid and completely ionized at physiological pH levels, crossed the blood–brain barrier in amounts sufficient effectively to inhibit

brain GABA–KGA transaminase. This was reflected in the increased concentrations of GABA which were observed. In Table 1 are shown the brain GABA levels which resulted from administration of AOAA at various doses in five species of animals. While there was a definite accumulation of GABA in all species, the dog proved to be most refractory and unpredictable in its response. The guinea pig, while not extensively studied, was most sensitive to the toxic effects of AOAA.

TABLE 1. DOSE OF AMINO-OXYACETIC ACID AND RESULTING GABA LEVELS IN FIVE SPECIES OF ANIMALS

Species	Dose of AOAA (mg/kg)	Route of administration	Interval before sacrifice (hr)	GABA in brain (μmoles/g)	Number of animals/group
Rat	none	—	—	2·5	2
Rat	6·25	i.v.	5·5	2·5	2
Rat	12·5	i.v.	5·5	5·2	2
Rat	25	i.v.	5·5	6·8	2
Rat	50	i.v.	5·5	10·0	2
Rat	none	—	—	4·4	2
Rat	25	oral	6	3·7	2
Rat	50	oral	6	4·3	2
Rat	100	oral	6	4·7	2
Rat	200	oral	6	9·0	2
Rat	6·25	subcut.	6	5·6	2
Rat	12·5	subcut.	6	7·6	2
Rat	25	subcut.	6	10·5	2
Rat	50	subcut.	6	10·8	2
Mouse	none	—	—	2·7	5
Mouse	6·25	subcut.	6	7·3	5
Mouse	12·5	subcut.	6	8·6	5
Mouse	25	subcut.	6	13·0	5
Mouse	50	subcut.	6	12·2	5
Cat	none	—	—	1·9	1
Cat	10	subcut.	6	3·0	1
Cat	20	subcut.	6	5·6	1
Cat	30	subcut.	6	10·2	1
Guinea pig	none	—	—	2·6	2
Guinea pig	10	subcut.	6	7·7	2
Guinea pig	20	subcut.	6	9·2	2
Dog	none	subcut.	4	1·7	1
Dog	50	subcut.	4	2·0	1
Dog	100	subcut.	4	5·0	1

As shown in Table 2, the peak levels of GABA which were reached in from 6 to 8 hr were followed by a slow decline. Twelve hours after administration of the inhibitor the brain levels in the rat, mouse, cat, and guinea pig were markedly elevated, as compared to those of the controls. Even 24 hr after dosage with AOAA, there was still some elevation of GABA in these species.

Gross physical effects seen following administration of AOAA were qualitatively similar in all species studied. In those animals able to do so, i.e. the cat and dog, there was usually some emesis; this occurred shortly after either oral or parenteral

administration. Then there ensued a gradual sedation and paralysis of the skeletal musculature. There was no marked analgesia or anesthesia as the animals, except when under very high doses, could be aroused. The palpebral, corneal, pinna and nasal reflexes were present even when the animals were very markedly sedated. These effects persisted for 12 hr or more, after which the animals returned to normal. All gross signs of the toxicity of AOAA disappeared within 24 hr.

TABLE 2. TIME CURVES OF GABA LEVELS FOLLOWING THE ADMINISTRATION OF AMINOOXYACETIC ACID IN FIVE SPECIES OF ANIMALS

Species	Dose of (mg/kg)	Route of administration	Interval before sacrifice (hr)	GABA in brain (μmoles/g)	Number of animals/group
Rat	none	i.v.	—	2·0	2
Rat	37·5	i.v.	1	3·8	2
Rat	37·5	i.v.	2	6·9	2
Rat	37·5	i.v.	4	6·4	2
Rat	37·5	i.v.	8	11·7	2
Rat	37·5	i.v.	12	9·0	2
Rat	37·5	i.v.	23	5·6	2
Rat	50	s.c.	1	3·1	2
Rat	50	s.c.	2	4·0	2
Rat	50	s.c.	4	6·2	2
Rat	50	s.c.	6	8·0	2
Rat	50	s.c.	8	7·8	2
Rat	50	s.c.	12	7·8	2
Rat	50	s.c.	24	3·8	2
Rat	50	s.c.	48	1·4	2
Mouse	none	s.c.	—	2·4	5
Mouse	30	s.c.	1	2·3	5
Mouse	30	s.c.	2	4·3	5
Mouse	30	s.c.	4	6·3	5
Mouse	30	s.c.	8	7·3	5
Mouse	30	s.c.	12	6·5	5
Mouse	30	s.c.	24	3·7	5
Cat	none	s.c.	—	1·9	1
Cat	30	s.c.	1	2·5	1
Cat	30	s.c.	2	2·7	1
Cat	30	s.c.	4	8·0	1
Cat	30	s.c.	6	10·2	1
Cat	30	s.c.	8	5·9	1
Cat	30	s.c.	12	4·7	1
Dog	none	s.c.	—	1·7	1
Dog	40	s.c.	1	1·8	1
Dog	40	s.c.	2	1·7	1
Dog	40	s.c.	3	2·3	1
Dog	40	s.c.	4	5·8	1
Dog	40	s.c.	6	6·4	1
Dog	40	s.c.	8	5·8	1
Guinea pig	25	s.c.	—	2·6	2
Guinea pig	25	s.c.	1	5·4	1
Guinea pig	25	s.c.	2	7·7	1
Guinea pig	25	s.c.	4	11·5	1
Guinea pig	25	s.c.	6	13·8	1
Guinea pig	25	s.c.	8	10·7	1
Guinea pig	25	s.c.	12	11·6	1
Guinea pig	25	s.c.	24	9·6	1

Mechanism of inhibition

As the *E. coli* transaminase had served as a valid model for the brain enzyme *in vivo*, kinetic studies were conducted with this preparation. In order to decrease the reaction rate, the pH was decreased from 8·2 to 7·4 and the temperature of the incubation was decreased to 30 °C. Under these conditions the reaction was linear and zero order over a 15-min period. The reaction was then run at seven different concentrations of GABA and four different concentrations of AOAA, in addition to a set of controls run at seven different concentrations of GABA in the absence of inhibitor. When the reciprocals of substrate concentration were plotted against reciprocals of velocity, in the manner described by Lineweaver and Burk[11] the intercept of the slopes remained constant, but the slope was increased by $(1 + I/K_i)$ with increasing concentrations of inhibitor, a finding which demonstrates that the inhibition was competitive. The calculated Michaelis–Menten constant (K_m) for GABA was $2{\cdot}76 \times 10^{-2}$, indicating a rather low affinity of the enzyme for GABA. On the other hand, the K_i for AOAA under these conditions was $7{\cdot}52 \times 10^{-6}$. The ratio between these two values is greater than 3600, which indicates that there is a rather remarkable difference between the affinity of the enzyme for the inhibitor, as opposed to its natural substrate, GABA.

These experiments were repeated at seven different concentrations of α-ketoglutaric acid and three different concentrations of AOAA, together with appropriate controls. The calculated K_m for KGA was $9{\cdot}66 \times 10^{-3}$, and the K_i under these conditions was $9{\cdot}16 \times 10^{-6}$. This value is in reasonable agreement with the preceding value of $7{\cdot}52 \times 10^{-6}$.*

DISCUSSION

Welch and Bueding[12] and Hunter and Lowry[13] in their discussions of effects of drugs on enzymes, concur on three important criteria which must be met before an enzyme inhibition observed *in vitro* may be said to occur *in vivo*. These points are: (1) there must be definitive proof that the inhibitor is selectively inhibiting the same enzyme in experimental animals, (2) in animals this inhibition must occur at approximately the same concentration of inhibitor as that observed *in vitro*, and (3) a direct relationship between increasing concentrations of inhibitor and observable effects relative to an increasing degree of inhibition of the enzyme must be demonstrable.

In this instance of brain γ-aminobutyric-α-ketoglutaric acid transaminase, these criteria have all been met. With regard to the first point, inhibition of this transaminase *in vivo* should result in increased concentrations of brain GABA. That this occurs has been demonstrated by a highly specific enzyme assay system, and also by paper chromatograms.

The second postulate is more difficult to assess, because as the inhibition is strictly competitive in nature, the degree of inhibition of the enzyme will be a function of absolute amounts of enzyme and substrates relative to concentrations of inhibitor. Under the conditions of assay used for testing compounds, in which substrate concentrations are far in excess of those found *in vivo*, and in which a transaminase preparation from cat brain is used, a 92 per cent inhibition of the enzyme was observed

* The author wishes to express his gratitude to Dr. E. R. Garrett, of the Department of Physical and Analytical Chemistry of The Upjohn Company, for his generous help in calculating these constants.

at an AOAA concentration of 1×10^{-5} M. G. R. Zins, of these laboratories, in as yet unpublished studies with ^{14}C-labelled AOAA observed concentrations of 1×10^{-5} M of this compound or its metabolites in the brains of rats within 1 hr after intravenous administration of a dose of 20 mg/kg. If the inhibitor were present entirely in unchanged form at the site of the enzyme, with the markedly lower substrate concentrations present, this level of AOAA should induce complete inhibition of the enzyme. That a very marked inhibition at this dose does in fact occur may be derived from the data of Table 1, which show that an intravenous dose of 25 mg/kg of AOAA to rats caused at least twofold increase in brain GABA.

The data of Table 1 also fulfill the third criterion by demonstrating that in several species there is an increasing accumulation of GABA with increasing doses of AOAA.

So far in this discussion only one aspect of the effects of AOAA has been dealt with, namely its influence as an inhibitor of GABA–KGA transaminase *in vivo*. When AOAA is given in toxic doses to all species of animals studied, convulsions are observed which terminate in a respiratory death. These effects cannot be attributed solely to the inhibition of GABA–KGA transaminase. Preliminary observations show that the free amino acid profile in livers of dogs is markedly altered by a convulsive dose of AOAA and thus other enzymes are probably affected. A dilemma is therefore posed as to what effects in the intact animal are caused by increasing cerebral concentrations of GABA as opposed to other and unrelated actions.

One result of inhibiting GABA–KGA transaminase, possibly of great importance, is a concomitant decrease in synthesis of succinic semialdehyde which would follow from increasing concentrations of brain GABA. As GABA and succinic semialdehyde are the only compounds unique to the GABA pathway, some of the gross effects observed in intact animals could well be attributed to this reciprocal effect. The importance of GABA in relationship to succinic semialdehyde, and the discrete role of succinic semialdehyde in brain have yet to be elucidated.

There is a marked discrepancy between the effects of hydroxylamine on GABA levels in rats, as reported by Baxter and Roberts[8], as contrasted to the effects seen with AOAA. With hydroxylamine, peak levels of GABA are observed within from 1 to 2 hr after administration compared to peak levels of GABA observed at from 6 to 8 hr with AOAA. The kinetics of inhibition by hydroxylamine have not been described but the work of Baxter and Roberts[9] is strongly suggestive that the inhibition is not competitive but is due to oxime formation between hydroxylamine and pyridoxal phosphate on the active surface of the enzyme. With mammalian GABA–KGA transaminase preparations, these authors observed that inhibition of the enzyme with hydroxylamine could be reversed by increased concentrations of pyridoxal phosphate. Inhibition of the mammalian enzyme by AOAA cannot be reversed by pyridoxal phosphate. Thus hydroxylamine and AOAA apparently inhibit the enzyme by two different mechanisms, which may be the reason why the results on GABA levels are different.

The observations presented in this report delineate one important physiological property of GABA. It has not been possible to demonstrate GABA in the cerebrospinal fluid of normal dogs or of dogs treated with AOAA, in which it was possible to show a markedly elevated brain level of GABA. It appears, therefore, that GABA is confined to the brain cells, because if appreciable quantities left the cells, one would expect the amino acid to appear in the cerebrospinal fluid. These results also suggest

that GABA is not utilized at extracellular sites. Inhibition of the transaminase should favor other reactions in which GABA may be involved, but the very high levels of GABA which are attained in most species following inhibition of the transaminase, and the length of time during which these concentrations are maintained, point to the conclusion that the transaminase reaction and subsequent pathway are the principal route for the utilization of GABA.

REFERENCES

1. E. ROBERTS and S. FRANKEL, *J. Biol. Chem.* **187**, 55 (1950).
2. J. AWAPARA, A. J. LANDUA, R. FUERST and B. SEALE, *J. Biol. Chem.* **187**, 35 (1950).
3. E. ROBERTS, In: S. R. KOREY and J. I. NURNBERGER (Editors), *Neurochemistry* Vol. I, pp. 11–15. Paul B. Hoeber, New York (1956).
4. E. ROBERTS and S. FRANKEL, *J. Biol. Chem.* **188**, 789 (1951).
5. E. ROBERTS and S. FRANKEL, *J. Biol. Chem.* **190**, 505 (1951).
6. S. P. BESSMAN, J. ROSSEN and E. C. LAYNE, *J. Biol. Chem.* **201**, 385 (1953).
7. E. ROBERTS and H. M. BREGOFF, *J. Biol. Chem.* **201**, 393 (1953).
8. C. F. BAXTER and E. ROBERTS, *Proc. Soc. Exp. Biol., N.Y.* **101**, 811 (1959).
9. C. F. BAXTER and E. ROBERTS, *J. Biol. Chem.* **233**, 1135 (1958).
10. W. B. JAKOBY and E. M. SCOTT, *J. Biol. Chem.* **234**, 937 (1959).
11. H. LINEWEAVER and D. BURK, *J. Amer. Chem. Soc.* **56**, 658 (1934).
12. A. D. WELCH and F. BUEDING, Biochemical aspects of pharmacology. *Currents in Biochemical Research* (Edited by D. E. GREEN) pp. 399–411, Interscience, New York (1946).
13. E. F. HUNTER and O. H. LOWRY, *Pharm. Rev.* **8**, 89 (1956).