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HIPPURIC ACID SYNTHESIZING SYSTEM DURING DEVELOPMENT

KINETIC STUDIES AND INHIBITION WITH SALICYLIC ACID*

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Abstract—*p*-Aminohippurate synthesis was investigated in mouse liver suspensions. It was found to be an age-dependent reaction with minimal activity in the neonatal period and adult values attained at 1 month of age. Sodium salicylate strongly inhibited the reaction; this inhibition was not suppressed by K or Mg ions. Kinetic studies indicated that the salicylate inhibition of *p*-aminohippurate synthesis is competitive in nature. Developmental kinetic experiments disclosed slightly different K_m values in young versus adult animals; however, K_i values at 7 and 68 days differed markedly.

CONJUGATION with the simple neutral amino acid, glycine, is an important mechanism for the metabolism of certain aromatic carboxylic acids such as benzoic acid or *p*-aminobenzoic acid (PAB). This reaction is of particular significance in the overall elimination of salicylate from the organism because the enzyme system appears to be easily saturated at therapeutic concentrations of this frequently used analgesic agent. A limited capacity to form salicyluric acid in man may play an important etiologic role in salicylate intoxication in infants and children, where low enzymic activity may be even more incapacitating. Brandt[1] found very limited *p*-aminohippuric acid (PAH) synthesis in liver of young rats, principally due to low glycine acyltransferase activity.[2] More recently, Levy and Amsel[3] demonstrated the competitive inhibition *in vivo* by benzoate of salicylurate excretion in man. The nature of the biochemical reaction has not been comprehensively investigated, particularly with respect to the kinetics *in vitro* of the interaction between benzoic acid and salicylic acid for the formation of their respective glycine conjugates, and to the changing pattern for this interaction with development of the experimental animal.

MATERIALS AND METHODS

Analytical methods. PAH was determined by the method of Cohen and McGilvery.[4] Salicyluric acid was measured by two methods: (1) a colorimetric method[5] based on the differences in partition coefficients of salicylic acid–salicyluric acid in systems of ethylene dichloride-acidified water and carbon tetrachloride-acidified water respectively; (2) a spectro-fluorimetric method, by a modification of the method of Schachter and Manis,[6] based on the differences in fluorescence between salicylic and salicyluric

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acids at acid and alkaline pH levels. Primary filter No. 7–60 (activation wavelength = 365 mμ) and secondary filter No. 2A (emission wavelength = 415 mμ) were used in a Turner model 111 fluorometer. Two ml of the acidified specimen (see below) were brought to pH 11·8 with 3 ml of 0·2 N NaOH. No extraction procedure was found necessary.

Preparation of the enzyme. Mice of the Swiss–Webster strain were fed standard laboratory diet and water *ad lib.* and used at various ages as indicated in the results. Adult mice were killed by cervical dislocation; livers were perfused with cold 0·25 M sucrose and removed. Young animals were decapitated; livers from littermates were pooled in order to obtain sufficient tissue. The livers were homogenized with 9 vol. of cold 0·25 M sucrose for 2 min in a glass homogenizer with a Teflon pestle.

Whole homogenates were employed, unless otherwise indicated, as the enzyme source. Mitochondria and soluble fraction (104,000 *g* supernatant) were obtained by differential centrifugation.[7]

Incubation procedure. Unless otherwise specified, the following conditions were employed in the enzymatic assay for both salicylurate and *p*-aminohippurate synthesis. The incubation mixture contained: potassium phosphate buffer (pH 7·56), 25 μmoles; $MgCl_2$, 5 μmoles; glycine, 45 μmoles; fumarate, 2·5 μmoles; ATP, 2·5 μmoles; *p*-aminobenzoic acid or salicylic acid, 3 μmoles; and 0·3 ml of a 10 per cent liver homogenate. The final volume was 1 ml. The mixture was incubated for 30 min at 37° in a Dubnoff metabolic shaker. At the end of the incubation period, the flasks were placed on cracked ice and 4 ml of 0·2 N trichloroacetic acid was added to each flask to stop the enzymatic reaction.

Protein assays were performed by the method of Lowry *et al.*[8] with bovine serum albumin (Sigma Chemical Company) as the standard.

RESULTS

The mean values for the enzyme activity at various stages of development are shown in Fig. 1. Very low levels of activity were observed from birth to 14 days; then activity rose gradually until adult levels were achieved at around 32 days.

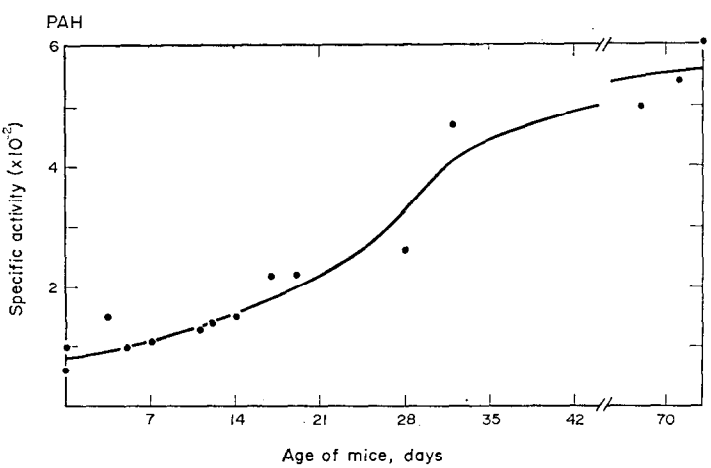

FIG. 1. Development of the PAH synthesizing system. Specific activities (μmoles PAH/mg protein/30 min) are presented as a function of age. Each point represents the results of duplicate analysis.

No formation of salicyluric acid in the incubation with mouse liver homogenates was demonstrated (either by the spectrophotometric or the spectrofluorimetric methods) when conditions similar to those for PAH synthesis were used. Even when the following conditions were changed, salicylurate was not detected: ATP concentration increased to 5×10^{-3} M; addition of CoA, 2×10^{-3} M; prolongation of the incubation time to 120 min and addition of salicylic acid in amounts ranging from 1 to 6 μmoles (total incubation volume, 1 ml).

In the absence of salicylate, 11·1 per cent of *p*-aminobenzoate was converted to *p*-aminohippurate; the addition of equimolar concentrations of sodium salicylate and *p*-aminobenzoate resulted in a 2·3 per cent conversion. No salicylurate formation was demonstrated in these experiments.

Kinetic experiments in the presence of sodium salicylate (at concentrations of 1×10^{-5} and 1×10^{-4} M) indicated a purely competitive inhibition, which is presented in Fig. 2 in a double reciprocal Lineweaver–Burk[9] plot.

Table 1 indicates that Mg^{2+} and K^+ were unable to overcome the salicylate inhibition, when added to the incubation mixture. However, the addition of Mg^{2+} increased the activity of the PAH-synthesizing enzyme system without affecting the salicylate inhibition.

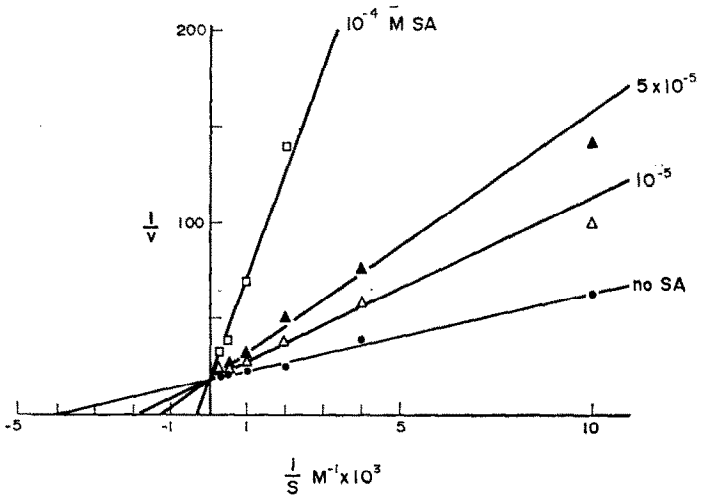

FIG. 2. Lineweaver–Burk plot of the PAH synthesizing enzyme system in the adult mouse. Activity (v) is expressed as in Fig. 1. Increasing concentration of salicylate produces increased inhibition. K_i was calculated from the extrapolated intercept on the ordinate. SA = salicylate.

Results of kinetic studies during development are shown in Table 2. A slightly different K_m was noted in the young (7 days) as compared to the adult animal ($5{\cdot}7 \times 10^{-4}$ M vs. $2{\cdot}5 \times 10^{-4}$ M respectively). However, a significantly different K_i was observed at these two ages ($3{\cdot}4 \times 10^{-4}$ M vs. $1{\cdot}9 \times 10^{-5}$ M respectively). The K_m and K_i values obtained from a Lineweaver–Burk reciprocal plot and from a Dixon[10] plot ($1/v$ vs. i), compared favorably with values calculated by using Marquardt's[11] technique for the least squares estimation of nonlinear parameters. This technique utilizes a computer program designed to estimate K_m and V_m from the observed activity at

TABLE 1. EFFECT OF IONS ON THE SALICYLATE INHIBITION OF *p*-AMINOHIPPURATE SYNTHESIS

	Specific activity (μmoles PAH/mg protein/30 min)	
(Mg^{2+})	No SA	With SA (1×10^{-4} M)
0	0·053	0·034
1×10^{-3}	0·086	0·040
5×10^{-3}	0·090	0·040
1×10^{-2}	0·091	0·038

p-Aminobenzoate concn., 2×10^{-3} M

	Specific activity (μmoles PAH/mg protein/30 min)	
K^+	No SA	With SA (5×10^{-3} M)
0	0·030	0·024
1×10^{-1}	0·034	0·028
5×10^{-2}	0·029	0·020

p-Aminobenzoate concn., 1×10^{-3} M.

TABLE 2. KINETIC STUDIES DURING DEVELOPMENT

Age (days)	No. of expts.	K_m (M)	K_i (SA as inhibitor)* (M)
7	3	$5{\cdot}7 \pm 0{\cdot}17 \times 10^{-4}$	$3{\cdot}4 \pm 0{\cdot}25 \times 10^{-4}$
14	2	$2{\cdot}0 \pm 0{\cdot}49 \times 10^{-4}$	$3{\cdot}6 \pm 0{\cdot}58 \times 10^{-5}$
32	1	$2{\cdot}7 \pm 0{\cdot}54 \times 10^{-4}$	$2{\cdot}8 \pm 0{\cdot}54 \times 10^{-5}$
68	1	$2{\cdot}5 \pm 0{\cdot}25 \times 10^{-4}$	$1{\cdot}9 \pm 0{\cdot}16 \times 10^{-5}$

* K_i values were calculated from kinetic experiments in the presence of 5×10^{-5} M salicylate in the incubation mixtures.

various substrate concentrations (nonlinear relationship) with a 95 per cent confidence level.

Fractional ultracentrifugation was employed in an attempt to compare enzymic activity found in the total homogenate, mitochondrial and supernatant fractions. In adult animals, activity was found chiefly in the mitochondrial fraction.

The stability of the enzyme was determined in adult animals. There was a loss of 23 per cent of the initial activity after 5 hr (tissue kept at 4°) and of 74 per cent of the initial activity in 24 hr (tissue kept frozen).

DISCUSSION

The interaction *in vivo* between salicylic acid and *p*-aminobenzoic acid was first documented in 1946 when Dry *et al.*[12] were able to increase greatly the serum concentration of salicylic acid in a patient by simultaneous administration of *p*-aminobenzoic acid.

Amsel and Levy[13] studied the interaction between benzoate and salicylate in two normal adults. They showed that the urinary elimination of salicylurate decreased during the administration of benzoate and that this phenomenon was not due to inhibition of tubular secretion. These authors applied enzyme kinetic methods to their data and interpreted their results as indicating that benzoate competitively inhibits the formation of salicylurate. They also demonstrated[13] that the administration of glycine increased the rate of hippuric acid formation without any effect on the synthesis of salicyluric acid. Their conclusion was that the benzoic inhibition of the formation of salicyluric acid was not due to the competition for glycine.

We could not show conversion of salicylate to salicylurate in mouse liver homogenates at any of the ages studied, as one might expect from a competitive inhibitor. There is no clear explanation for this phenomenon, since it is possible that the conditions for salicylurate formation in the mouse are entirely different from those required for hippurate formation, or that the reaction may proceed at a very slow rate. The methods employed included a very sensitive spectrofluorimetric determination able to detect salicylurate in concentrations as low as 5×10^{-7} M in the presence of a 1000- to 2000-fold excess of salicylate. Using tissue slices, Little *et al.*[14] had difficulties in demonstrating salicylurate formation in rat, rabbit and dog.

Salicylic acid is a very effective inhibitor of *p*-aminohippurate formation. In the double reciprocal plot, at all ages, it is a competitive "dead-end" inhibitor.

The developmental curve of the hippuric acid synthesizing system in the mouse liver is similar to the one described in the rat. Brandt[2] found very low activity in young rats and proposed that the rate-limiting step in this reaction was probably glycine acyltransferase activity. Human newborn infants also have a limited capacity for the formation of hippuric acid.[15] In our experiments, kinetic studies at different ages showed a similar K_m for *p*-aminohippurate formation but different K_i values, when salicylic acid was used as inhibitor. This implies that some properties of the enzyme system have changed with age. Possible changes might involve one or a combination of the following parameters: affinity of the inhibitor for tissue proteins, interaction at the mitochondrial membrane level, metabolism of salicylic acid to metabolites other than salicylurate, allosteric or structural changes in the enzyme, etc.

In summary, we have demonstrated that salicylate is a competitive inhibitor of *p*-aminohippurate formation *in vitro* in mouse liver at any age, and that the K_i values (with salicylate) in young and adult animals differ significantly at the ages studied. We were unable to demonstrate salicylurate formation *in vitro* under the assay conditions employed.

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