SOME FACTORS AFFECTING THE ACETYLATION OF 
\textit{p}-AMINOBENZOIC ACID IN THE RAT*

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The acetylation reaction involving detoxification of various aromatic amines both \textit{in vitro} and \textit{in vivo} has become of new importance with the discovery by Lipmann et al. (1) that the coenzyme necessary for this coupling contains pantothenic acid. In an earlier paper (2), we gave evidence demonstrating that pantothenic acid is necessary for normal acetylation of \textit{p}-aminobenzoic acid (PAB) by rats. Although many studies have been made on the general reaction (3), the lack of agreement on results suggests that the conditions under which the measurements are made may be predominant in determining the results. It appears desirable, therefore, to reexamine some of the factors which previous work has suggested might affect the extent of the over-all coupling reaction when PAB is administered to rats. Studies reported here consist of three parts: (1) an examination of the effect of thiamine and riboflavin deficiencies on acetylation to see whether low acetylation is specific for pantothenic acid deficiency; (2) a measure of the effect of added acetate on the acetylation by deficient animals in order to eliminate low acetate supply as a possible cause of low acetylation; and (3) a measure of the effect of the amount of PAB administered on the degree of acetylation.

EXPERIMENTAL

The ability of growing rats to acetylate doses of PAB given intraperitoneally was measured by analysis of the 24 hour urine under conditions reported previously (2). All rats weighed around 100 gm. originally. Controls were fed a purified diet containing 73 per cent glucose, 18 per cent vitamin-free casein, 4 per cent corn oil (Mazola), 4 per cent Salts 4 (4), 1 per cent cod liver oil, and the following vitamins per 100 gm.: thiamine chloride 400 \( \gamma \), riboflavin 800 \( \gamma \), pyridoxine hydrochloride 400 \( \gamma \), nicotinic acid 4000 \( \gamma \), calcium pantothenate 2000 \( \gamma \), and chlorine chloride 100 mg. Animals made deficient received the same diet with the appropriate vitamin omitted. In either pantothenic acid or riboflavin deficiency, the ani-

mals survived for long periods, but in thiamine deficiency it was necessary after 2 1/2 weeks to supply small amounts of thiamine to the animals in order to keep them alive. They were given 15 to 50 \( \gamma \) of the vitamin per 100 gm. of diet thereafter and received intraperitoneal injections of 25 to 100 \( \gamma \) of thiamine when critical polynouritic symptoms appeared. PAB and thiamine injections were never made at the same time. Those animals that died were replaced by others which were made deficient in the same manner as the original group.

All animals received the appropriate purified diet for 1 month before acetylation values were determined. At the end of this time, all the groups showed values which did not change appreciably over the length of the experiment for a given dose. The PAB was given intraperitoneally in single injections except in the case of the 10 mg. doses, which were given in two injections of 2 cc. each \( \frac{1}{2} \) hour apart. In measuring the response to acetate, 3 or 6 per cent of the glucose in the diet was replaced by that amount of sodium acetate.

**Results**

Fig. 1 illustrates graphically both the effect of size of dose of PAB and of the three vitamin deficiencies upon the per cent acetylation of the PAB administered. It can be seen that low acetylation results from vitamin deficiencies other than pantothenic acid, but this is clear-cut only at the proper PAB dose level. Under each of the four nutritional conditions, the per cent acetylation of varied amounts of PAB is apparently described by two linear curves. A change in slope occurs at about the 2.5 mg. level, after which the decrease in acetylation with increasing doses is much less than at doses less than 2.5 mg. At 10 mg., all groups are approaching the same value, and none of the deficient animals shows a difference from the controls which is of statistical significance. At the lower levels (0.5 and 1 mg.), thiamine-deficient animals give values identical with the controls, and even at 2.5 and 5 mg. the difference is not striking. Values from riboflavin-deficient animals are significantly lower than from controls at all levels except at 10 mg., while values obtained during pantothenic acid deficiency are consistently below all others. The latter animals reached their minimum at 2.5 mg. and did not go below this when the dose was increased 4-fold. Such a behavior was exhibited by none of the other groups. The pantothenate curve thus appears to be distinctly different from the others, a fact which suggests that the mechanism causing lowered acetylation in this one group may be unique.

Previous work (2) on a small number of animals indicated that 3 per cent sodium acetate in the diet of pantothenic acid-deficient animals could increase acetylation significantly if a 2.5 mg. dose of PAB was given. This
suggested that the acetate supply might be the limiting factor for the reaction under some conditions. Since both thiamine and riboflavin are known to function in systems involved in the formation of metabolic acetate, it

![Graph showing the influence of nutritional deficiencies and size of dose upon the acetylation of p-aminobenzoic acid.](image)

**Fig. 1.** The influence of nutritional deficiencies and size of dose upon the acetylation of p-aminobenzoic acid.

**Table I**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Dietary sodium acetate</th>
<th>No. of trials</th>
<th>No. of animals</th>
<th>Acetylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>per cent</td>
<td>16</td>
<td>5</td>
<td>57.2 ± 1.8</td>
</tr>
<tr>
<td>Thiamine-free</td>
<td>51.7 ± 2.4</td>
<td>17</td>
<td>9</td>
<td>49.8 ± 2.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>47.2 ± 2.3</td>
<td>19</td>
<td>8</td>
<td>43.7 ± 2.5</td>
</tr>
<tr>
<td>Riboflavin-free</td>
<td>38.8 ± 0.95</td>
<td>21</td>
<td>9</td>
<td>35.8 ± 1.4</td>
</tr>
<tr>
<td>&quot;</td>
<td>43.5 ± 3.4</td>
<td>18</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pantothenic acid-free</td>
<td></td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* Per cent bound ± the standard error of the mean.

appeared likely that low acetylations in these deficiencies might be due to low acetate supply. In order to examine this possibility, all three groups of deficient rats were placed on a 3 per cent sodium acetate diet and the acetylation values determined. As can be seen from Table 1, in no instance did the dietary acetate have an effect. Not even when 6 per cent
sodium acetate was fed to the pantothenic acid-deficient group did they respond. Thus we have been unable to repeat the previous observations on pantothenic acid-deficient animals.

To examine the possibility of poor utilization of pantothenic acid by the riboflavin-deficient group, these animals were injected with either 1 or 2.5 mg. of calcium pantothenate simultaneously with 1 mg. of PAB. Table II shows that there was no effect.

**DISCUSSION**

It is apparent that a reduction in the extent that \(p\)-aminobenzoic acid is acetylated is not specific for any one nutritional deficiency. Whether the cause of the lowered acetylating ability is common in the three deficiencies is unknown, but it appears unlikely that the same defect is involved in each case. It does seem certain that reduced food intake, while common to the three deficiencies, is not the direct cause. When the controls were fasted for a 72 hour period, and the acetylation tested during the last 24 hours of the fast, a value of 60.5 ± 2.1 per cent acetylation of a 2.5 mg. dose was obtained, compared to 57.2 ± 1.8 per cent for the same animals not fasted. Furthermore, the food intake of the severely thiamine-deficient animals is practically zero, but such animals showed essentially the same per cent acetylation of a given dose as they did when only mildly deficient. The intake of food by the riboflavin- and pantothenic acid-deficient animals was usually greater than that by those deficient in thiamine, although their ability to acetylate amine was less.

The importance of the size of the dose of the amine to be acetylated in evaluating the possible presence of some metabolic defect is clearly shown by the data. At all doses of 5 mg. or less, it is apparent that the pantothenic acid- and riboflavin-deficient animals gave results below normal,

**Table II**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Calcium pantothenate injected</th>
<th>No. of trials</th>
<th>No. of animals</th>
<th>Acetylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>mg.</td>
<td>10</td>
<td>5</td>
<td>67.3 ± 0.75</td>
</tr>
<tr>
<td>Riboflavin-free</td>
<td>1 mg.</td>
<td>22</td>
<td>8</td>
<td>56.2 ± 2.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.5 mg.</td>
<td>8</td>
<td>8</td>
<td>58.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>59.1 ± 1.6</td>
</tr>
</tbody>
</table>

* Per cent bound ± the standard error of the mean.
but this was not evident when a low dose (0.5 or 1 mg.) was given to the thiamine-deficient group. This group showed differences from normal only in the intermediate range of the doses tested, and all of the groups approached the same value as the amount of PAB was increased to the still relatively low dose of 10 mg. The size of the dose must be considered, therefore, in attempts to compare the results of various studies upon the factors affecting the extent of acetylation of a foreign amine. Many of the data in the literature have been obtained at doses far above those used in this study. Bloomberg (5) reported that a 25 mg. dose of PAB was in the maximum range that could be completely acetylated by the human. This dosage level is of course several times less per unit of body weight than the lowest level we have tested in rats, which was 0.5 mg. to a 250 gm. animal. We have never observed individual acetylation values much above 85 per cent in any of our studies. In addition, Bloomberg gave the PAB orally, which might be expected to yield higher acetylation values owing to a less rapid rate of absorption than after intraperitoneal injection. He found a maximum of but 50 per cent absorption from the intestines, which would mean that a 25 mg. dose would amount to only a 12.5 mg. dose available for acetylation and urinary excretion. The combined data of Torda and Wolff (6) and of Bloomberg (5) show a decrease in the extent of acetylation in the human being with increasing dosage comparable to that found in this study. Also at a comparable dose per unit of body weight, the extent of the acetylation of PAB that they observed is in the same range as has been found with the rat.

The shape of the curve showing a gradual decrease in the per cent acetylated with increasing doses is undoubtedly due to several factors, which may include the rate of the acetylation reaction itself as well as the time that the test substance is in the body (cf. Beyer et al. (7)). More rapid kidney excretion of the larger doses and the different rates of elimination of the free and conjugated forms (7, 8) are certainly the major factors. It would be expected that changes in kidney function would invalidate the test as a measure of acetylation rates, but a comparison of the total amounts of the amine excreted by the animals subjected to the various deficiencies does not suggest that this is the explanation of the results obtained.

On the basis of the work of Lipmann and associates (1, 9) one can ascribe the lowered acetylation by pantothenic acid-deficient rats to a relative lack of coenzyme A, which is required for acetylation. Olson and Kaplan (10) showed that the amount of the coenzyme in various tissues falls during the development of the deficiency. According to their data, the coenzyme A content of the tissues of riboflavin-deficient animals is normal, and, as shown in the present paper, additional supplies of pantothenic acid fail to increase the acetylating ability of the riboflavin-deficient...
animals. It is unlikely that the amount of coenzyme A is the determining factor in riboflavin deficiency, and the same is probably true of the thiamine-deficient animals.

Since feeding acetate in fairly large amounts failed to modify the extent of acetylation in any of the animals, it would appear that the supply of acetate per se is not a limiting factor. Bernhard (11) and Bloch and Rittenberg (12) have clearly shown that dietary acetate can serve in the acetylation of PAB, and the latter workers concluded that acetate is probably the sole precursor of the acetyl group for this compound. If this is true in the deficient animals as well, then it would appear that the defect in acetylation in thiamine- and riboflavin-deficient animals might be in the formation of the enzyme for acetylation, or in the activation of the acetate prior to the acetylation. Presumably a high energy phosphate bond must be produced before the coupling can take place (13), and it is possible that interference with the metabolic pathways of carbohydrate at several points might thus have an influence upon the extent of acetylation.

Whether differences in the degree of acetylation with the different deficiencies can be interpreted is open to question, since one cannot measure the relative severity of the thiamine, riboflavin, and pantothenic acid deficiencies. However, the relatively small decrease in acetylation observed in thiamine deficiency may be considered as consistent with the suggestion of Bloch and Rittenberg (12) that the major portion of the acetate arises from the oxidation of fatty acids, which does not require cocarboxylase.

**SUMMARY**

Acetylation studies have been made on normal rats and rats depleted of thiamine, riboflavin, and pantothenic acid, with p-aminobenzoic acid as the test substance.

1. In riboflavin- and pantothenic acid-deficient rats acetylation is significantly less than normal when 5 mg. doses of PAB or less are administered, the pantothenic acid-deficient animals showing consistently lower values.

2. Thiamine-deficient rats show slightly less than normal acetylation at 2.5 and 5 mg. PAB doses. At the levels tested above and below these amounts, the values obtained were normal.

3. When the amount of PAB was varied, all groups demonstrated a decrease in the per cent acetylated as the dosage increased. This decrease is represented graphically in each instance by linear curves which show a change in slope at about 2.5 mg. of PAB. Differences in the ability to acetylate the amine due to the nutritional state are evident only within a limited dose range, and all animals approach the same acetylation value at the maximum dose tested (10 mg. per rat). Pantothenic acid deficiency
was unique in that this minimum value was obtained with a dose of 2.5 mg. per rat.

4. Supplementation of the diet of any of the deficient animals with sodium acetate had no effect upon the acetylation of a 2.5 mg. dose of PAB.

5. Excess calcium pantothenate did not alter the low acetylation values given by riboflavin-deficient rats.

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