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ACETYLATION OF *p*-AMINOBENZOIC ACID BY HUMAN BLOOD

FREDERIKA MANDELBAUM-SHAVIT and S. H. BLONDHEIM

Metabolic Laboratory and Department of Medicine B, Hadassah University Hospital and Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Abstract—Acetylation of *p*-aminobenzoic acid was studied in human blood cell lysates. The rate of acetylation with acetyl-coenzyme A was 3.4 nmoles per min per 0.5 ml lysate (corresponding to 0.5 ml blood with a 50% hematocrit). In the presence of a coenzyme A generating system, the rate was only 0.1 nmole per min per 0.5 ml lysate. *N*-Acetyltransferase (acetyl-CoA : arylamine *N*-acetyltransferase, EC 2.3.1.5) activity exhibited a temperature optimum within the range of 34–37° with a Q_{10} of 2 between 24–34°. Heating for 3 min at 50° caused 50 per cent inactivation of enzymatic activity. The pH activity profile showed an optimum at pH 6.0–6.5. *p*-Chloromercuribenzoate was a potent inhibitor causing 50 per cent inhibition at 9 μM. The apparent K_m value for *p*-aminobenzoic acid was 0.4 mM and for acetyl-coenzyme A, 0.3 mM. The enzyme activity with *p*-aminosalicyclic acid was about 50 per cent that obtained with *p*-aminobenzoic acid. Acetylation of sulfamethazine was either very low or in several individuals undetectable. Isonicotinic acid hydrazide at a concentration 10 times that of *p*-aminobenzoic acid did not interfere with acetylation of the latter, nor did trimethoprim, another compound with an amine moiety. Folic acid and amethopterin competitively inhibited the acetylation of *p*-aminobenzoic acid, with respective K_i values of 0.01 mM and 0.06 mM. It is concluded that the activity of *N*-acetyltransferase in human blood may have important clinical implications.

Drug acetylating enzymes have been studied extensively in human and other mammalian species [1, 2]. The subject of most of these studies was *N*-acetyltransferase of liver and only more recently has attention been directed toward the characterization and determination of the activity of that enzyme in extrahepatic tissues [2–5].

The acetylating ability of human blood cells was first demonstrated in 1955 [6] and studied later by other workers using an acetyl-coenzyme A generating system with guinea pig whole blood [7] and human and rabbit red cell hemolysates [8, 9]. Recently a new polymorphic mechanism for acetylation of *p*-aminobenzoic acid was found in rabbit blood cells, which was shown to be reciprocally related to the activity of hepatic *N*-acetyltransferase [2, 4, 5].

The importance of acetylation as a major inactivation step for many drugs prompted us to study the specificity and mechanism of action of *N*-acetyltransferase in human blood. It was also of interest to compare the rate of acetylation of *p*-aminobenzoic acid (*p*ABA) in the presence of acetyl-coenzyme A with that dependent upon the activity of acetyl-coenzyme A synthetase (acetate: CoA ligase AMP-forming, EC 6.2.1.1).

MATERIALS AND METHODS

Chemicals. CoASAc was prepared from CoASH by the method of Simon *et al.* [10], as modified by Ochoa [11]. CoASH, *p*ABA, isonicotinic acid hydrazide, *p*-aminosalicylic acid (sodium salt), folic acid and trimethoprim were purchased from Sigma Chemical Co. Amethopterin was obtained from Lederle Laboratories Division.

Blood cells. Freshly drawn blood in heparin was centrifuged at 3,000 *g* for 10 min, plasma was removed and the cells were washed twice in cold saline. The cells were lysed by rapid freezing in liquid nitrogen and thawing at room temperature. The cell debris was removed by centrifugation in a Sorvall centrifuge at 30,000 *g* for 15 min. The supernatant was diluted 1:2 in 50 mM potassium phosphate buffer (pH 7.0) and used as enzyme source.

White cells were prepared from 25 ml blood mixed with 5 ml of a solution of 5% dextran, 0.04% heparin and 0.7%NaCl. Red cells were sedimented by standing at room temperature for about an hour, or until a distinct separation of the red cell layer had occurred. White cells were isolated from the plasma by centrifugation at 3,000 *g* for 10 min and then washed twice with 5 ml of cold saline. The separated red cells were washed by the same procedure. Red and white cell counts were performed on each preparation. Both lines of cells were lysed by 4 cycles of freezing in liquid nitrogen and thawing at room temperature. A 30,000 *g* supernatant was used in enzyme assays. After storage for 4 weeks at −20°, the lysate retained approximately 90 per cent of its activity.

Enzyme assays

N-Acetyltransferase. The reaction mixture in a total volume of 1.0 ml contained 100 mM potassium phosphate buffer (pH 6.5), 0.2 mM *p*ABA, 0.4 mM CoASAc and enzyme. Addition of CoASAc initiated the reaction. Appropriate controls for possible hydrolysis of CoASAc were run for each enzyme preparation. The reaction mixtures were incubated in a water bath at 34° and the reaction was terminated by adding a 0.2 ml aliquot to 2 ml of 5% trichloroacetic acid and centrifugation.

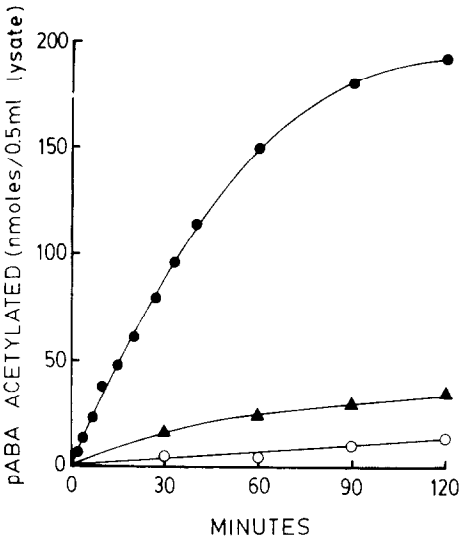

Fig. 1. Time course of *p*ABA acetylation by lysates of human blood cells. Washed cells were lysed and diluted in one volume of 50 mM phosphate buffer (pH 7.0), see Methods. 0.5 ml lysate corresponding to 0.5 ml blood with a 50% hematocrit was used for determination of the enzymatic activity. The reaction mixtures for determination of *N*-acetyltransferase and acetyl-CoA synthetase activities were as described in Materials and Methods. 0.2 ml portions were removed at various time intervals and added to 2.0 ml 5% trichloroacetic acid. *p*ABA acetylated in the presence of CoASAc (●), in the presence of CoASH (○) and with whole blood lysate (46% hematocrit) in the presence of CoASH (▲). Points represent means of experiments carried out in duplicate with three different blood samples.

The aromatic amine (non-acetylated *p*ABA) was determined by the procedure of Bratton and Marshall [12]; 0.2 ml of each of the following reagents: 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% *N*-1-naphthylethylenediamine dihydrochloride were added at 3 min intervals to 2 ml of the trichloracetic acid-supernatant. The color developed for 10 min and absorbance was measured in a Bausch & Lomb Spectronic 710 spectrophotometer at 540 nm, against water as blank. Acetylation was measured by subtracting the readings obtained after incubation from those of a reaction mixture without CoASAc or prior to incubation. The same method was used for the determination of sulfamethazine and a procedure of Marshall [13] for quantitation of *p*-aminosalicylic acid.

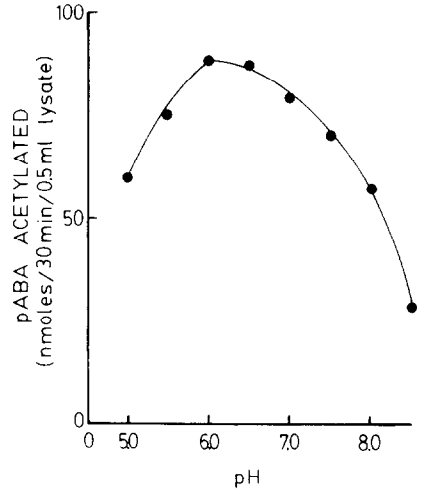

Fig. 2. Effect of pH on *n*-acetyltransferase activity. The activity was determined following the procedure described in Materials and Methods, with variations in the pH values of the potassium phosphate buffer (200 mM) used. 0.2 ml cell lysate was added and the reaction incubated for 30 min at 34°. Points are means of experiments with three separate blood samples.

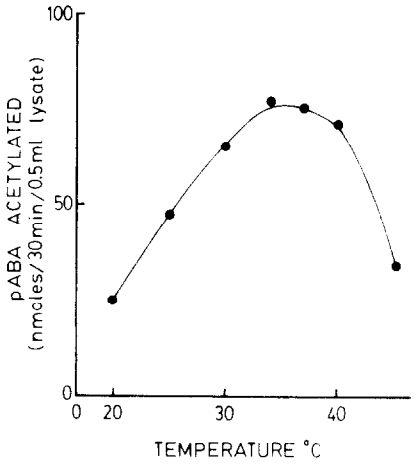

Fig. 3. Effect of temperature on *N*-acetyltransferase activity. Reaction mixtures were incubated for 30 min in water bath thermostated at various temperatures.

Acetyl-CoA synthetase. The reaction mixture in 1.0 ml consisted of: 100 mM potassium phosphate buffer (pH 7.0), 0.2 mM *p*ABA, 0.4 mM CoASH, 10 mM sodium acetate, 3 mM ATP, 2.5 mM $MgCl_2$ and enzyme. The mixture was incubated at 34° and the reaction terminated as above.

RESULTS

Time course of p*ABA acetylation.* In the presence of CoASAc, acetylation was linear for up to about 30 min of incubation. Within this range 3.4 nmoles of acetyl-*p*ABA were synthesized per min per 0.5 ml of washed blood cells lysate (Fig. 1) Under identical experimental conditions only 0.1 nmole of *p*ABA was acetylated in a reaction mixture with a CoASAc generating system. With a whole blood lysate instead of washed lysed cells, the rate of acetylation was 5 times greater. Addition of plasma to a cell lysate restored the activity to that of a whole blood lysate (not shown). Thus the low acetylation in lysates of washed cells was not due to inactivation of acetyl-CoA synthetase during preparation of the cell-free extract. The activity of *N*-acetyltransferase acetylation in the presence of CoASAc was not affected by a plasma factor.

Effect of pH on N-acetyltransferase activity. The pH activity profile showed an optimum at pH 6.0–6.5. The activity decreased sharply both at more acidic or alkaline pH ranges (Fig. 2).

Effect of temperature on the enzyme activity and heat inactivation. The optimal temperature for *N*-acetyltransferase activity was in the range between

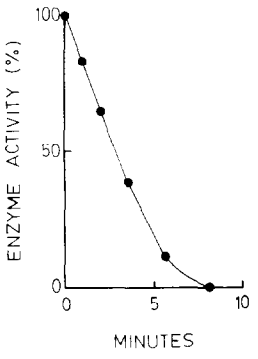

Fig. 4. Thermal inactivation of *N*-acetyltransferase. The enzyme was incubated in a water bath at 50°. Samples were removed at the indicated time intervals and stored in ice until assayed. The reaction mixtures containing 0.5 ml of the heat-treated lysate were incubated for 30 min at 34°. Points are means of experiments with three blood samples.

34–37°, with a Q_{10} value of about 2 between 24 and 34° (Fig. 3).

Thermal inactivation of enzyme activity was followed at 50°. Heating for 3 min caused about 50 per cent inactivation (Fig. 4).

Enzyme inactivation by p-*chloromercuribenzoate.* Enzymatic activity was inhibited by a sulfhydryl inhibitor. Preincubation of the reaction mixture for 5 min with *p*CMB (9 μM) caused 50 per cent inhibition of enzymatic activity (Fig. 5).

Substrate specificity of N-*acetyltransferase.* Acetylation of *p*ABA was almost twice that of *p*-aminosalicylic acid (Table 1). The enzymatic activity with sulfamethazine was very low and with some lysate preparations was undetectable.

Examining the effect of structurally related compounds on the acetylation of *p*ABA it was found that isonicotinic acid hydrazide at a concentration 10 times greater than that of *p*ABA did not affect

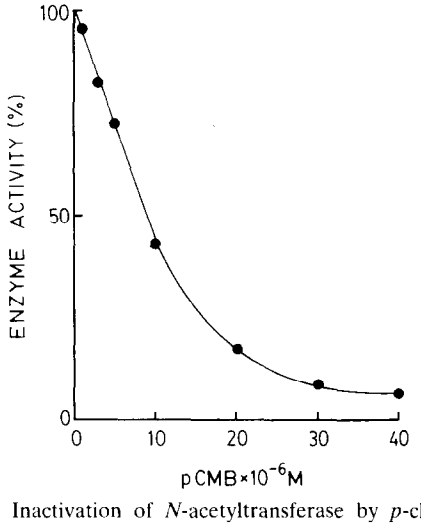

Fig. 5. Inactivation of *N*-acetyltransferase by *p*-chloromercuribenzoate. The reaction mixtures were preincubated for 5 min with various concentrations of *p*CMB, prior to addition of CaASAc (0.2 mM). Other experimental conditions were as in Fig. 4. Points are means of experiments with four blood samples.

Table 1. Substrate specificity of *N*-acetyltransferase

Compound	Compound acetylated* (nmoles/30 min/ 0.5 ml lysate)
p-Aminobenzoic acid	77.0 ± 2.08
p-Aminosalicylic acid	42.8 ± 0.85
Sulfamethazine	2.0 ± 0.43

The reaction mixture consisting of 100 mM potassium phosphate buffer (pH 6.5), 0.2 mM CoASAc, 0.1 mM of the indicated substrate and 0.5 ml of lysate of blood cells was incubated in a water bath at 34°.

* The values are means ± S.D. of experiments performed in duplicate with four separate blood samples.

acetylation of the latter (Table 2). Another compound with an amine moiety, trimethoprim (2,4-diamino-5-(3, 4, 5-trimethoxylbenzyl) pyrimidine), also did not interfere with acetylation of *p*ABA, even at a concentration 5 times that of *p*ABA. On the other hand a folic acid analog, amethopterin, very effectively competed with *p*ABA and at an equimolar concentration caused 72 per cent inhibition of acetylation of the latter. Kinetic analysis of this phenomenon revealed that both folic acid and amethopterin competitively inhibited acetylation of *p*ABA. This was shown by adding a constant concentration of folate or amethopterin to increasing concentrations of *p*ABA. The apparent K_i values for folate and amethopterin were 0.01 mM and 0.06 mM respectively (calculated from Fig. 6).

Kinetics of p*ABA acetylation.* The rate of acetylation of *p*ABA increased with increasing concentration of the substrate. In the presence of a constant concentration of CoASAc (0.4 mM) the K_m for *p*ABA was 0.4 mM (Fig. 6) and with a constant concentration of *p*ABA (0.2 mM), the K_m for CoASAc was 0.3 mM (Fig. 7)

Acetylation of p*ABA by red and white blood cells.* To determine which line of blood cells was respon-

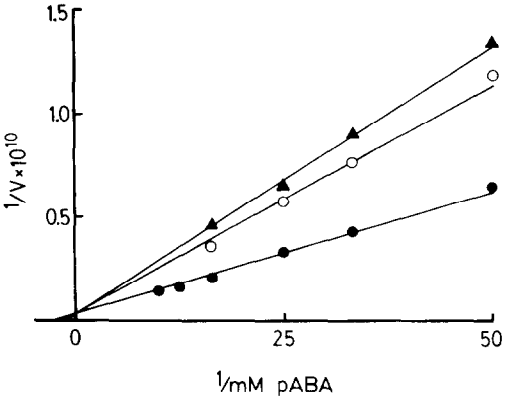

Fig. 6. Lineweaver–Burk plot of *p*ABA acetylation with *p*ABA as the variable substrate. Lysate (0.2 ml) was incubated for 15 min at 34° in 100 mM phosphate buffer (pH 6.5), 0.4 mM CoASA ...ing concentrat... *p*ABA. V = rate of *p*AB... in mole... per 0.2 ml blood cells lysa... ...or in the presence of 0.01 mM ... 0.1 mM amethopterin (▲). E... experiments with four ... ...m...

Table 2. Effect of structurally related compounds on acetylation of *p*ABA

Compound added (mM)	Acetyl-*p*ABA* (nmoles/30 min/0.5 ml lysate)	Per cent inhibition
None	70.0 ± 2.43	0
Isonicotinic acid hydrazide 1.000	70.0 ± 2.60	0
Trimethoprim 0.500	70.5 ± 2.10	0
Amethopterin 0.100	19.6 ± 0.82	72.0
Amethopterin 0.050	44.4 ± 1.60	36.6
Amethopterin 0.025	57.4 ± 1.95	18.0

The reaction mixture was as described in legend for Table 1. Compound examined was added simultaneously with *p*ABA and the reaction initiated by addition of CoASAc.

* The values are means ± S.D. of experiments performed in duplicates with four blood samples.

sible for the process of acetylation, the activity of *N*-acetyltransferase was determined in lysates of red and white cells separately. The activity in white cells was about 100 times greater than in red cells (Table 3).

DISCUSSION

Acetylation of aromatic amines is an accepted measure of the capacity of *N*-acetyltransferase to metabolize and thus inactivate a variety of important therapeutic compounds, such as sulfonamides, *p*-aminosalicylic acid, isonicotinic acid hydrazide, hydralazine, procainamide and others [1, 2]. *N*-Acetylation is also of importance in biotransformation of arylamines to carcinogens [14]. Activity of hepatic *N*-acetyltransferase varies between individuals and has been used to determine a 'slow' or 'rapid' acetylator phenotype [15, 16].

The increasing attention directed toward extrahepatic tissues as contributors to the total acetylating capacity of the rabbit [2–5] has aroused renewed interest in the process of acetylation in blood. Studies on the acetylating activity of the blood of 'slow' and 'rapid' acetylator rabbits revealed a reciprocal relation to the hepatic *N*-acetyltransferase activity [2, 4, 5]. No such relation has been reported for man.

Considering the possible importance of the contribution of blood enzymatic activity to the total process of acetylation, we extended studies on the mechanism of action of *N*-acetyltransferase in human blood.

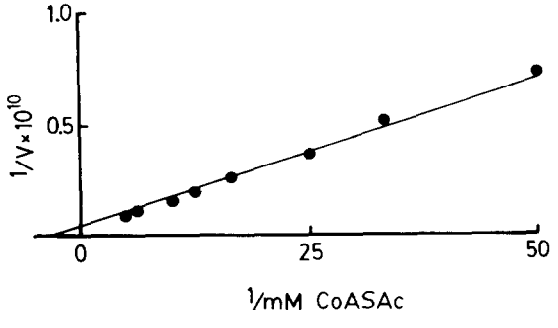

Fig. 7. Lineweaver–Burk plot of *p*ABA acetylation with CoASAc as the variable substrate. Experimental conditions were as in Fig. 6, with 0.2 mM *p*ABA and varying concentrations of CoASAc.

We have shown that lysates of washed human blood cells acetylated 3.4 nmoles of *p*ABA per min per 0.5 ml lysate, which is a considerably lower rate than that found in the blood of rabbits [2]. When acetylation was determined in the presence of CoASAc generating system it was strikingly lower: 0.1 nmole of *p*ABA was acetylated per min per 0.5 ml lysate. With lysates of whole blood or upon addition of plasma to washed cell lysates, a 5-fold higher rate of acetylation was obtained (Fig. 1). Thus a plasma factor enhanced acetylation in the presence of the CoASAc generating system, apparently affecting acetyl-CoA synthetase, but not the activity of *N*-acetyltransferase.

Comparing other properties of *N*-acetyltransferase of human blood to those reported for the blood of rabbits, it was found that the pH activity profile exhibited a single optimum at pH 6.0–6.5 in contrast to the two peaks found for the enzyme of the rabbit [2]. The optimal temperature for the enzymatic activity in human blood was within the range of 34–37° (Fig. 3), while that for the rabbit was apparently 27° [4]. The thermal stability of the enzyme studied (Fig. 4) was lower than that of the rabbit enzyme [2]. Sulfhydryl groups were essential for human enzyme activity, as well as for that of the rabbit [2].

Studies of substrate specificity demonstrated that acetylation of sulfamethazine was strikingly low (Table 1), as also reported for the blood cells of rabbits [3]. Isonicotinic acid hydrazide at a concentration 10 times greater than that of *p*ABA did not affect acetylation of the latter (Table 2), which is in agreement with the findings of Motulsky *et al.*, who used human red cells with a CoASAc generating system [17].

Another compound with an amine moiety, trimethoprim, widely used in combination with sulfonamides as an antibacterial agent [18], did not interfere with the acetylation of *p*ABA (Table 2).

4-Amino analogs of folic acid, aminopterin and amethopterin, were found to inhibit acetylation of sulfanilamide in pigeon liver extracts, while folic acid was ineffective [19–21]. It was reported subsequently that these analogs also inhibit acetylation of *p*ABA in human red cells [17]. Due to the importance of these drugs in cancer chemotherapy we studied the mechanism of this inhibition. Amethopterin at an equimolar concentration with *p*ABA was found to cause 72 per cent inhibition of acetylation (Table 2). Both folic acid and amethopterin competitively

Table 3. Acetylation of *p*ABA by red and white blood cells

Blood cells	Acetyl-*p*ABA* (nmoles/10^6 cells)
Red	0.1 ± 0.02
White	11.0 ± 1.09

Blood cells were separated and prepared for enzymatic assay as described in Materials and Methods. The reaction mixture consisting of 100 mM potassium phosphate buffer, 0.4 mM CoASAc, 0.2 mM *p*ABA and enzyme was incubated for 1 hour at 34°.

* The values are means ± S.D. of experiments performed in duplicate with three separate blood samples.

inhibited acetylation of *p*ABA with respective K_i values of 0.01 mM and 0.06 mM (Fig. 6). Acetylation of *p*ABA followed Michaelis–Menten kinetics with an apparent K_m for *p*ABA of 0.4 mM and for CoASAc of 0.3 mM (Figs. 6 and 7). Thus the affinity of *N*-acetyltransferase of human blood cells for folate and amethopterin appears to be markedly higher than for *p*ABA.

Acetylation of *p*ABA in white blood cells was about 100 times that found in the red cells (Table 3), which approximates the activity ratio reported for rabbit lymphocytes and erythrocytes [5].

N-Acetyltransferase in human blood appears to be a significant contributor to the process of drug detoxication. This reaction may also serve as a trap for blood acetate, which in the presence of substrates (drugs) being acetylated, would not be available for other metabolic processes, such as incorporation into blood lipids. We have recently found (unpublished results) that *p*ABA interferes with the incorporation of labelled acetate into human blood lipids.

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