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The Influence of Age on the Activity of Acetylsalicylic Acid-Esterase and Protein-Salicylate Binding

A. Windorfer jun., W. Kuenzer and R. Urbanek
Universitats Kinderklinik, Freiburg i. Brg., Federal Republic of Germany

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Summary. The activity of acetylsalicylic acid-esterase (ASA-esterase) in blood and the degree of protein-salicylate binding were estimated in different age groups. The activity of ASA-esterase was assayed by the amount of salicylate released after incubation with ASA under standard conditions. Lower activity of the enzyme was found in neonates (48—49% of ASA hydrolysed after 60 min) than in older children (60—64% hydrolysed) and male adults (78—83% hydrolysed). The erythrocytes of adult females were less active than those from males. Protein-binding of salicylic acid (SA) was also lower in neonates than in adults (30.7% at SA 68.4 µg/ml, and 52% at 10.28 µg/ml SA, respectively). Even if the protein concentration in adult plasma were reduced to the level of the neonates by dilution, and if bilirubin were added to the mixture, the protein-binding was still greater in plasma from adults than neonates. These observations suggest that in the neonatal period the protein-affinity of certain substances may differ from that in adults.

Key words: Salicylate, acetylsalicylic acid esterase, acetylsalicylic acid, protein binding, age effect, sex difference.

The recent discovery that acetylsalicylic acid (ASA) inhibits thrombocyte aggregation in human blood (Weiss and Aledort, 1966; O'Brien, 1968; MacMillan, 1968; Zucker and Peterson, 1968; Quick, 1968; Menon, 1970), has given new importance to this drug in certain pediatric diseases (Jobin and Delage, 1970; Sutor *et al.*, 1972).

The first steps in the metabolism of ASA are accomplished by the drug metabolizing systems of the liver and by an esterase in erythrocytes. These enzymes split ASA into salicylic acid (SA) and acetyl groups and the latter react quickly with certain proteins; for example, the inhibition of thrombocyte aggregation mentioned above is due to the acetylation of proteins in thrombocyte membranes (Evans *et al.*, 1976; Breddin *et al.*, 1971; De Weck, 1971). Menguy *et al.* (1972) have recently published data about the activity of this esterase in human blood; erythrocytes from females were less active than those from males.

In 1968 Ganshorn and Kurz demonstrated quantitave differences in the plasma protein binding of certain drugs, including salicylates, in newborn children and adults. In the present study, both esterase activity and salicylate binding by plasma proteins have been measured by the method of Potter and Gruy (1964) in male and female subjects of different ages, including children.

Material and Methods

Blood from boys and girls of different ages has been examined, as well as samples from male and female adults. The children, who were patients in the University hospital, were healthy at the time of the study.

The adults were healthy volunteers. Only babies with a total serum bilirubin of less than 8 mg/100 ml were selected for the group of premature infants and neonates of normal weight. Blood was collected at the same time as samples required for diagnostic purpose. The following age groups were examined:

premature, female: 1—4 days old: weight 1550—2300 g: n = 11,
premature, male: 1—4 days old: weight 1600—2300 g: n = 10,
newborns, female: 1—5 days old: weight 2700—3900 g: n = 9,
newborns, male: 1—5 days old: weight 2700—4100 g: n = 10,
infants, female: 1—10 months old: weight 5—10 kg: n = 8,
infants, male: 1—10 months old: weight 5—11kg: n = 8,
children, female: 5—8 years old: weight 13—25 kg: n = 10,
children, male: 5—8 years old: weight 14—25 kg: n = 8,
adults, female: 18—25 years old: weight 55—63 kg: n = 10,
adults, male: 25—33 years old: weight 70—85 kg: n = 10.

The experiments were divided into two parts:

A) Estimation of ASA-esterase activity and salicylate-protein binding. 1.6 ml of each blood

sample was added to a tube containing sodium heparinate 0.02 ml and the mixture was incubated with 200 µg/ml ASA for 60 min at room temperature. Blanks were prepared with blood or plasma but not ASA. After incubation, the amount of SA released was measured by gel filtration and fluorescence-photometry according to the method of Potter and Guy (1964), i.e. at the end of incubation, the samples and blanks were centrifuged at 3000 rpm. 0.1 ml aliquots of the clear supernatant were dropped carefully onto small Sephadex columns (10 cm Sephadex G-25 fine). Washing with phosphate buffer (0.15 M, pH 7.4) eluted protein bound SA in the first 1.5 ml. The next 2 ml of buffer removed free SA and ASA from the column. The two elutes were diluted to 4 ml with buffer of pH 7.4 and their fluorescence measured in a spectrofluorimeter (Aminco Bowman and Eppendorf fluorimeters). This method only assayed free and bound salicylate, and ASA was measured after hydrolysis to SA with two drops of strong NH_4OH. ASA-esterase activity was estimated by the amount of ASA metabolized to SA after incubation for 60 min with 200 µg/ml ASA. The protein-binding of SA was calculated by expressing the amount of protein-bound SA as a percentage of the total SA released by hydrolysis of the added ASA.

Standard curves prepared with pure SA and ASA were used to estimate the concentrations of these compounds, and, because of the native fluorescence of protein, a special standard curve was constructed for protein-bound SA.

All samples were examined on the day they were collected.

B) In order to measure the effect of protein and bilirubin concentrations on protein-binding, pools of adult and neonatal human blood and plasma were prepared. The adult plasma was diluted with buffer to give different protein concentrations and increasing amounts of SA were added to the samples. After dilution, other samples were mixed with a bilirubin solution (pH 9) to produce two different bilirubin concentrations (albumin: bilirubin molar ratios of 1:0.25 and 1:0.5), and the same amounts of salicylate were then added. The mean and standard deviation of each group was calculated and the significance of the differences examined by Student's t-test.

Results

The values of protein-bound SA in the different age groups are shown in Table 1. Preliminary experiments had shown very little protein-binding of ASA. The significantly low values of protein-bound SA in neonates, especially prematures, is apparent in the table, whereas infants between one and ten months of age already had similar values as older children and adults. No differences between the sexes were found in any age group. Fig. 1 shows that

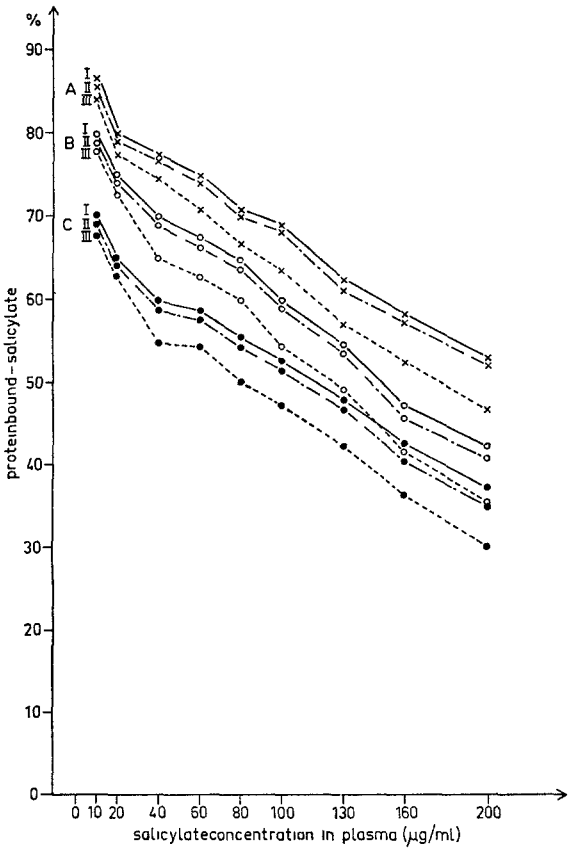

Fig. 1. Estimation of protein-salicylate binding in human plasma (adults). Different protein concentrations were obtained by diluting plasma with buffer. Bilirubin was added to give two different concentrations in some samples. Salicylate-binding with and without bilirubin was measured at increasing salicylate concentrations

A = proteinconc. 6.5g%, B = proteinconc. 5.0g%, C = proteinconc. 3.5g%

A I, B I, C I = plasma
A II, B II, C II = plasma with bilirubin (albumin-bilirubin 1 mol: 0.25 mol)
A III, B III, C III = plasma with bilirubin (albumin-bilirubin 1 mol: 0.5 mol)

protein-binding was dependent on the plasma concentrations of both salicylate and protein. The neonates had lower protein concentrations than the other groups. In addition, as is shown below, the amount of salicylate released on incubation of ASA with blood from neonates was less than that obtained using blood from older children. Therefore, a comparison was made between protein-binding in neonates and adults at the same plasma concentrations of protein and salicylates, using adult plasma diluted

with buffer and added salicylate. These results, too, are shown in Table 1. The differences between the age groups were significant.

In order to study the influence of protein and bilirubin, pools of adult plasma of different protein concentrations were prepared and with various amounts of added bilirubin and salicylate. Increasing bilirubin and decreasing protein concentration caused decreased protein-salicylate binding (Fig. 1). However, a definite effect of bilirubin plasma from

Table 1. *Acetylsalicylic acid-esterase activity in blood from different age groups (for details see 'Materials and Methods')*

Patients	Esterase-activity (percent ASA hydrolysed)	Salicylate formed after 60 min incubation (μg/ml)	Protein-bound salicylate (%)	Average protein-salicylate binding in adult plasma of comparable protein concentration (%)
prematures, female	49.6±6.3	68.4±4.2	31.7±5.1	69
prematures, male	48.1±4.8	67.1±3.8	32.9±3.5	68
newborns, female	64.8±5.5	102.8±6.3	51.6±4.7	65
newborns, male	61.3±4.2	104.5±2.8	49.2±3.1	66
infants, female	75.8±5.1	121.3±6.1	65.4±5.2	65
infants, male	78.1±4.3	124.5±5.3	64.5±2.7	64
children, female	80.5±4.5	131.1±7.1	62.5±5.1	63
children, male	83.8±4.3	133.6±6.1	62.2±4.0	62
adults, female	69.7±3.9	115.3±3.3	67.8±6.3	67
adults, male	82.3±6.1	132.3±6.3	62.8±3.8	62

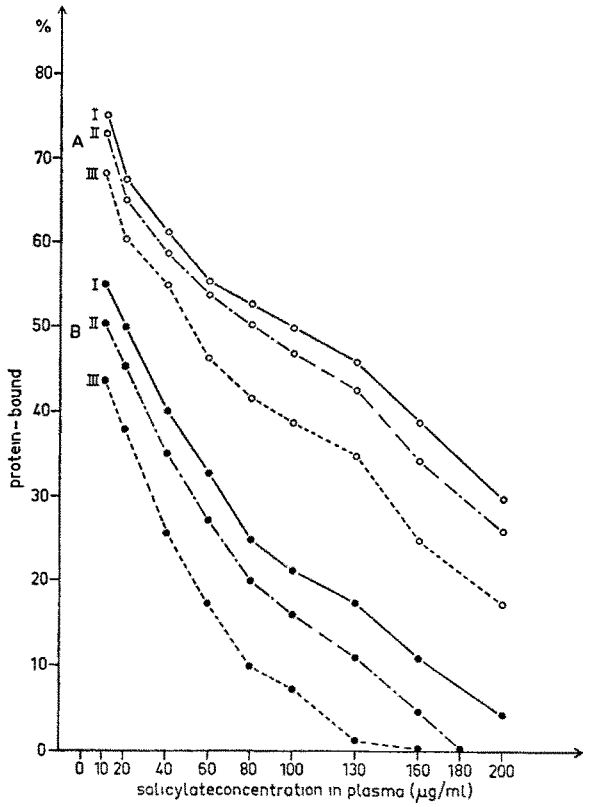

Fig. 2. Estimation of protein-salicylate binding in plasma of prematures and newborns. Bilirubin was added in two different concentrations. Salicylate-binding was measured with and without bilirubin at increasing salicylate concentrations

A = newborn (proteinconc. 5.8 g%)
B = premature (proteinconc. 5.3 g%)
A I, B I = plasma
A II, B II = plasma with bilirubin (albumin-bilirubin 1 mol: 0.25 mol)
A III, B III = plasma with bilirubin (albumin-bilirubin 1 mol: 0.5 mol)

adults was found only at the higher bilirubin concentration. The same concentrations of bilirubin and salicylate produced in plasma from neonates (prematures and normal weight newborns) had different effects. In these two age groups protein-binding was lower than in adult plasma at the same protein concentration. The most striking difference was found at high salicylate concentrations when, in contrast to adults, even a low bilirubin concentration caused a marked decrease of protein binding. The higher bilirubin concentration produced even greater displacement of salicylate from protein.

The activity of ASA-esterase, too, was dependent on age (Table 1). Blood samples from adult males, older children and infants more than one month of age hydrolysed 80% of the added ASA. The activity of the enzyme was lower in prematures and other neonates. There was no apparent sex difference in the various groups of children, whereas in adults enzyme activity in the erythrocytes of females was significantly lower than in males.

An effect of bilirubin on ASA-esterase was sought by adding various amounts of bilirubin to the blood samples, and it was found not to have any effect on this enzyme. The time course of ASA esterase activity in blood from subjects of different ages is shown in Fig. 3; there was a lower rate of hydrolysis of ASA in prematures and newborns.

Discussion

Ganshorn and Kurz (1968) demonstrated lower protein binding of certain substances, including

salicylate in the plasma of newborn children. Similar results were obtained by Ehrnebo *et al.* (1971) for diphenylhydantoin, phenobarbitone, ampicillin and benzylpenicillin. Chignell *et al.* (1971) also detected differences in the protein-binding of sulphapheazole in plasma from adult and newborn humans. The present experiments have revealed comparable differences for salicylate, namely low protein-binding in prematures, a rather higher value in newborns of normal weight, and a further increase in older children and adults. The quantitative values for protein-binding in adults are similar to those reported by Kucera and Bullock (1969).

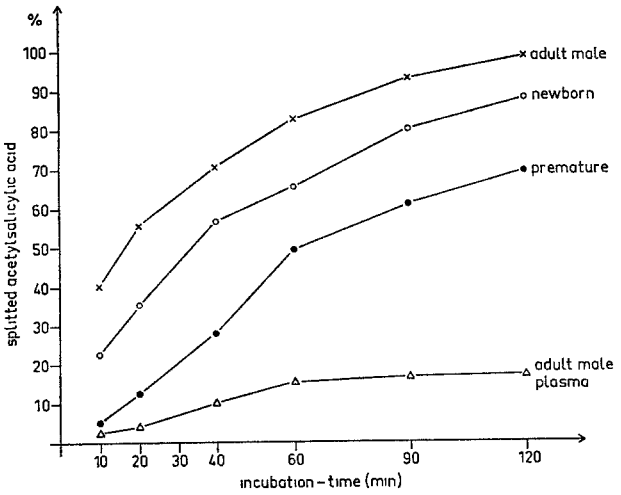

Fig. 3. Estimation of ASA-esterase by hydrolysis of ASA added to blood from adult males and premature and newborn infants

Chignell *et al.* (1971) suggested that the decrease in protein-binding in neonates might be due to displacement of drugs from protein by endogenous substances, such as bilirubin. An attempt was made to confirm this hypothesis by diluting adult plasma and adding bilirubin to it. However, the values for protein-binding obtained were still higher than those in premature and newborn children; mixing samples from neonates with bilirubin produced a larger decrease in protein-salicylate binding than in adults. The lower bilirubin concentration employed which had almost no effect in adults, displaced measurable amounts of salicylate from protein in the plasma of neonates. At comparable protein concentrations in plasma from adults and neonates, there was significantly lower protein-salicylate binding in the neonates. It is possible that other endogenous substances, which occur at higher concentrations in the neonatal period than later, for example free fatty acids, may contribute to further decrease in protein-binding at this age. Salicylate is bound to albumin and other proteins, and bilirubin only to albumin. Free fatty acids in the concentrations found in neonates are bound exclusively to proteins other than albumin (Jacobsen *et al.*, 1972). Since bilirubin causes greater displacement of salicylates from protein in neonates than in adults, it can be assumed that different binding affinities exist, at least for albumin, at different ages. It has been shown elsewhere (1973), using identical molar ratios of albumin to bilirubin that the displacement of drugs from albumin by bilirubin occurs more easily in prematures than older children; and this supports the hypothesis advanced that both endogenous and exogenous substances are bound to a lesser degree in neonates than in adults. It is impossible to rule out other endogenous compounds, e.g. products of steroid metabolism, as the reason for the increased displacement of drugs or bilirubin in plasma from foetuses and neonates.

The other parameter measured, the activity of ASA-esterase, also differed in the various age groups. It is interesting that its activity in premature children was particularly low, and that infants more than one month old had already developed as much activity as adult males. Like Menguy *et al.* (1972), the present study showed marked sex dependence of ASA-esterase in blood from adults, namely lower activity in females than in males. There was no difference between the sexes in the pre-pubertal period. Until now dependence of enzyme activity on age and sex has been known only for the components of the microsomal system of the liver. However, whereas this enzyme complex is lipophilic and membrane-bound, ASA-esterase is a hydrophilic enzyme. Further knowledge of the latter is required to explain these peculiarities. The lower ASA-esterase activity and protein-binding in neonates emphasise the quantitatively different nature of pharmacologically important processes in the neonatal period compared with other age groups. It is important to bear this in mind when neonates are given drugs.

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Dr. A. Windorfer jun.
Universitats-Kinderklinik
78 Freiburg i. Brg.
Federal Republic of Germany