ring to rotate into a position behind the hydrogen at carbon 4 of pyridine, as it could in conformation I of nicotine itself. In conformation III of nicotine *N*-oxide, the oxygen interferes with the hydrogen at carbon 4 of pyridine, and thereby prevents the molecule from assuming the conformation III of nicotine itself. The observed moment of nicotine *N*-oxide is considerably less than that calculated for either type of free rotation. Conformation III is closest to the observed moment. The reason the observed moment is 0.25 D. below that calculated for III is probably to be found in the above-mentioned proximity of the $\overset{+}{N}—\overset{-}{O}$ group to carbon 4 of the pyridine ring, which has a partial positive charge due to resonance, thus tending to reduce the moment between $\overset{+}{N}$ and $\overset{-}{O}$.

The same situation exists in (L)-nicotine *N,N'*-dioxide where the observed moment again comes closest to that calculated for conformation III. Here, however, the observed moment is 0.25 D. above the calculated one, and this is understandable because the resonance in the pyridine-*N*-oxide ring places a partial negative charge on carbon 4 which would now enhance the $\overset{+}{N}—\overset{-}{O}$ dipole. In both compounds, the evidence indicates the compounds exist in the conformation with the smallest dipole moment which is favored both from dipole interaction and from steric considerations.

EXPERIMENTAL

The dielectric constants were measured with a WTW Dipolmeter model DM-01 with a DF L-2 4-ml. cell with a thermostated jacket maintained at a temperature of 25.00° ± 0.03°. The P_{E_2} values are molar refractions calculated from refractive indices of the solutions measured with the D sodium line at 25.00° ± 0.03°. Densities were measured with a pycnometer of approximately 2-ml. capacity. Solvents were refluxed over sodium twice and distilled twice from a 1-M. column. Dipole moments were calculated using the Halverstadt-Kumler (8) method, for which programs were developed for use on both the IBM 1620 and 1401 computers. The results are given in Table IV.

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Acetylsalicylic Acid Hydrolysis in Human Blood and Plasma I

Methodology and *In Vitro* Studies

By PHILLIP A. HARRIS and SIDNEY RIEGELMAN

The *in vitro* hydrolysis of acetylsalicylic acid in 90 vol. per cent human blood and plasma was studied at therapeutically significant levels (below 15 mcg./ml.) by a spectrophotofluorometric method. Several new analytical aspects are presented. The discrepancies with earlier investigations are discussed.

IT IS WELL KNOWN that acetylsalicylic acid (ASA) rapidly hydrolyzes in aqueous solution, and its hydrolysis is accelerated by esterases found in the blood or plasma (1). Morgan and Truitt (2) recently reviewed the literature relating to the hydrolysis of ASA in blood and plasma. The hydrolysis would be expected to be a second-order process dependent upon both the enzyme and the substrate concentrations. At low aspirin and fixed enzyme concentrations, the reaction should reduce to a pseudo first-order reaction. Some of the previous investigations on this subject have been run in highly diluted plasma and at high aspirin levels (2). The excessive concentration of ASA might swamp the enzyme and cause

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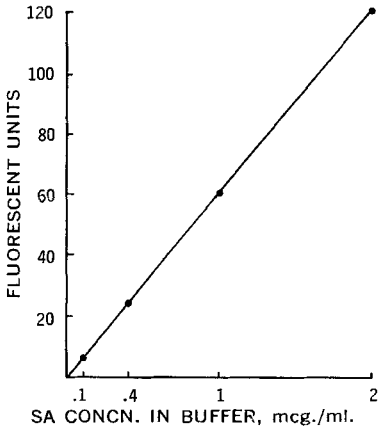

Fig. 1—Standard curve for salicylic acid in phosphate buffer (0.1 M, pH 7) using the Aminco Bowman spectrophotofluorometer. The activating wavelength was 315 mμ (uncorrected) and the emission wavelength was 420 mμ (uncorrected). (See Footnote 1.)

the reaction to follow zero-order kinetics. High dilution of the blood and plasma may have been required for the analytical approach utilized, but extrapolation back to undiluted blood cannot be made with certainty. These circumstances make it difficult to apply the results to an *in vivo* system.

The object of this paper is to report a method of studying ASA hydrolysis in virtually undiluted blood and plasma (90 vol. %) and at therapeutically significant concentrations (below 15 mcg./ml.).

EXPERIMENTAL

Rate Study—The subjects were four healthy males and one healthy female, ranging in age from 26 to 42 years. Blood was drawn from the antecubital vein into a heparanized syringe. The experiment was started soon after drawing the blood and lasted in most instances for 1 hr. All rate studies were carried out with U.S.P. acetylsalicylic acid which we found to contain less than 0.1% free salicylic acid (SA).

The blood sample was divided into two parts. One part was used for the study of the hydrolysis of ASA in whole blood. The other was centrifuged at 2200 RCF for 20 min., and the separated plasma was then used for the study of the hydrolysis of ASA in plasma. From each 10 ml. of blood or plasma, 1 ml. was removed for a blank analysis. Then 1 ml. of ASA in isotonic NaCl solution was added to the remainder, resulting in a 10% dilution of the blood. Two initial concentrations of ASA (13 mcg./ml. and 6.5 mcg./ml.) in the blood or plasma were used. The rate studies were carried out at 37°, and in most cases data points were obtained at 5, 10, 20, 30, 45, and 60 min.

Spectrophotofluorometric Assay—One-milliliter samples of blood or plasma were withdrawn from the test medium, mixed immediately with 1 ml. isotonic NaCl solution, and extracted with 5 ml. ether containing oxalic acid (8 mg./ml.). The extraction with acidified ether serves several functions: (*a*) the oxalic acid partitions into the aqueous phase rendering it acidic to a pH of approximately 2; (*b*) the esterases in the blood and plasma are immediately inactivated; (*c*) whole blood mixes well with the ether before clumping occurs; and (*d*) under these pH conditions this volume of ether virtually completely extracts the ASA and SA. These phenomena were verified by preliminary experiments using spectrophotometric and spectrophotofluorometric measurements.

The sample of ether and blood or plasma was centrifuged and 3 ml. of ether was withdrawn and extracted with 3 ml. phosphate buffer (0.1 *M*, pH 7). Nitrogen gas was bubbled through the buffer for 2 or 3 min. to remove residual ether, since the presence of ether affects the SA fluorescence in the buffer.

The fluorescence of salicylic acid in pH 7 phosphate buffer was determined using the Aminco-Bowman spectrophotofluorometer by activating at a wavelength of 315 mμ (uncorrected) and reading the emission at 420 mμ (uncorrected).[1] (ASA is not fluorescent under these conditions.) The slits were placed in the following order: 1/2, 1/16, 1/8, 1/8, 1/16, 1/8, and 3/16, respectively.

Buffer solutions containing both ASA and SA from blood or plasma samples were analyzed fluorometrically for free SA within 2 to 3 hr. after extraction. At pH 7 and room temperature, ASA hydrolysis proceeds relatively slowly with a half-life of approximately 3 days (3). However, when heated to 100° hydrolysis is very rapid with a half-life of approximately 10 min. The buffer solutions were heated at 100° in stoppered tubes for 1.5 hr., which completely hydrolyzes the intact ASA, and analyzed for total SA (*i.e.*, that liberated by hydrolysis of ASA and the free SA).

The fluorescence of each sample was determined at three dilutions. First, 1.0 ml. of undiluted sample was read; then 1.0 ml. of buffer was added and the sample read again; finally, another 1.0 ml. of buffer was added and the sample read again. This was done so that quenching corrections could be applied. The recovery of ASA and SA was greater than 98%, with somewhat better recovery of ASA than of SA due to its more favorable partition characteristics.

RESULTS AND DISCUSSION

Spectrophotofluorometric Assay—Figure 1 is a plot of fluorescence against various concentrations of SA in pH 7 phosphate buffer. The fluorescence reading of SA is linear with concentration over the range of concentrations studied. The concentrations used in this standard curve were the same magnitude as those encountered in the actual experiments after the dilution involved in the extractions.

The lower limit of detection of SA using the Aminco-Bowman spectrophotofluorometer is approximately 1.0 ng./ml. However, the blank readings obtained from blood and plasma are equivalent to a 25 ng./ml. of SA and remain constant before and after heating. The lowest amount of

[1] The Aminco-Bowman spectrophotofluorometer used in this study was not standardized to precise wavelength, and the values of excitation reported are probably 5 to 10 mμ too high.

free SA determined was at least 125 ng./ml. However, this is a false evaluation of the accuracy of the method, since the relative reproducibility of the readings is important and the total method is a difference method requiring an assessment of the relative error of the reading of the free salicylate and the total salicylate. It was the authors' experience that there was an over-all error of approximately 1 to 2% in the procedure. Therefore, the authors would not be able to discriminate accurately less than 2 parts of ASA in the presence of 100 parts of SA regardless of the concentrations.

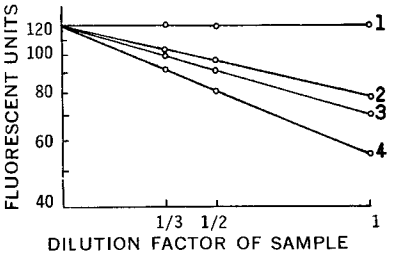

Fig. 2—Semilogarithmic plots illustrating the method of quenching correction. Key: 1, a standard buffer solution of SA; 2, 3, and 4, standard buffer solutions of SA shaken with ether containing quenching components from blood.

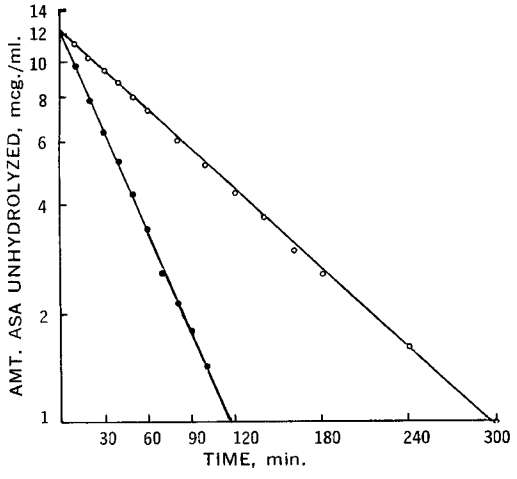

Fig. 3—Semilogarithmic plots of the hydrolysis of ASA carried out for 100 min. with blood and 300 min. with plasma. Initial ASA concn. was 13 mcg./ml. Key: ●, blood; ○, plasma.

It was found that the SA fluorescence in whole blood extracts was highly quenched. This was true of plasma extracts also but to a much lesser extent. A quenching correction procedure was developed in this laboratory analogous to that developed by Peng (4) for quenching corrections in scintillation counting of radioactive samples. It was observed that quenching obeys the Beer-Lambert law. A 1.0-ml. sample of the quenched solution was diluted in three increments and the fluorescence readings corrected for dilution (*i.e.*, if diluted $^1/_2$ the reading is multiplied by 2, if diluted $^1/_3$, by 3, *etc.*). The readings were plotted on semilogarithmic paper with the abscissa as the dilution factor and the ordinate as the corrected fluorescence reading. A line was drawn through these points and the intercept on the ordinate was taken as the true fluorescence in the absence of quenching components from the blood. If a standard buffer solution of SA in the absence of quenching components from the blood is diluted and read by this procedure, there is no change in the fluorescence reading. This is shown in Fig. 2 as the horizontal line along with three samples representing blood extractions with acidified ether subsequently shaken with the standard buffer solution of SA. Each sample varied in the degree of quenching as shown by the different slopes obtained. This is apparently a function of the amount and manner of shaking in the ether extraction of blood which can release varying amounts of quenching components. In assessing the true value by extrapolation, it was found that the method was reproducible with a range of ±1% of the standard.

Rate Data—It is apparent from Fig. 3 that the hydrolysis of ASA in blood and plasma appears to obey pseudo first-order kinetics for more than three half-lives. A summary of all the half-lives obtained, including repeated determinations at different times with the same subject, is shown in Table I. Figure 4 shows typical plots of hydrolysis data at initial ASA concentrations of 13 and 6.5 mcg./ml. in blood and plasma from three subjects

TABLE I—HALF-LIVES OF ASA HYDROLYSIS IN MINUTES AT 37° C. IN 90 VOL. PER CENT HUMAN BLOOD AND PLASMA

Subject	13 mcg./ml. Initial ASA Concn. Blood	13 mcg./ml. Initial ASA Concn. Plasma	6.5 mcg./ml. Initial ASA Concn. Blood	6.5 mcg./ml. Initial ASA Concn. Plasma
A	38.5	62.5		
B	35	56	27.5	80
	30.5	72		
	28	68		
C	33	70	28	65
	32	68.5	31	70
	27	67		
D	31	57	30.5	63
	31.5	58	31	68
	30	67		
	32.5	80		
E	35	65		
Mean ± S.E.	32.0 ± 0.91	65.9 ± 1.98	29.6 ± 0.77	69.2 ± 2.96

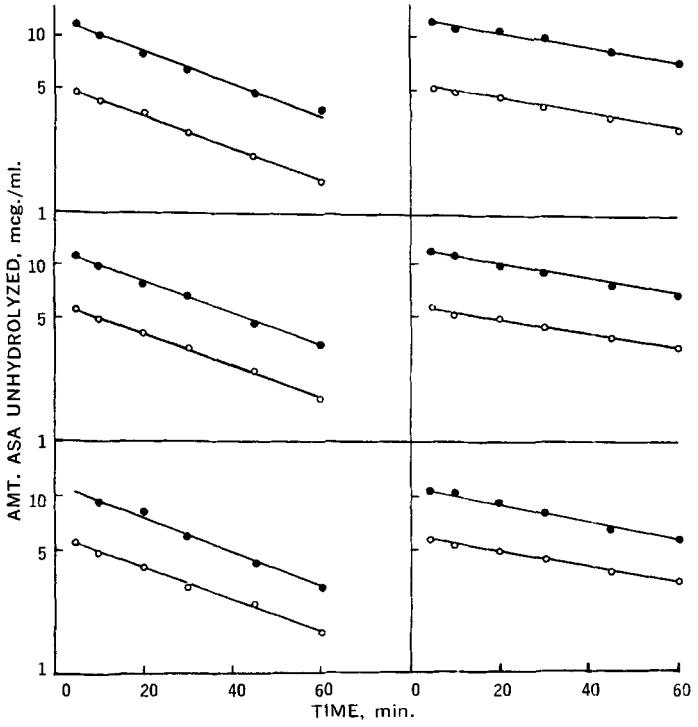

Fig. 4—Typical plots of hydrolysis of ASA in blood (left) and plasma (right) from three subjects. Hydrolysis was followed for 60 min. at two initial levels of ASA in both the blood and plasma. Key: ●, 13 mcg./ml.; ○, 6.5 mcg./ml.

It is to be noted that while the half-lives in these systems are not exactly reproducible, they are reasonably close indicating that the hydrolysis in this concentration range obeys first-order kinetics. However, this system is first order only at constant enzyme concentration. Since the blood and plasma were diluted reproducibly in each experiment, the differences in hydrolysis rates in repeated experiments in the same subject might be explained in terms of day-to-day variations of enzyme concentration in the blood.

Table I shows an average value of approximately 30 min. for the half-life of hydrolysis in whole blood and slightly over 1 hr. in plasma. It is important to note that the studies carried out in this investigation are at the levels of ASA that might be expected in the body from normal doses (5).

However, the results do not agree with the recent report of Morgan and Truitt (2), wherein they studied the hydrolysis of ASA in thirtyfold dilutions of serum and blood and at a concentration of 2.4×10^{-3} *M* ASA (433 mcg./ml.), a level 33 times the concentrations used in this study. Their reported average rate constants for ASA hydrolysis at 25° are 4.74×10^{-2} hr. ($t_{1/2}$ = 14.7 hr.) and 11.7×10^{-2} hr.$^{-1}$ ($t_{1/2}$ = 5.9 hr.) for serum and blood, respectively. They also showed that at 37° the rate is about twice that at 25°. These half-lives can only be compared to our own by linearly correcting for enzyme dilution by dividing by 30 and by 2 to correct for temperature. If we adopt such a correction procedure, even though Morgan and Truitt used a 3.3% serum concentration, we obtain half-lives of approximately 15 min. and 6 min for serum and blood, respectively. These are approximately 1/4 to 1/5 the estimates of the half-lives for hydrolysis obtained in the present study. Edwards (6) studied the hydrolysis of ASA in guinea pig plasma from 1 to 50 vol. %. Below approximately 15%, there was considerable departure from linearity.

The observation that the hydrolytic rate in whole blood is about twice as rapid as in plasma suggests the presence of higher concentrations of the hydrolyzing enzyme(s) or additional enzyme(s) in the red blood cells or in the leucocytes. Red blood cells are known to possess a high content of acetylcholinesterase. A recent review (7) includes a discussion of esterases found in leucocytes.

SUMMARY

An assay for acetylsalicylic acid is reported based on a spectrophotofluorometric assay of the free and total salicylate content. Using this assay the hydrolytic rate of acetylsalicylic acid in 90 vol. % human whole blood and plasma at levels of 13 and 6.5 mcg./ml. was studied. The hydrolysis was found to obey pseudo first-order kinetics. The mean half-lives of hydrolysis were 32 ± 0.91 min. (mean ± S.E.) for blood and 65.92 ± 1.98 min. for plasma at the 13 mcg./ml. level. At the 6.5 mcg./ml. level the values were 29.6 ± 0.77 min. for blood and 69.2 ± 2.96 min. for plasma.

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