Evaluation of Acetylsalicylic Acid Esterase in Aspirin Metabolism

Interspecies Comparison

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The hydrolysis of acetylsalicylic acid *in vitro* by serum from various species of laboratory animals and humans has been estimated by spectrophotometric measurement of salicylic acid appearance. Various animal species differ widely in their serum aspirin hydrolysis rates, with cats and rabbits approximating the human rate. Rats greatly exceed the rate for man, while dogs show a much slower rate. Human serum samples *in vitro* show considerable individual differences in acetylsalicylic acid esterase activity. A variety of serum concentrations, temperatures, and buffers have been compared. *In vivo* aspirin hydrolysis was also compared in 20 humans using an adaptation of the Brodie method, which measured aspirin blood levels at various time intervals following oral administration of buffered and unbuffered aspirins.

CLINICAL preference for the analgesic action of the acetyl ester of salicylic acid over other salicylate forms in the management of pain has had a long and controversial history. Since the therapeutic introduction of aspirin (ASA) in the middle of the 19th century, there have been as many reports claiming more effective analgesia for it when compared with sodium salicylate, as there have been those disclaiming superior analgesia (1–5). In part, these discrepancies have arisen because of a lack of sensitivity in the methods for measuring analgesia in both animals and man, and until an objective method is devised, this controversy will probably continue. To date one of the most widely accepted methods for comparing ASA preparations has been the determination of blood salicylic levels because of the rapid hydrolysis of ASA to salicylic acid (SAL) by the acetylesterases in the plasma and tissues. This method of comparison has been accepted even though the duration of action for an analgesic or anti-inflammatory ASA dose is much shorter than the measurable SAL levels. This discrepancy has led many investigators to believe that the unhydrolyzed acetylester is the active form of the drug. In spite of this rapid enzymatic hydrolysis, several investigators have detected intact ASA in blood (6–8) and others have confirmed its presence by studying the radioactively labeled drug (8–12). However, with the variability and difficulty of the ASA measurements, as well as of the pain response measurements, it has not yet been possible to correlate ASA levels with analgesia.

The hydrolyzing enzyme, acetylsalicylic acid esterase (ASA esterase), despite its specific name, is probably not a single enzyme but belongs to a group of enzymes known as acetylesterases. It differs from acetylcholine esterase in that it is slightly inhibited by eserine, inhibited by isoflurophate (DFP), unaffected by cyanide and calcium ions, and destroyed by boiling (13–16), and it has been clearly differentiated from the pseudocholine esterase of serum (17–19). The role of this enzyme in aspirin metabolism has been recognized, but it has not been adequately measured in animals and man (20–22).

The hypothesis that the entire ASA molecule is the active moiety in aspirin analgesia has made it important to study further the factors controlling blood aspirin concentration. These factors include (*a*) rate of absorption, (*b*) rate of distribution to tissues, (*c*) rate of hydrolysis, and (*d*) rate of kidney excretion. This study concerns the measurement of hydrolysis.

The rate of hydrolysis can be tested *in vitro* free of the other factors which control blood aspirin concentration. Although tissues such as liver and kidney have been shown to hydrolyze acetylsalicylic acid (23–25), these would not influence the enzymatic activity of ASA esterase in blood samples tested by adding exogenous aspirin. The relative activity of serum from different laboratory animals in hydrolyzing aspirin was measured in order to select an animal species comparable to man. The individual variability of acetylsalicylic acid esterase activity of human sera *in vitro* has been measured using 20 different serum samples, and *in vivo* hydrolyzed and unhydrolyzed blood ASA

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levels (26, 27) have been analyzed in the same 20 subjects.

EXPERIMENTAL

The continuous spectrophotometric method of Hofstee (28) for the determination of esterases was adapted to measure the hydrolysis of acetylsalicylic acid to salicylic acid at 300 mμ. As the aspirin is hydrolyzed, a stoichiometric amount of salicylic acid appears; thus, continuous hydrolysis can be followed in buffered samples of serum or blood. However, the hydrolytic rate is maximal and continuous for an adequate period of time only when supratherapeutic concentrations of aspirin are added to the serum (final cell concentration = 2.4×10^{-3} *M*).

Varying amounts of serum, water, and aspirin were combined to find the optimum combination for maximum differences between the spontaneous and enzymatic hydrolysis rate. The total volume in the Beckman cell of 3.0 ml. and the 1.0-ml. volume of the buffer (pH 8.0 Tris) were kept constant. It was found that the enzymatic rate did not exceed the spontaneous rate of hydrolysis in an aqueous solution at pH 8.0 sufficiently to permit accurate quantitation of enzyme activity. It was then decided to adjust the Tris buffer to a pH of 7.0 for a slower aqueous hydrolysis rate. When Tris buffer was compared with sodium phosphate and barbital buffers at the values of pH 7.0, 7.4, and 7.8, it was apparent that Tris buffer produced slightly faster hydrolysis rates than the other two buffers (Table I).

As a preliminary human trial, serum and plasma in 0.1-ml. volumes were used as the enzyme fraction in the quartz cells. From these values (Fig. 1) it was evident that serum contained more of the active enzyme than plasma, as was also mentioned by Mulinos and Ardam (20). When whole blood was later compared using the method of Cotty *et al.* (26), the rate of hydrolysis exceeded those of both serum and plasma.

Varied concentrations of pooled human sera yielded a linear relationship with hydrolysis rate at both 25° and 37° (Fig. 2). Serial dilutions of the serum from each of the animal species tested (rats, dogs, cats, and rabbits) were also checked for a linear extrapolation of hydrolysis rate with enzyme

TABLE I.—EFFECT OF VARIOUS BUFFER SYSTEMS ON SERUM ASPIRIN HYDROLYSIS RATES *In Vitro* (25°)[a]

pH	Media	Tris	Na Phosphate	Barbital
	H_2O	0.012	0.011	0.011
7.0	Serum	0.022	0.015	0.011
	Boiled serum	0.012	...	0.011
	H_2O	0.012	0.012	0.012
7.4	Serum	0.018	0.013	0.014
	Boiled serum	0.010	0.012	0.009
	H_2O	0.012	0.014	0.011
7.8	Serum	0.021	0.014	0.018
	Boiled serum	0.011	...	0.011

[a] $E^{300\,m\mu}/10'$ $\left(E = \frac{\Delta A\ 10' - A_{H_2O}}{\text{total SAL } A}\right)$.

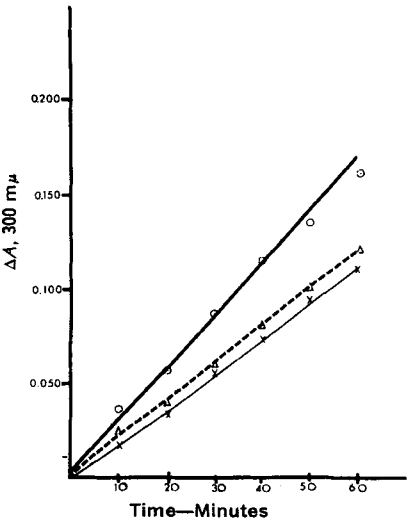

Fig. 1.—Comparison of acetylsalicylic acid esterase activity of pooled human serum and plasma with water hydrolysis. Key: X, water; O, serum; Δ, plasma.

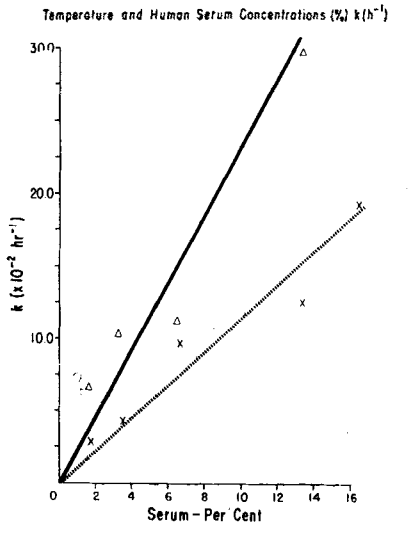

Fig. 2.—Human serum aspirin hydrolysis rates at varied concentrations and temperatures. Key: X, 25°; Δ, 37°.

concentration. Room temperature (24–26°) was selected for the subsequent studies because it permitted a sufficiently slow rate of hydrolysis for accuracy. Samples were kept outside the Beckman cell compartment between readings because the lamp heat tended to raise temperatures to about 31° inside.

The final cell amounts selected for the serum comparisons were as follows:

Tris buffer, pH 7.0	1.0 ml.
Serum or whole blood	0.1 ml.
Acetylsalicylic acid	1.0 ml.[1]
Distilled water	0.9 ml.
Total	3.0 ml.

[1] Final cell concentration = 2.4×10^{-3} *M*.

TABLE II.—FIRST-ORDER VELOCITY CONSTANTS (k)[a] FOR DOGS, CATS, RABBITS, AND RATS. ASPIRINASE HYDROLYSIS RATES—25°C., pH 7.3

Species	No.	k ($\times 10^{-2}$)	S.E.
Dogs	10	2.9810	0.33
Cats	8	5.1816	0.83
Rabbits	10	5.5060	0.67
Rats	10	8.5294	0.51

[a] $k = \frac{2.303}{t} \cdot \log \frac{a}{a - x}$.

A Beckman DU spectrophotometer set at 300 mμ was used for all measurements with the cells arranged as follows: cell *1*, buffer for the reference standard; cell *2*, spontaneous (buffer + water + ASA) hydrolysis; cells *3*, *4*, *5*, cells containing the various serum samples with buffer + water and ASA.

Simultaneous comparison of the reaction rates in the cells was computed by reading the ΔA every 5–10 min. for 1 hr. or longer. This time interval seemed adequately spaced in all species tested with the exception of the rat. In this case, the readings were made at 1-min. intervals for the first 10 min., then at 5-min. intervals until after the 20-min. period.

After the changes in absorbance (ΔA) *versus* time were plotted for each species, the first-order velocity constants (k) for the rate of ASA esterase hydrolysis were calculated and recorded in Table II. This rate is expressed as:

$$k = \frac{2.303}{t} \log \frac{a}{a - x} \text{ in hr.}^{-1}$$

where t = 1 hr., $a = A_{max.} - A_{initial}$, and $x = \Delta A_{hr.}$

To correct for the slight temperature variations from day to day, the spontaneous hydrolytic

TABLE III.—CALCULATION OF FIRST-ORDER VELOCITY CONSTANT (k)[a] FOR 18 SUBJECTS ASPIRINASE HYDROLYSIS—*In Vitro* SERUM—25°C. = pH 7.3

Subject	a	x	k ($\times 10^{-2}$)
A	1.35	0.018	1.3357
B	1.248	0	0
C	1.431	0.048	3.4107
D	1.375	0.102	7.7151
E	1.343	0.066	5.0436
F	1.303	0.090	7.1623
G	1.403	0.060	4.3757
H	1.147	0.066	5.9187
I	1.295	0.066	5.2278
J	1.222	0.018	1.4739
K	1.428	0.108	7.8763
L	1.404	0.096	7.0702
M	1.365	0.030	2.2109
N	1.431	0.042	2.9709
O	1.373	0.120	9.1429
P	1.324	0.024	1.8194
Q	1.426	0.051	3.6387
R	1.331	0.114	8.9587
		Mean	4.7418
		S.E.	0.65

t = 1 hr.
$a = A_{max.} - A_{initial}$
$x = \Delta A_{hr.}$

[a] $k = \frac{2.303}{t} \log \frac{a}{(a - x)}$.

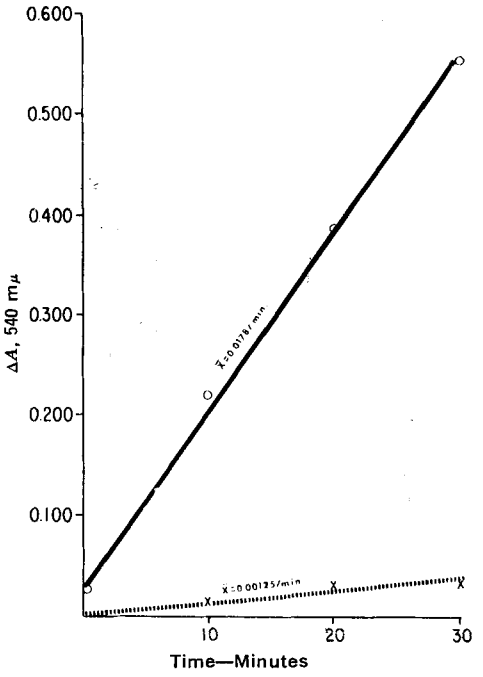

Fig. 3.—Average human blood acetylsalicylic acid esterase hydrolysis rate. Key: ×, water; ○, blood.

(aspirin + buffer + H_2O) rate of change, as measured by the absorbance changes, was subtracted from the absorbance changes for the hydrolytic total (aspirin + buffer + H_2O) rate of change before final calculation.

For the second phase of this study, the modified Brodie method for total plasma salicylates (27, 29) as revised by Cotty *et al.* (26) to measure both total and free salicylate in the same whole blood sample was used. Known concentrations of salicylic acid or aspirin were added to pooled human blood and carried through this determination to prepare standard reference curves. The experimental absorbances were then read from these curves as mg.% SAL. Any blank (control sample) values found in any of the 20 subjects was subtracted from the person's drug values before final calculation and tabulation. These control blank values, when present, were never more than 0.3 mg.%.

RESULTS

Man.—*A.*—Twenty healthy subjects, with no history of gastrointestinal diseases and who repeatedly participated in salicylate test panels, were used in this phase of the study. A blood sample of 5.0 ml. was withdrawn from each subject. After serum separation, the control samples were placed in 10-mm. Beckman quartz cells in the order previously described for an estimate of the variability of human serum hydrolysis rate.

The k values for 18 of these subjects' control serum with *in vitro* salicylate are shown in Table III. These rates varied from complete lack of enzymatic action in subject *B* to 9.1429 × 10^{-2} in subject *O*. This latter rate is translated as a complete hydrolysis in 10–11 hr. of this large

TABLE IV.—COMPARISON OF TOTAL SALICYLATE (T), FREE SALICYLATE (F), AND ASA BLOOD LEVELS IN 20 SUBJECTS AFTER 2 TABLETS OF BUFFERED ASPIRIN

mg.% SAL

Subject	10 min. T	10 min. F[a]	10 min. ASA	20 min. T	20 min. F[a]	20 min. ASA
A	0.71	0.30	0.41	1.87	1.22	0.65
B	0.94	0.61	0.33	2.15	1.67	0.48
C	0.95	0.55	0.40	2.54	1.56	0.98
D	0.42	0.15	0.27	1.67	0.95	0.72
E	0.64	0.23	0.41	1.50	0.90	0.60
F	0.70	0.21	0.49	1.50	0.76	0.74
G	1.50	0.61	0.89	3.22	2.38	0.84
H	0.15	0.15	0	0.35	0.32	0.03
I	0.42	0.21	0.21	2.40	1.34	1.06
J	0.20	0.03	0.17	0.70	0.27	0.43
K	1.60	0.87	0.73	3.00	1.67	1.33
L	0.72	0.27	0.45	1.22	0.67	0.55
M	0.80	0.32	0.48	1.56	1.06	0.50
N	0.55	0.33	0.22	1.55	0.91	0.64
O	0.25	0.11	0.14	0.32	0.32	0
P	1.00	0.52	0.48	1.30	0.93	0.37
Q	0.40	0.04	0.36	0.85	0.49	0.36
R	0.05	0.21	−0.16	1.65	1.18	0.47
S	...	...	...	0.25	0.42	−0.17
T	1.02	0.42	0.60	1.92	1.25	0.67
Mean	...	...	0.36	...	...	0.56

mg.% SAL

Subject	30 min. T	30 min. F[a]	30 min. ASA	45 min. T	45 min. F[a]	45 min. ASA
A	2.30	2.07	0.23	2.85	2.55	0.30
B	3.08	2.32	0.76	2.80	2.43	0.37
C	2.80	2.23	0.57	2.30	2.14	0.16
D	2.87	1.76	1.11	3.58	2.55	1.03
E	2.10	1.44	0.66	2.40	1.85	0.55
F	1.58	1.15	0.43	1.55	1.28	0.27
G	3.82	3.31	0.51	3.22	3.00	0.22
H	0.95	0.61	0.34	1.05	0.93	0.12
I	3.72	2.74	0.98	3.72	2.90	0.82
J	1.20	0.68	0.52	2.08	1.40	0.68

mg.% SAL

Subject	30 min. T	30 min. F[a]	30 min. ASA	45 min. T	45 min. F[a]	45 min. ASA
K	3.20	2.42	0.78	4.22	3.34	0.88
L	2.40	1.38	1.02	2.54	2.01	0.53
M	2.05	1.29	0.76	2.22	1.79	0.43
N	3.40	2.28	1.12	3.42	3.34	0.08
O	0.60	0.53	0.07	0.85	0.91	−0.06
P	1.40	0.99	0.41	1.56	1.14	0.42
Q	1.30	0.76	0.54	1.40	1.33	0.07
R	1.65	1.33	0.32	1.70	1.59	0.11
S	0.75	0.80	−0.05	1.25	1.33	−0.08
T	2.52	1.67	0.85	3.36	2.57	0.79
Mean	...	...	0.60	...	...	0.38

mg.% SAL

Subject	90 min. T	90 min. F[a]	90 min. ASA	240 min. T	240 min. F[a]	240 min. ASA
A	2.59	2.53	0.06	1.68	2.51	−0.83
B	2.36	3.34	−0.98	1.00	1.75	−0.75
C	2.10	2.04	0.06	1.20	1.73	−0.53
D	3.42	3.02	0.40	2.68	1.98	0.70
E	2.68	2.34	0.34	1.65	1.32	0.33
F	1.50	1.19	0.31	0.80	0.65	0.15
G	2.82	2.87	−0.05	2.22	1.82	0.40
H	1.42	1.46	−0.04	1.38	1.06	0.32
I	3.80	3.09	0.71	2.80	2.26	0.54
J	2.75	2.57	0.18	2.05	2.05	0
K	3.40	2.81	0.59	2.10	2.24	−0.14
L	2.77	2.28	0.49	2.87	2.85	0.02
M	2.60	3.27	−0.67	1.25	1.25	0
N	2.90	3.06	−0.16	2.38	2.28	0.10
O	1.00	1.14	−0.14	1.38	1.57	−0.19
P	1.78	1.73	0.05	1.56	1.56	0
Q	1.50	1.33	0.17	1.70	1.52	0.18
R	1.90	2.20	−0.30	1.25	1.33	−0.08
S	1.41	1.59	−0.18	1.20	1.18	0.02
T	2.87	2.69	0.18	1.77	1.67	0.10
Mean	...	...	0.05	...	...	0.02

[a] All F readings were adjusted by a factor of 0.76 to compensate for high spectrophotometer readings due to interfering substances of phenolic nature. The factor was determined by the ratio of free and total salicylate at the times where no further hydrolysis could occur.

amount of aspirin substrate. These data also show that a wide variability between individual aspirin hydrolysis rates may be an important factor in aspirin blood levels. This variability was previously noted for aspirin plasma levels by Mandel (10).

In vitro measurement of human whole blood hydrolysis rates of aspirin was possible using the method of Cotty *et al.* Data averaged from 11 subjects are shown in Fig. 3, with the calculated rate for blood greatly exceeding that for serum ($k = 11.7 \times 10^{-2}$).

B.—After an overnight fast and withdrawal of a control (blank) blood sample (5.0 ml.), the same 20 subjects were given either 2 tablets of aspirin[2] or of a buffered aspirin[3] to be swallowed whole with 100 ml. of cold water. Subsequent blood samples were taken at 10, 20, 30, 45, 90, and 240 min. After an interval of at least 3 days, the same subjects then received the other aspirin product in a crossover design. Blood samples were determined by the Cotty modification of the familiar Brodie colorimetric salicylate method. In Tables IV and V are shown the total and free salicylate as well as the aspirin blood levels which are calculated from the difference (total − free × mol. wt. ASA/mol. wt. SAL) for each subject with each product. (Any blank values were subtracted from the person's drug values before calculation and tabulation.) These show clearly that the buffered aspirin product which produced higher total salicylate levels at all time periods than the unbuffered product, also had higher ASA levels at all time periods. Figure 4 shows the graphic representation of the ASA data. Tables IV and V also demonstrate the variability of the salicylate blood levels which are inherent in the study of any biological subject.

Animals.—Ten animals of each species—cats, rabbits, dogs, and rats—were used in this study. These were all healthy, laboratory housed, and unpremedicated in any way. A 1.0–2.0-ml. blood sample was withdrawn from the jugular vein of the cats and dogs and by heart puncture from the rats and rabbits. After serum separation, the spontaneous and enzymatic hydrolysis rates were determined in the manner previously described for man.

In order to facilitate comparisons of the slope constants of all of the species tests, Fig. 5 was prepared. The mean curve of spontaneous aqueous hydrolysis and its 95% confidence limits, were

[2] Bayer.
[3] Bufferin.

TABLE V.—COMPARISON OF TOTAL SALICYLATE (T), FREE SALICYLATE (F), AND ASA BLOOD LEVELS IN 20 SUBJECTS AFTER 2 TABLETS OF PLAIN ASPIRIN

mg.% SAL

Subject	10 min. T	10 min. F[a]	10 min. ASA	20 min. T	20 min. F[a]	20 min. ASA
A	0.30	0.02	0.28	0.50	0.29	0.21
B	0.75	0.08	0.67	1.30	0.42	0.88
C	0.20	0.05	0.15	0.20	0.38	−0.18
D	0	0.04	−0.04	0.75	0.11	0.64
E	0.25	0.15	0.10	1.20	0.56	0.64
F	0.25	0	0.25	...	0.30	...
G	0.68	...	...	1.30	1.06	0.24
H	0.17	0.11	0.06	0.45	0.23	0.22
I	...	...	...	1.48	1.02	0.46
J	0.20	0.11	0.09	1.15	0.72	0.43
K	0.35	0.38	−0.03	1.40	1.10	0.30
L	0.50	0.15	0.35	1.20	0.72	0.48
M	0.15	0.11	0.04	0.95	0.46	0.49
N	0.25	0.24	0.01	1.05	0.61	0.44
O	0	0.05	−0.05	0.10	0.09	0.01
P	0.30	0.11	0.19	0.58	0.34	0.24
Q	0.45	0.23	0.22	0.50	0.49	0.01
R	...	...	...	...	...	...
S	0.10	0.03	0.07	0.40	0.15	0.25
T	0.25	0.19	0.06	0.90	0.65	0.25
Mean	...	...	0.14	...	...	0.33

mg.% SAL

Subject	30 min. T	30 min. F[a]	30 min. ASA	45 min. T	45 min. F[a]	45 min. ASA
A	1.05	0.68	0.37	1.80	1.22	0.58
B	1.55	0.65	0.90	1.60	1.25	0.35
C	0.85	0.99	−0.14	2.10	1.73	0.37
D	1.30	0.53	0.77	2.60	1.66	0.94
E	1.40	0.90	0.50	1.45	1.41	0.04
F	1.05	0.83	0.22	1.40	0.93	0.47
G	2.25	1.56	0.69	2.70	2.07	0.63
H	0.85	0.65	0.20	1.05	0.80	0.25
I	1.66	1.73	−0.07	3.04	2.31	0.73
J	...	1.44	...	2.65	2.36	0.29

mg.% SAL

Subject	30 min. T	30 min. F[a]	30 min. ASA	45 min. T	45 min. F[a]	45 min. ASA
K	2.32	2.52	−0.20	2.92	3.76	−0.84
L	1.60	1.09	0.51	2.75	2.20	0.55
M	1.03	0.81	0.22	1.47	1.14	0.33
N	1.78	1.08	0.70	2.10	2.42	−0.32
O	0.35	0.21	0.14	0.55	0.24	0.31
P	0.90	0.38	0.52	0.95	0.65	0.30
Q	0.70	0.72	0.02	0.95	0.97	−0.02
R	...	...	...	...	...	...
S	0.40	0.34	0.06	0.70	0.65	0.05
T	1.02	0.80	0.22	1.66	1.19	0.47
Mean	...	...	0.31	...	...	0.29

mg.% SAL

Subject	90 min. T	90 min. F[a]	90 min. ASA	240 min. T	240 min. F[a]	240 min. ASA
A	2.25	2.09	0.16	1.85	2.49	−0.64
B	2.60	2.66	−0.06	2.00	2.36	−0.36
C	2.00	1.60	0.40	1.25	1.10	0.15
D	4.55	3.42	1.13	0.20	0.19	0.01
E	1.85	1.90	−0.05	1.65	1.44	0.21
F	1.50	1.63	−0.13	1.20	0.96	0.24
G	3.70	3.44	0.26	2.58	2.58	0
H	1.80	1.64	0.16	1.85	1.14	0.71
I	3.54	3.28	0.26	2.36	2.10	0.26
J	2.50	2.39	0.11	1.74	2.28	−0.54
K	2.65	4.59	−1.94	2.00	2.60	−0.60
L	3.30	3.46	−0.16	2.65	2.60	0.05
M	2.07	1.81	0.26	1.25	1.25	0
N	3.60	3.44	0.16	2.10	2.74	−0.64
O	0.70	0.61	0.09	0.75	0.70	0.05
P	1.05	1.14	−0.09	1.10	1.54	−0.44
Q	1.10	1.86	−0.76	1.80	1.66	0.14
R	...	...	...	...	...	...
S	1.00	0.85	0.15	1.65	1.28	0.37
T	2.56	2.34	0.22	1.75	1.98	−0.23
Mean	..	...	0.01	...	...	−0.06

[a] All F readings were adjusted by a factor of 0.76 to compensate for high spectrophotometer readings due to interfering substances of phenolic nature. The factor was determined by the ratio of free and total salicylate at the times where no further hydrolysis could occur.

plotted for 1 hr. on this figure. This shows that cats and rabbits approximate the human rate, while dogs have a much slower rate, and rats a much faster aspirin hydrolysis rate than man.

DISCUSSION

Despite the numerous but scattered references to an enzyme hydrolyzing aspirin in the literature, there exists no interspecies study comparable to the foregoing data, except for an abstract prepared by Mulinos and Ardam (21) of an apparently unpublished study. A preliminary interspecies study was also presented by Vincent and Lagreu (13) but the object of this study was to differentiate between acetylcholine esterase and acetylsalicylic acid esterase.

There is no clear priority to the observation of an *in vivo* hydrolysis for aspirin. Although it was noted by Lester *et al.* (6) that plasma could hydrolyze the drug, they gave no data and they did not speculate whether the process was enzymatic. The report of Vandelli and Scaltriti (23) recognized the presence of an ASA esterase in several tissues other than blood, and Smith *et al.* (30) also recognized tissue hydrolysis of aspirin. The report by Augustinsson (17) clearly differentiated the enzyme from the pseudocholinesterase of plasma.

Vincent and Lagreu (13) measured the hydrolytic

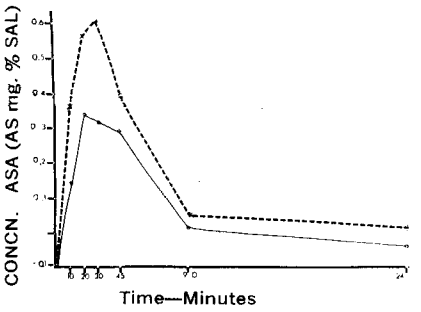

Fig. 4.—Average blood aspirin levels for 20 subjects after 0.6 Gm. plain or buffered acetylsalicylic acid tablets. Key: – – –, buffered aspirin; ——, plain aspirin.

activity of guinea pig serum for aspirin and found it to be 10 times as active as human serum. The uncompleted report of Mulinos and Ardam, previously cited, gives some basis for comparison with the findings of this investigation. Both studies found that the rat hydrolyzed ASA rapidly and that the rate for dogs was comparable to humans. However, Mulinos and Ardam found a more rapid rate for rabbits than was found here.

The distribution of the ASA esterase activity among body tissues has received more attention

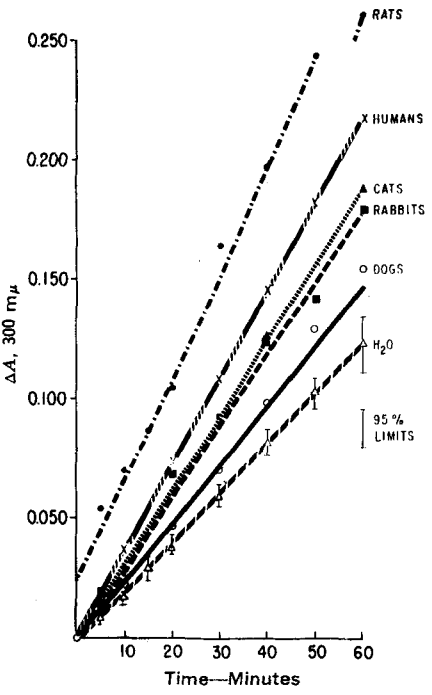

Fig. 5.—A comparison of aspirin hydrolysis rates of serum from humans with 4 laboratory animal species and water hydrolysis.

than its interspecies variation. Serum is almost as active as whole blood. The serum activity has been used as a test of liver function and in altered hepatic disorders (24, 25). Beckman (22) found lymph to contain ASA esterase activity. Bastide (19) also found whole blood to exceed serum in hydrolytic activity. Leonards (8) reported that the hydrolysis of aspirin in plasma *in vitro* is slower than the disappearance of aspirin in whole blood *in vivo* after intravenous injection into dogs.

The data reported here satisfy several objectives of the study. They show that either dog, cat, or rabbit serum *in vitro* hydrolyzes aspirin slowly enough to be comparable to man. The very much faster rate of aspirin hydrolysis in the rat may explain why experimental analgesia is difficult to obtain in the species with anything less than toxic doses of aspirin. They also show for the human data that a considerable variation exists between subjects in the activity of serum in splitting aspirin. The close to tenfold difference in ASA esterase activity could partially account for wide differences in the blood aspirin levels among the 20 subjects. There is also presented evidence that the aspirin blood levels of a buffered aspirin exceed those of a plain aspirin at all the time periods tests, *i.e.*, 10, 20, 30, 45, 90, and 240 min., which was to be expected from previously reported plasma and blood salicylate levels with these same products (26, 31, 32). The modifications of the Brodie colorimetric SAL method for these determinations may help identify the form of salicylate which produces analgesia, since aspirin, as such, can now be measured during the analgesic period.

This study can also provide a comparison basis for the activity of intact animals and humans in the disposition of aspirin. If serum activities for hydrolysis *in vitro* are known, and the rate of absorption is known from serial aspiration measurement of residual amounts in the gastrointestinal tract, then it should be possible to account for the distribution of aspirin out of the blood stream to various organs. Thus, the completed work is an integral part of the over-all study of the metabolic disposition of aspirin in man and other animals.

SUMMARY

Two established methods for the determination of salicylates have been modified to (*a*) measure the hydrolytic activity of serum samples from a variety of species to which aspirin was added *in vitro* and (*b*) determine *in vivo* blood levels of aspirin in 20 subjects.

Serum activity was corrected for the spontaneous hydrolysis rate in control buffer-aspirin solutions. Studies of serum dilutions for all species tested showed a linearity of hydrolysis rates with increasing amounts of enzyme, thus establishing that extrapolation of the data to whole serum was valid.

Serum samples were tested for dogs, cats, rabbits, rats, and humans. It was found that rats exhibit a faster aspirin hydrolysis than man, while the other three species were equal to or slower than the human rate. Considerable individual variation was observed in human ASA esterase activity *in vitro*. The implications of these findings for studies of the metabolic disposition of aspirin and the extrapolation to *in vivo* hydrolysis is discussed. A comparison of blood ASA levels, in 20 subjects, was made with a buffered and a plain aspirin, with the buffered aspirin producing higher ASA blood levels at all times tested. These higher ASA blood levels were comparable with previously reported plasma salicylate levels for the same buffered product.

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Kaliuretic Properties of Furosemide and Hydrochlorothiazide by *In Vivo* Liquid Scintillation Counting

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A simple and concise analytical technique was developed for the determination of the kaliuretic properties of diuretic agents. The kaliuretic properties of furosemide and hydrochlorothiazide were compared in electrolyte and nonelectrolyte supplemented rats. ^{42}Potassium was employed as a tracer, and whole body liquid scintillation counting was utilized. The per cent of ^{42}K retention was determined for drug and control groups at various time intervals and comparisons were made for differences in potassium depletion due to drug action. Whole-body liquid scintillation counting was found to be applicable for the study of the kaliuretic properties of diuretic agents.

In the search for new and improved diuretics, the excretion and depletion of body electrolytes by these agents must be evaluated. Many new diuretic agents increase urinary potassium excretion and may cause hypokalemia. The technique of determining electrolyte content in urine samples by flame photometry is subject to complications and errors, such as the interference of trace ions present in the urine. There exists a need for the development of new and improved analytical techniques for the study of potassium excretion as affected by diuretic and allied medicinal agents.

The development of large-volume liquid scintillation counters such as the Purdue University Small Animal Counter (PUSAC) has made possible the detection and measurement of minute amounts of γ-emitting radioactivity administered to an intact test subject (1–3). Following equilibration of an isotope with normal body electrolyte, whole body radioactivity may be determined and subsequent measurements made over a period of time. Direct comparison of radioisotope retention in treated and in control animals allows simple evaluation of drug effect on the ion of interest. An alteration in the excretion of the tracer isotope, and thereby normal body electrolyte, may be noted easily as a change in the whole body radioactivity contained within the animal. Data obtained are indicative of the direct effect of a medicinal agent upon the excretion of the ion from the entire body of the animal.

This study dealt with the development of an *in vivo* tracer method for the investigation of potassium metabolism using ^{42}K in conjunction with whole body liquid scintillation counting. The effects of furosemide[1] and hydrochlorothiazide,[2] diuretic compounds, on potassium metabolism in the fasted rat were studied. In the first phase of study (*Experiment A*), the effects of the diuretic agents on potassium excretion in fasted animals not receiving electrolyte supplement were investigated. In the second phase of investigation (*Experiment B*), replacement electrolyte supplement (sodium and potassium) was administered to the fasted rats while studying potassium excretion as affected by the diuretic agents. The supplement was given to simulate the ingestion of electrolyte through food, as occurs in the human, and perhaps allow greater kaliuretic drug action.

EXPERIMENTAL

Experiment A.—The effects of furosemide and hydrochlorothiazide were investigated at dosage

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[1] Furosemide is 4-chloro-*N*-(2-furyl-methyl)-5-sulfamoylanthranilic acid. This compound was supplied by Lloyd Brothers, Inc., Cincinnati, Ohio, and is marketed as Lasix.

[2] 6 - Chloro - 3,4 - dihydro - 7 - sulfamoyl - 2H - 1,2,4-benzothiadiazine-1,1-dioxide. The compound was supplied by Merck Sharp & Dohme, West Point, Pa., as Hydrodiuril