References and Notes

1. E. C. Dougherty, E. L. Hansen, W. L. Nicholas, J. A. Mollett, E. A. Yarwood, *Ann. N.Y. Acad. Sci.* **77**, 176 (1959).
2. W. L. Nicholas, E. C. Dougherty, E. L. Hansen, *ibid.*, p. 278.
3. F. W. Sayre, R. T. Lee, R. P. Sandman, G. Perez-Mendez, *Arch. Biochem. Biophys.* **118**, 58 (1967).
4. F. W. Sayre, E. L. Hansen, E. A. Yarwood, *Exp. Parasitol.* **13**, 98 (1963).
5. M. Rothstein and P. Götz, *Arch. Biochem. Biophys.*, in press.
6. R. J. Cole, thesis, University of Maryland (1967); M. Rothstein, *Comp. Biochem. Physiol.*, in press.
7. E. C. Dougherty, *J. Parasitol.* **39**, suppl., 32 (1953).
8. K. Block, *Evolving Genes and Proteins*, V. Bryson and H. J. Vogel, Eds. (Academic Press, New York, 1965), p. 53.
9. E. L. Hansen, E. J. Buecher, Jr., E. A. Yarwood, *Nematologica* **10**, 623 (1964).
10. An analysis of the lipids extracted from active liver preparations has revealed that cholesterol is present [W. F. Hieb and R. P. Sandman, *Fed. Proc.* **26**, 797 (1967)].

11. We thank Dr. R. J. Harvey for the initial culture of *E. coli* (ML30 strain). Supported in part by PHS grants GM 13766 and AI 07145. W.F.H. is a predoctoral candidate on leave from the Department of Nutritional Sciences, University of California, Berkeley.

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Acetylation of Human Serum Albumin by Acetylsalicylic Acid

Abstract. *Human serum albumin is acetylated when exposed to acetylsalicylic acid (aspirin) under physiologic conditions in vitro. Indications are that a similar phenomenon occurs in vivo after ingestion of aspirin.*

Previous studies have shown that treatment of human serum albumin (HSA) with acetylsalicylic acid (aspirin) permanently enhanced the capacity of albumin to bind a marker anion, I^{131}-labeled acetrizoate (*1–4*). The treatment in vitro or in vivo of albumin by salicylic acid did not produce this effect. The permanent alteration of the capacity of albumin to bind the marker anion indicated that the structure of HSA was irreversibly altered by aspirin. We now report that aspirin acetylates HSA under physiologic conditions in vitro and that acetylation of HSA in vivo is probably responsible for the reported enhancement of acetrizoate binding observed in the serum of many patients (*1*) or normal subjects (*4*) ingesting aspirin in a therapeutic dosage.

The inherent chemical instability of the aspirin molecule, as exemplified by its spontaneous hydrolysis in water to acetic and salicylic acids, suggested that either the acetyl or the benzoate portion of aspirin might interact with HSA. Accordingly, HSA was treated with aspirin labeled with C^{14} either at the carboxyl group or at the acetyl group; sodium salicylate labeled with C^{14} at the carboxyl group was used as a control (*5*) (Fig. 1). Human serum albumin (Squibb, fraction V); 14 mg/ml, $2.0 \times 10^{-4}M$ was dialyzed against $0.10M$ sodium phosphate buffers, *p*H 5.0 through 10.0. Duplicate 2.0-ml samples of these albumin preparations were added to 2.0 ml of $1.0 \times 10^{-3}M$ solutions of aspirin labeled in the carboxyl or acetyl position, or of sodium salicylate labeled in the carboxyl position. The final reaction concentrations of the salicylates were chosen to be comparable to therapeutic blood levels. The mixtures were incubated at 37°C for 24 hours and then were dialyzed at 4°C for 48 hours against multiple changes of $0.15M$ NaCl containing $0.01M$ sodium salicylate as carrier to remove ionically bound aspirin. No radioactivity was detected in the final dialyzate. Duplicate 0.10-ml samples were counted for C^{14} activity in a Packard Tri-Carb liquid scintillation counter. Samples of 1.0 ml were dialyzed against three 1-liter volumes of $8M$ urea (*p*H 5.5) or $6M$ guanidine (*p*H 6.4) at 25°C for 48 hours, against $0.10M$ NaH_2PO_4 for 24 hours, and then against phosphate-buffered saline, *p*H 7.0, for 24 hours. The samples treated with urea and guanidine were counted for C^{14} activity. Protein concentrations were determined before and after treatment with urea or guanidine by the micro-Kjeldahl method of nitrogen determination. In addition to the above, duplicate 1.0-ml samples were dialyzed free of the sodium salicylate with borate-saline buffer, *p*H 7.4 and acetrizoate binding was determined by equilibrium dialysis with $1.0 \times 10^{-7}M$ I^{131}-labeled acetrizoate (*6*).

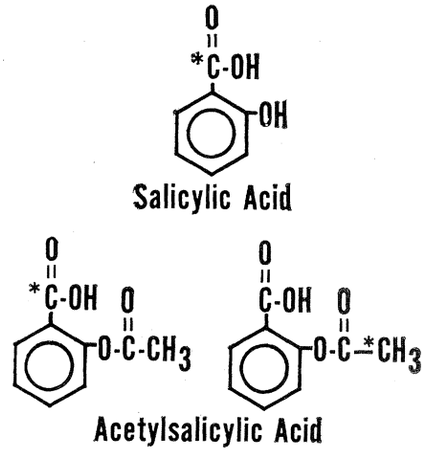

Fig. 1. Structural formulas of salicylic acid and acetylsalicylic acid showing position of carbon-14 label (*).

Human serum albumin treated with aspirin labeled in the carboxyl position retained a maximum of less than 2 moles of C^{14}-carboxyl per 100 moles of HSA (Table 1). It is possible that this extremely low binding could be due to the formation of a salicylamide derivative (*7*); alternatively the <1 percent trace contaminant that may have been in the aspirin labeled in the carboxyl position could account for this binding. The C^{14} activity of HSA treated with salicylic acid labeled in the carboxyl position was negligible. In striking contrast, the HSA treated with aspirin labeled in the acetyl position had significant C^{14} activity, indicating that acetylation of HSA occurred. The degree of acetylation was identical before and after treatment with either $8M$ urea or $6M$ guanidine and varied with the *p*H of the reaction mixture, tending to increase with decreasing hydrogen ion concentration. At *p*H 7.3 (that is, physiologic *p*H) an average of 1.2 acetyl groups were covalently bound per molecule of HSA, and a maximum binding of three acetyl groups per molecule was observed at *p*H 9 to 10. The degree of acetylation of HSA was determined by assuming that 100 percent of the C^{14} activity was associated with HSA rather than with a trace serum contaminant in the purified fraction V preparation. This assumption was verified by autoradiography of an immunoelectrophoretic pattern of HSA acetylated with acetylsalicylic acid-1-C^{14}(acetyl). Carbon-14 activity was associated with the entire albumin line and not with other serum proteins (Fig. 2), even when subjected to electrophoresis with whole normal human serum as carrier.

Binding of the marker anion, acetrizoate, was similarly enhanced by treatment with aspirin labeled in either the carboxyl or acetyl position. The acetrizoate data obtained with the latter is presented in Table 1. Near maximum binding was seen at *p*H 7.0, where there was an average of one acetyl group per HSA molecule. Despite the addition of two more acetyl groups at *p*H 9.0, there was a minimum increase in acetrizoate binding.

The striking difference in residual C^{14} activity which remained on the HSA molecule after exposure to aspirin labeled in the acetyl position as compared with aspirin labeled in the carboxyl position indicated that aspirin

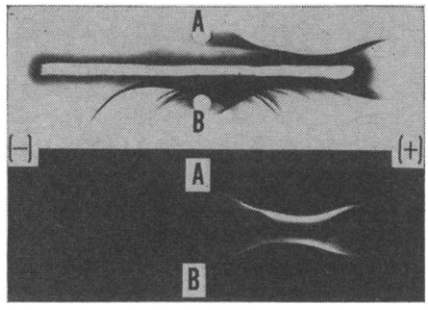

Fig. 2. Immunoelectrophoretic pattern (top) and autoradiograph (bottom) of acetyl-1-C^{14}-HSA. (A) Acetyl-1-C^{14}-HSA; (B) acetyl-1-C^{14}-HSA mixed with normal human serum carrier. Immunoelectrophoretic pattern developed with goat antiserum to whole human serum (Hyland).

acetylated HSA and that this structural alteration was responsible for enhanced acetrizoate binding. To test further the premise that acetylation alone could increase acetrizoate binding, we acetylated HSA with acetic-l-C^{14}-anhydride, and the resulting acetyl-HSA, with one to ten acetyl groups per HSA molecule, bound increased amounts of acetrizoate comparable to albumin acetylated by aspirin (*8*). Since there is a definite cause and effect relationship between ingestion of aspirin and enhanced acetrizoate binding by HSA (*4*) and since sodium salicylate does not enhance acetrizoate binding in vitro or in vivo, we postulate that ingestion of aspirin results in the acetylation of HSA in vivo.

The possible physiologic consequences of acetylation of HSA by aspirin are not known. The gamma G globulin class of immunoglobulins in the serums of many apparently normal persons as well as patients with a variety of disease states can bind I^{131}-labeled HSA previously treated with aspirin (*9*). Highly acetylated rabbit serum albumin has an increased catabolic rate and can induce the formation in the rabbit of antibodies to the acetylated albumin (*10*). In addition, the increased affinity for acetrizoate of albumin acetylated by aspirin suggests that the transport of biologically important anions structurally related to acetrizoate may be altered in vivo (*2*). Furthermore, preliminary experiments indicate that aspirin acetylates still other human serum proteins in vitro (*11*).

It remains to be determined whether aspirin can acetylate in vivo host components other than serum albumin. Should this prove to be the case, an even greater variety of physiologic or pathologic results might be expected. There is an increasing awareness that ingestion of aspirin is sometimes associated with a number of untoward reactions. Such reactions to aspirin are found in one-fifth as many instances as penicillin reactions (*12*); this figure probably substantially underestimates the incidence of these reactions inasmuch as "aspirin intolerance," one of the more common reactions, is frequently not causally related to aspirin either by patients or by physicians. The syndrome of "aspirin intolerance," characterized by asthma, rhinitis, and nasal polyps (*13*), is induced by ingestion of aspirin but not sodium salicylate. The pathogenesis of this syndrome is unknown. The capacity of aspirin, but not of sodium salicylate, to acetylate serum proteins is viewed as indicating that acetylation is likely to play a role in "aspirin intolerance."

In addition to the well-established syndrome of "aspirin intolerance," aspirin ingestion has been implicated in a variety of other human pathologic states, such as abnormal renal function in rheumatoid arthritis, analgesic nephropathy, massive gastric hemorrhage, abnormal hemostasis due to hypoprothrombinemia and impaired platelet function, pancytopenia, and encephalopathy. In view of the widespread and often indiscriminate use of aspirin by children and adults, and because the consequences of acetylation of serum proteins by aspirin remain unclear, the biologic significance of this property of aspirin warrants further investigation.

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Table 1. Binding of acetyl-1-C^{14}-aspirin and carboxyl-C^{14}-aspirin to HSA at *p*H 5.0 to 10.0. The ratios of bound acetrizoate to free acetrizoate shown are those of HSA treated with acetyl-1-C^{14}-aspirin. Control HSA had a ratio of 5.32 to 6.33 after previous dialysis at *p*H 5.0 to 10.

Reaction mixture *p*H	Acetyl-C^{14} (mole/mole of HSA)			Carboxyl-C^{14} (mole/mole of HSA)		Ratio of bound to free acetrizoate
	After dialysis	After urea	After guanidine	After dialysis	After urea	
5.0	.32	.31	.31	< .01	< .01	7.85
	.45	.44	.43	< .01	< .01	7.25
6.0	.34	.35	.34	< .01	< .01	10.9
	.37	.36	.34	< .01	< .01	10.4
7.0	.90	.95	.89	.010	.010	15.5
	.95	.93	.96	.010	.010	15.1
7.3	1.23	1.23	1.26	.010	.010	
	1.23	1.22	1.27	.011	.010	
8.0	2.15	2.20	2.10	.013	.014	15.9
	2.08	2.20	2.06	.013	.012	15.9
9.0	3.02	2.82	3.53	.015	.015	16.4
	3.12	3.24	3.20	.014	.015	16.2
10.0	2.94	3.08	3.05	.010	.012	15.1
	2.99	2.97	3.03	.013	.013	14.4

References and Notes

1. R. S. Farr, G. P. Rodnan, E. C. Lasser, *J. Clin. Invest.* **40**, 1037 (1961); G. P. Rodnan, P. W. Garver, R. S. Farr, *Arthritis Rheum.* **5**, 316 (1962).
2. R. T. Reid and R. S. Farr, *Arthritis Rheum.* **7**, 747 (1964).
3. R. S. Farr, R. T. Reid, P. Minden, *J. Clin. Invest.* **45**, 1006 (1966).
4. P. K. Hench and R. S. Farr, *Arthritis Rheum.* **10**, 283 (1967).
5. Salicylic-7-C^{14} acid; specific activity 2.31 mc/mmole, radiochemical purity > 99 percent, Lot No. 277-192 purchased from New England Nuclear Corporation, Boston, Mass. Acetylsalicylic-carboxyl-C^{14} acid; specific activity 0.69 mc/mmole, radiochemical purity > 99 percent, Lot No. 277-199 purchased from New England Nuclear Corporation, Boston, Mass. Acetylsalicylic-acetyl-1-C^{14} acid; specific activity 2.38 mc/mmole, radiochemical purity > 99 percent, provided by Merck Sharp and Dohme Research Laboratory, Rahway, N.J.
6. R. N. Pinckard and D. M. Weir, in *Handbook of Experimental Immunology*, D. M. Weir, Ed. (Blackwell, Edinburgh, 1968), p. 493.
7. M. A. Schwartz and G. L. Amidon, *J. Pharm. Sci.* **55**, 1464 (1966).
8. R. N. Pinckard, D. Hawkins, R. S. Farr, *Fed. Proc.* **27**, 543 (1968).
9. P. Minden and R. S. Farr, *Arthritis Rheum.* **10**, 299 (1967).
10. J. S. Ram and P. H. Maurer, *Arch. Biochem.* **72**, 119 (1957).
11. R. N. Pinckard, D. Hawkins, R. S. Farr, in preparation.
12. Registry of Adverse Drug Reactions. Report of the Drug Reaction Registry Subcommittee of the Great Philadelphia Committee for Medical-Pharmaceutical Sciences, *J. Amer. Med. Ass.* **203**, 85 (1968).
13. M. Samter and R. F. Beers, Jr., *J. Allergy* **40**, 281 (1967).
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