These results indicate that the masking of radiosensitive groups by specific chemical reactions may be a more effective way of reducing certain effects of ionizing radiation than the addition of free radical acceptors (*5*).

F. E. LITTMAN
E. M. CARR
J. K. CLAUSS

Stanford Research Institute,
Menlo Park, California

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24 January 1957

Effect of Multiple Recrystallization on Derivatives of Mixtures of D- and L-Alanine

Among the problems encountered in tracer studies with biological systems is the difficulty of isolating a compound that is present in minute amounts. To reduce the difficulty of this problem, it is a common practice to add as carrier the non-labeled material sought and then to proceed with the isolation. As a matter of convenience, the DL racemate is sometimes added, although the compound sought may be solely of the D or L configuration. A question arises about the degree of significance of the isomeric nature of the carrier. This is of particular importance if the radioactivity of the isolated material is to be interpreted as representative of the activity of the compound as it exists in the biological preparation.

In the same regard, it is often desirable to determine the amount of the compound in the system. One manner in which this is done involves establishment of the isotope dilution. Again, the significance, if any, of the isomeric form may become an important consideration. This is especially true if the compound is to be isolated as a derivative. It was from interest in the latter case that the present investigation (*1*), which employed a particular system involving the isolation of alanine as the azobenzene sulfonate derivative, was initiated.

Table 1. Radioactivity (count/min) of alanine azobenzene sulfonate.

No. of recrystallization	Source of derivative		
	Parent DL-alanine	Diluted DL-alanine	1/3 D- to L-alanine
1	113	55	52
2	112	56	49
3	110	57	45
4	109	56	42
6	112	56	39

The magnitude of changes in activity during multiple recrystallization of derivatives of alanine having equal activity but differing in the ratios of the D to L isomers has been investigated. Other derivatives prepared from alanine of the same and of unequal amounts of D and L configuration have also been studied.

Two mixtures of alanine were prepared from a parent source of racemic alanine-1-C^{14}. The first was prepared by diluting the radioactive material with an equal amount of DL-alanine. The second mixture contained equal amounts of the radioactive racemate and L-alanine. The mixtures were dissolved in water to insure complete mixing and were then precipitated by the addition of ethanol. In this manner, two alanine mixtures were obtained with equal radioactivity contributed from the D and L isomers, but with different total proportions of the isomers. The first mixture was racemic, while the second contained the D and L isomers in a ratio of 1/3. The alanine salt of azobenzene-*p*-sulfonic acid (*2*) was prepared from each of these mixtures as well as from the parent racemic alanine. The salts were recrystallized once from water, dried, and assayed for radioactivity. Each of the compounds was subsequently recrystallized several times from water and counted following each recrystallization.

The radioactivity was measured on pressed solid mounts of the derivative plated at infinite thickness. Recrystallized samples were mounted in the same aluminum planchet to maintain identical counting-surface area. All counts were made with a thin end-window Geiger-Müller tube. The values reported are expressed as total counts per minute for the sample less background. Since the same derivatives are compared, these values are both proportionate to the activity per milligram and per millimole of derivative. The results of these determinations are shown in Table 1. The activities of the salts of the parent and diluted racemate did not change, while the activity of the 1/3 D- to L-alanine derivative steadily decreased. Based on the mean activity for the salt of the parent alanine, the salt of the racemate after six recrystallizations had 100 percent of the theoretical activity, while the activity of the 1/3 D to L mixture had dropped to 70 percent.

Another alanine mixture was prepared in the manner already described in which the D- and L-isomers were present in a ratio of 2/3, respectively. From this mixture, the phenyl isocyanate (*3*) and *p*-toluenesulfonyl (*4*) derivatives were prepared. After five recrystallizations, neither of these derivatives showed a change in radioactivity. The fact that no apparent shift in activity was observed may be dependent on the nature of the derivatives or the concentrations of the two isomers, which in this case were more nearly equal.

As a final experiment, two alanine mixtures were prepared from a source of racemic alanine-1-C^{14} in such a manner that one contained the isomers in a ratio of 1/3 D to L and the other 1/3 L to D. The mixtures had equal amounts of C^{14} in each isomeric form. Again the azobenzene-*p*-sulfonic acid salt was made from each of the mixtures and recrystallized. In Fig. 1 are shown the changes in radioactivity with subsequent crystallizations. As a control, the same derivative was prepared from a DL-alanine-1-C^{14} source and was likewise subjected to recrystallization. The data are included in Fig. 1. The radioactivity of the 1/3 D to L and the 1/3 L to D derivatives showed a consistent and similar decrease. The results with the alanine azobenzene sulfonates may be explained in each case by an enrichment of the isomer originally present in the larger amount.

The isomeric nature of the carrier appears to be worthy of consideration in the preparation of a derivative from an optically active compound. The degree of importance varies with the derivatives of choice, the number of crystallizations, and the ratio of the D and L isomers. In many situations, analytic results will not be affected to a significant degree by excessive crystallization. However, the im-

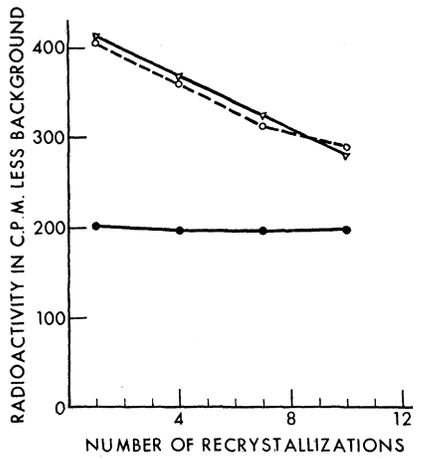

Fig. 1. Effect of successive recrystallization on the radioactivity of alanine azobenzene sulfonates; △, derivative prepared from 1/3 D- to L-alanine; ○, derivative from 1/3 L- to D-alanine; ●, derivative from a racemic mixture.

portance is intensified in certain instances and should be considered in any situation where multiple recrystallization is to be encountered.

DONALD S. WIGGANS
WILLIAM W. BURR, JR.
Biochemistry Department,
University of Texas,
Southwestern Medical School, Dallas

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Changes of Thixotropic Behavior in Actomyosin Solutions Induced by Cardiac Glycosides

Actomyosin solutions are thixotropic, which literally means that they "change by touching." An internal structure formed by the fibrous protein molecules is partially broken by agitation of the fluid, and, for example, the viscosity is accordingly diminished. As long as the flow remains constant, the structure will not reform. As soon as flow stops, thixotropic structure is built up again.

For measuring changes in viscosity, the thixotropy has to be kept at a steady state. In making these measurements with an Ostwald viscosimeter, we found that numerous active and inactive cardiac glycosides reduce the viscosity of actomyosin solutions parallel to the degree of biological fixation on heart muscle. The binding of some glycosides to actomyosin was proved by ultrafiltration and determination of the dissociation constants. One receptor group per molecule of actomyosin (molecular weight taken as 10^6) was found, probably belonging to myosin (*1*).

The thixotropy of actomyosin that had been extracted from rabbit skeletal muscle was studied in a rotational viscosimeter at 0°C. Eighteen milliliters of protein solution (3 mg/ml in Weber-Edsall fluid with barbiturate buffer) was placed in a narrow cylindrical slit in which another cylinder was rotated with constant speed. The breaking force on the cylinder exerted by the viscous solution was counteracted by a spring and was measured on a dial. It was proportional to the viscosity of the fluid.

We measured the coefficient of "thixotropic breakdown with time" (*2*):

$$B = \frac{U_1 - U_2}{\ln \frac{t_2}{t_1}}$$

where U_1 is the viscosity at time t_1 and U_2 is the viscosity at time t_2. The times t_1 and t_2 were 1 and 10 minutes, respectively. Both the coefficient for actomyosin (B_{AM}) and for actomyosin of the same concentration with 10^{-6} *M* glycoside (B_{AMG}), were determined. No alcohol was used to increase solubilities, for we found that even traces of alcohol caused fundamental changes in actomyosin. The difference in thixotropy ($B_{AM} - B_{AMG}$) was then compared with the biological activity (see Fig. 1). Potency is expressed as reciprocal of lethal Hatcher dose (moles per kilogram) determined in cats by W. R. Schalch of Sandoz AG. Basel. The probability that Δ-thixotropy ($B_{AM} - B_{AMG}$) is significant (< 0.02 to 0.05) was computed from the variances of the determinations of $B_{AM} - B_{AMG}$ using the *t* distribution.

There exists an interesting correlation between these two properties. Cardioactive glycosides diminish the thixotropy, whereas biologically inactive glycosides (potency = 0) augment it. Activities of lanataglycosides are approximately proportional to the decrease of thixotropy. In three cases (open bars in Fig. 1: digitoxin, acetyldigitoxin α, and acetyldigoxin α) in which the ion concentration in the solution was diminished by adding the glycosides in water instead of buffer solution, the effect was larger than was expected from the biological activities. This may be connected with the poor water solubility of these three glycosides, or else with the decrease of the ion concentration. A change in the active ion concentration or some other charge phenomenon along the actomyosin fiber may be responsible for the observed effect. Preliminary studies on the dialysis of actomyosin in 0.6*M* K solution have shown the concentration of free potassium ions to be diminished by adding 10^{-6} *M* glycosides (lanatosid A, B, C, scillaren A, cymarin, and strophanthin) but not by the inactive glycosides hexahydroscillaren A and allocymarin.

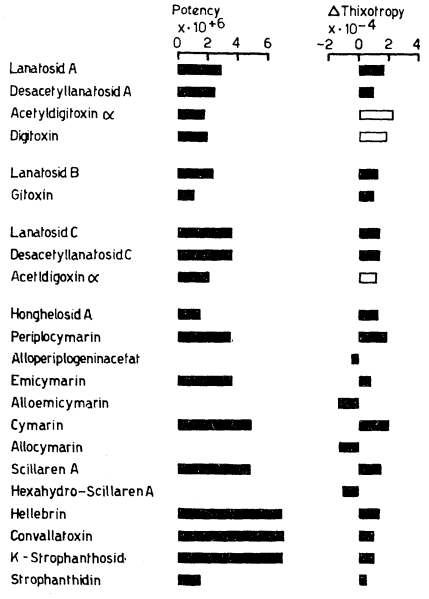

Fig. 1. Comparison of difference in thixotropy ($B_{AM} - B_{AMG}$) with biological activity.

It is therefore possible that there exists a direct mode of action of cardiac glycosides on actomyosin within the cell and not only in the cell membrane phase, as was suggested by previous workers (*3*).

PETER G. WASER
Department of Pharmacology,
Medical School, University of
Zürich, Zürich, Switzerland

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Withdrawal Hyperexcitability Following Chronic Administration of Meprobamate to Mice

Because of known side effects and potential "behavioral" toxicity, concern over the indiscriminate use of the so-called "tranquilizing agents" is readily justified. However, the added possibility that these drugs might also possess addiction liability has been little, if at all, explored. Recently, Lemere (*1*) reported that one patient who took 6.4 g of meprobamate (Equanil, Miltown) daily for 30 days exhibited a grand mal convulsion 10 hours after therapy was discontinued. In view of this report, the effects of the chronic administration of this agent and its abrupt withdrawal on the excitability of the central nervous system of mice, by use of the experimental design developed by McQuarrie and Fingl (*2*), were studied.

Since this investigation (*3*) was planned merely to determine whether tolerance develops during the chronic administration of meprobamate and whether abrupt withdrawal is followed by increased excitability of the central nervous system, only high dose levels were employed. This choice of dosage was justified also on the bases that large amounts are taken by patients who abuse drugs and that excessive quantities are usually necessary to demonstrate physical dependence on ethanol and barbiturates in man (*4, 5*). It must be emphasized that final assessment of the addiction liability of any drug must be based on quantitative comparison of the dosage schedules that induce withdrawal phenomena with those that are required to obtain therapeutic results.

Male albino mice (Carworth Farms, CF #1 strain, 25 to 35 g in weight) were