Table 1. Effect of diphenhydramine plus pentobarbital on sleeping time of mice (probability < 0.01).

Agent	Avg. sleeping time (min)
Diphenhydramine	0.0
Pentobarbital sodium	35.0
Diphenhydramine plus pentobarbital sodium	52.3

Table 2. Effect of diphenhydramine plus strychnine on mice.

Agent	No. fell (per 25)	No. died (per 25)
Diphenhydramine	0	0
Strychnine	8	4
Diphenhydramine plus strychnine	24	19

Subsequent to the publication of reports concerning these clinical and experimental observations, several investigators described the potentiation of barbiturate sleeping time in mice following prior administration of relatively small quantities of Benadryl (*4*). In studies directed toward the elucidation of a CNS site of action for the compound, it was observed that Benadryl would also cause a reappearance of spontaneous convulsive activity in the cat when it was administered on the decay curve of a strychnine-induced convulsion (*5*).

Recent experiments in this laboratory have shown that pretreatment of mice with a given quantity of Benadryl, which, by itself, effects no apparent alteration in the appearance or activity of the animals, will enhance the gross CNS effects of either pentobarbital or strychnine. The methods employed were as follows.

Groups of ten male white mice, NIH strain, weighing 18 to 22 gm each, were randomly divided into subgroups of five mice each. One subgroup of each group was given 20 mg/kg of Benadryl hydrochloride by subcutaneous injection, while the other functioned as a control group. Twenty-five minutes later the stimulant or depressant drug was administered to all ten animals in each group by intraperitoneal injection.

The mice that received 1.0 mg/kg of strychnine sulfate were then placed on a slanting wire screen, 0.25-in. mesh, and the numbers falling after development of convulsive activity in each group were determined, the end-point being the fall resulting from the loss of ability to cling to the screen. For those animals that received the barbiturate (50 mg/kg pentobarbital sodium), differences in sleeping times were ascertained, the return of the righting reflex being the end-point and the time being taken from the barbiturate injection.

The results are summarized in Tables 1 and 2. The disparity between the two groups that received strychnine in the number of animals dying was undoubtedly influenced to an unknown degree by the effect of the fall itself, so that this difference is probably not as important as the figures would indicate.

These studies demonstrate that diphenhydramine, at a single dosage level that, by itself, exerts no gross effect, can enhance the CNS activity of two compounds long recognized as being pharmacological antagonists. In an attempt to account for this diphenhydramine activity, two modes of action might be considered. The first would involve an inhibition of the biotransformation of the barbiturate and strychnine, similar to the action of β-diethylaminoethyl diphenylpropyl acetate hydrochloride (SKF 525-A) (*6*) to whose structure diphenhydramine bears considerable resemblance. The second possible mode of action would postulate a more direct effect on elements of the CNS, resulting in alterations in levels of neuronal activity that, in turn, could quantitatively affect the response to the second drug.

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Fractionation of Acrasin, a Specific Chemotactic Agent for Slime Mold Aggregation

The aggregation stage during slime mold development is a device whereby the individual myxamebas are brought together in a compact mass preliminary to the formation of the organized fruiting body. Several workers have shown (*1–3*) that the process is chemotactic and that the aggregative center and the cell streams radiating therefrom produce a specific attractive material that was given the generic name *acrasin* by J. T. Bonner (*2*).

Sometime ago, B. M. Shaffer obtained leachings from cells in postaggregative stages that could cause dispersed myxamebas to aggregate in the direction of the point of application (*3*). A small, early aggregate on a thin agar layer was dispersed mechanically. A tiny square plug of agar was cut out and inverted over a glass slide in a moist chamber. By these means, the dispersed cells existed in a film of water between the slide and the agar and adhered to the glass. Very small drops containing leachings from a single pseudoplasmodium were applied at short intervals to the meniscus around the plug and within 5 minutes evoked pronounced elongation and streaming of the test cells toward the point of application. Shaffer found that the attractive material was highly unstable and lost all activity within 15 minutes at room temperature, although, when it was frozen, it remained active for longer periods of time.

We can now report (*4*) the stabilization of acrasin and its fractionation into two components, neither of which attracts cells when alone but only in combination.

Dictyostelium discoideum was grown on glucose peptone agar with *Aerobacter aerogenes* by methods previously described (*5*) and incubated until the cells had aggregated. The entire plate contents were dumped into cold dilute HCl at *p*H 3.5. A clear solution was obtained by decantation and centrifugation. This solution, when neutralized, was shown to possess acrasin activity by the Shaffer procedure. Washings obtained from cells prior to aggregation or a long time thereafter were biologically inactive, as were preparations obtained by leaching at *p*H 7.

The washings at *p*H 3.5 retained activity indefinitely when they were kept in the acid condition but lost activity rapidly upon neutralization. The preparations were concentrated by vacuum distillation at 50°C, the *p*H being held constant at about 3.5 by periodic additions of base. The 200-fold concentrate was put through a loosely packed cellulose powder column with *p*H 3.5 HCl. A fraction of the eluate, distinguished by its fluorescence in the ultraviolet, showed strong biological activity and was stable thereafter at neutrality. The neutralized solution was taken to dryness and provided a fine yellow powder. The yield was 150 mg from 16 growth plates, each containing about 10^9 myxamebas.

Samples containing 6 mg of crude powder were run in ascending bar chromatograms on Whatman No. 1 paper cylinders (15 in. wide) with 80-percent ethanol. Two strongly fluorescent bands appeared with R_f values of 0.3 (fraction A) and 0.1 (fraction B). These were eluted with water and tested separately and in combination by the Shaffer procedure. Table

Table 1. Biological activity of fractions A and B after paper chromatography. The number of plus signs is a rough measure of the intensity of the response.

Final concn. (mg/ml)		Absorbance at 265 mμ		Biological activity
A	B	A	B	
1.3	0	.560		–
1.3	0.7	.560	.280	++++
1.0	1.0	.420	.420	++
0.7	1.3	.280	.560	–
0	1.3		.560	–

Table 2. Inactivation of acrasin by neutral extract (NE). The acrasin solution contained 3 mg/ml of crude powder and was mixed with neutral extract in the ratio of 10:1 (vol/vol).

Mixture	Biological activity	
	Zero time	4 hr
Acrasin + neutral extract	+++	–
Acrasin + boiled neutral extract		++
Acrasin + H_2O		++
H_2O + neutral extract		–

1 summarizes the data from one separation. Note that the relative proportions of the two components were critical for demonstration of biological activity. The absolute amounts are also important since excessive concentration or dilution of the mixture yielded negative results. Too great a supply of the agents would tend to diminish the relative concentration gradients in the system. Previous results (*2*) do indicate that the myxamebas depend on a gradient in order to know in which direction to move.

The A and B fractions have also been detected by spot chromatography, using a water-butanol-ethnol (50 : 30 : 17) solvent. The R_f values were 0.48 and 0.42, respectively.

The reason for the instability of acrasin in the crude state has been investigated. At the outset of this study, the most likely possibility was held to be the existence of an extracellular enzyme that catalyzes the destruction of acrasin. It was upon this preconception that we chose the extraction procedure with the expectation that *p*H 3.5 might be either in the isoelectric range of the enzyme or else far removed from its *p*H optimum. Subsequent experiments, while admittedly not crucial by rigorous biochemical criteria, strongly support this view.

To obtain the hypothetical enzyme, cells from eight growth plates were extracted with 50 ml of ice water (hereafter called neutral extract). A solution of crude powder from the acrasin concentrate (3 mg/ml) was mixed 10 : 1 with the neutral extract and incubated at 25°C. The mixture and appropriate controls were assayed for biological activity by the Shaffer procedure and were run in bar chromatograms with 80-percent ethanol. Table 2 shows the data. Although it was initially active, the mixture lost all activity after 4 hours of incubation. In contrast, boiled neutral extract failed to inactivate the crude powder. The chromatograms revealed that the inactivated acrasin had lost its B fraction while the A fraction had increased in quantity.

The neutral extract was subjected to ammonium sulfate precipitation. The precipitate that was brought down by adding between 50 and 55 percent ammonium sulfate was washed and redissolved in water. This preparation could inactivate the crude powder precisely as described in the previous paragraph, and it also caused the disappearance of the B fraction. Further, the crude powder that had been inactivated by these means was mixed with additional B fraction which had been eluted from paper chromatograms. The Shaffer test revealed that inactivated powder plus B fraction was active, while inactivated powder plus A fraction was not active. Thus the putative enzyme destroyed the B fraction of acrasin but left the A fraction intact.

Preliminary experiments have been performed on the partially purified A and B fractions themselves. The neutral extract was shown to have no effect on fraction A, but it converted fraction B into something that was identical with fraction A, both biologically and chromatographically.

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Properties of Acrasin

Although it had been suspected for nearly half a century that aggregating amebas of the cellular slime mold *Dictyostelium* oriented to a chemical gradient, Bonner (*1*) was the first to demonstrate this convincingly; he named the chemical *acrasin* but was unable to isolate it *in vitro*. Amebas could be made to orient to the gradient set up by an artificial source, which consisted of the cell-free liquid obtained from the immediate surroundings of a natural source, once a method had been devised for renewing it at frequent intervals with this liquid, if it was freshly collected (*2*). At room temperature, this acrasin solution lost its activity within a few minutes, an instability that was mainly responsible for the chemical's elusiveness.

In an experiment to confirm Runyon's finding (*3*) that the stimulus to aggregation would pass through cellophane dialysis membrane, natural sources were grouped on a raft of this material and liquid was periodically added to, and collected from, the underside of it; I found that it oriented the amebas; moreover, this cell-free acrasin solution, unlike that obtained earlier by filtering through a Millipore membrane, was stable. I concluded that acrasin activity was associated with molecules small enough to pass rapidly through cellophane and that loss of activity was due to reaction with larger molecules, possibly those of an enzyme, also present extracellularly.

During my tenure of a Dill fellowship at Princeton University in 1954, I was able to develop a procedure for extracting acrasin chemically. Cold methanol was poured over culture plates in which aggregation was general; this was dried in a vacuum at – 10°C, and the residue was extracted with a small volume of methanol, which was then filtered and again dried. An aqueous solution of the product had a very high acrasin activity, which was retained after boiling for more than 1 hour and after exposure to 0.1*N* hydrochloric acid or 0.01*N* sodium hydroxide. The solubility of dry acrasin decreased rapidly in passing up the alcohol series.

A cell-free aqueous extract of an aggregating culture had the power to inactivate acrasin but lost it after boiling: the fraction that could be precipitated with ammonium sulfate was shown to be responsible. These findings made it probable that the original instability was due to an extracellular enzyme (*4*).

The action of acrasin was not confined to the orienting of sensitive amebas: it also caused them to secrete acrasin. The functional significance of this is clear: it is the basis of a chain reaction that permits chemotactic attraction to operate over distances much greater than those over which diffusion processes are normally effective in biological systems.

Sussman and Noel (*5*) thought it likely that the capacity to initiate a center of aggregation was not identical with the ability to produce acrasin during aggregation; it now appears that the essential thing for initiation is for some cell or cell group to be able to start the production of acrasin and so to excite the system of acrasin relays. The rhythmic movements during aggregation described