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Effect of Sulphydryl Compounds and dl-Alanine on Rate of Development of Eggs of Physa and Lymnaea.

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Hammett[1, 2, 3] has recently proposed that the sulphydryl (–SH) group is "an essential stimulus to cell proliferation." He developed the idea from experiments in which an increased growth-rate by means of accelerated cell-division occurred in Zea mays root tips and in Paramecium as a result of treatment with sulphydryl compounds. In later work, Hammett and Reimann[4, 5] found similar compounds stimulating cell proliferation in the wounds of rats and man.

By making gross length experiments, mitotic counts and cell measurements, Hammett found that treated Zea root tips grew more rapidly than those of control plants and that this was the result of more rapid cell division rather than an increment in cell size. In Paramecium he also reported a markedly increased division rate in the treated organisms. His controls were grown in culture solutions prepared with equivalent concentrations of some related substance lacking the –SH group. For instance, his experiments on thio-glycollic acid (with some exceptions) were controlled with glycollic acid, and those with cysteine were controlled with alanine. In this way he sought to demonstrate the specificity of –SH as the stimulating factor. His results were very consistent and in every case where the reduced –SH grouping was present in a certain optimum range of concentration he found marked acceleration of division. Thio-glycollic acid, Na-dithio-diglycollate, cysteine, glutathione and cystin (to a slight extent) were reported as effective compounds in stimulating cell division. On the basis of these experiments Hammett stated the hypothesis that the –SH group is universally a "cell division hormone."[1]

Inasmuch as this hypothesis, if true, is one of profound biological

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1 Hammett, F. S., *Protoplasma*, 1929, **7**, 297.

2 Hammett, F. S., *Proc. Am. Phil. Soc.*, 1929, **68**, 151.

3 Hammett, F. S., *Arch. Path.*, 1929, **8**, 575.

4 Hammett, F. S., and Reimann, S. P., *J. Exp. Med.*, 1929, **50**, 445.

5 Reimann, S. P., and Hammett, F. S., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 20.

import, I set out to test the effect of some of these compounds on the development of the fresh water snails, *Physa heterostropha Say,* and *Lymnaea columella Say.* This is desirable material on which to work because the eggs can be obtained immediately after laying in the one-celled state and normally the rate of development of all the eggs of any one laying is remarkably constant. The egg masses can be cut into 2 or 3 pieces and each piece given a different treatment, with the assurance that under similar conditions they would all hatch at very nearly the same time.

Two criteria as to the relative rate of development of the controls and the treated eggs were tried. The first few cleavages may be followed by actual count, and with a small series this index was used. But since it was only a matter of a few hours until there were too many cells to count, this method was abandoned as inadequate. The time of hatching of treated and of control embryos was used as a criterion. This permitted treatment for 6 or 7 days, under laboratory conditions. An embryo was described as hatching when it had broken through the egg membrane. This entails microscopic examination, because after hatching an embryo may remain for some time on the egg mass and appear unhatched to the naked eye. To divide an egg mass into exactly 2 equal quantities was very difficult, consequently they were approximately halved and the average hatching time in any one solution was recorded as the hatching time of the mass.

The principal sulphydryl compound used was cysteine, procured fresh from the Eastman Kodak Company laboratories at frequent intervals. This product comes as cysteine hydrochloride. Concentrations ranging from 10^{-3} to 5×10^{-9} g. sulphur per cc. as cysteine were tried in about 200 experiments.

A mass of eggs in a 1-, 2- or 4-celled stage would be cut in two and one part placed in filtered stream-water and the other part dropped into a known concentration of the –SH compound. The –SH solutions were made up in the filtered stream-water and both control and test solutions identically buffered with phosphate buffers to a pH of 6.8. Fresh solutions were made daily and the eggs changed to 15 cc. of the freshly-prepared solution each day. This guarded against the instability of the compounds and prevented any accumulation of waste products. Care was taken that in the preparation of the solutions they should not become alkaline because of the well-known instability of the –SH group in alkaline solutions. The cultures were kept in a closed cupboard in an evenly heated

room so that any difference in temperature between control and test cultures was negligible.

It was found that a concentration of cysteine of 5×10^{-5} g. S. per cc. was slightly but definitely toxic. The more concentrated the solution the greater the toxicity noted. According to Hammett, the stimulating range of concentration is to be found in a dilution just below the toxic concentration. We were unable, however, to find any concentration in which the eggs would hatch faster than in the control cultures. In solutions diluted below the toxic level, control and test eggs developed almost simultaneously and any difference in hatching time was easily within the range of individual variation. This was in marked contrast to the precision of results noted when a slightly toxic culture solution was used, where although the treated eggs might lag in hatching by only a few hours an almost invariable difference was found.

In view of these negative findings Dr. Hammett was consulted and he suggested that reliable results could not be obtained unless the control solutions were made up with an equivalent concentration of a related compound lacking –SH. Consequently dl-alanine was obtained from Eastman Kodak Company and a series of experiments run, in which the alanine, made up in stream-water, was used as controls. This, too, was buffered to a pH of 6.8 as the previous controls had been.

In this series the cysteine-treated eggs hatched faster than the alanine-treated controls when a concentration of 4×10^{-5} g. S. per cc. or stronger was used. In other words, if the controls were adequate there was an actual stimulation of development by treatment with cysteine. But it was also noted that this apparent stimulation was marked in the series where cysteine to a concentration of 5×10^{-5} g. S. per cc. was used. And previous experiments had shown that such a concentration was slightly toxic when compared to stream-water controls. Therefore, it seemed probable that both substances were toxic, but one slightly more so than the other.

That such was the case was easy to demonstrate in another series of experiments in which egg masses were divided into 3 parts and one part cultured in cysteine solution, another in alanine solution, and the third in buffered stream water. With a concentration of 5×10^{-5} g. S. per cc., the stream-water cultures hatched first, the cysteine cultures second and the alanine-treated ones last. In 29 experiments with this concentration the stream-water cultures hatched first in 24 cases; and in 20 cases the cysteine cultures hatched

before the alanine-treated eggs. A more dilute concentration of 2×10^{-5} g. S. per cc. was below the toxic range of both compounds.

Thus it appears that for these forms the use of alanine controls would lead to entirely erroneous conclusions. Hammett states that all of his Paramecium experiments were controlled by this method, i. e., control solutions were made up of some compound lacking –SH that was as nearly as possible related to the compound being tested. In all cases in which cysteine was used in his experiments on root tips he states that alanine controls only were used. However, in his experiments on root tips with thio-glycollic acid, some were controlled with glycollic acid and others were not. He got a stimulation in either case.

In an earlier series of experiments done in the spring of 1930 the effect of thio-glycollic acid on the development of the eggs of *Physa* and *Lymneae* was studied in the same manner as the cysteine experiments described above, except that only stream-water controls were used. With this compound likewise no concentration was found that would stimulate the rate of development above that of the controls.

These results are in conformity with those of Dr. Sun[6] who was unable to accelerate the rate of cell division in the sea-urchin egg by treatment with H_2S.

In a private communication Dr. Hammett raised the objection that the presence of a jelly mass around the eggs might in some way prevent the entrance of –SH as such. He suggested that perhaps there were enzymes present which oxidized the cysteine before it got to the egg. This is a possibility, but it seems improbable. It can be demonstrated that at least part of the cysteine molecule goes almost immediately into the egg. When an unbuffered solution of fairly high concentration is used, it penetrates the egg and coagulates the albuminous part within 2 or 3 minutes after they are dropped into it. That the –SH is oxidized before this entry is made seems improbable.

These results, as well as the experiments of Sun, indicate that the sulphydryl group is not invariably a stimulus to cell division. They also call into question the legitimacy of the use of controls cultured in solutions of compounds even closely related to the compound being tested.

[6] Sun, T. P., *Anat. Rec.*, 1930, 47, 309 (Abstract).