THE "ACTIVATION" OF AMYLASE

by

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INTRODUCTION

In recent years a great deal of valuable work has been done at the Boyce Thompson Institute for Plant Research, on the breaking of the dormant period in potatoes and other plants. Denny and his co-workers, (4), (5), (6), (7), and (10) have performed very thorough experiments on the subject and have found startling differences in the times of germination for treated and untreated specimens. The chemicals used in the treatments included ethylene chlorhydrin, potassium thiocyanate, thiourea, and many others. Considerable work has also been done in determining the enzyme changes brought about by the treatments, (8), (9), and (11).

Other methods for breaking the rest period have been used by various workers. As early as 1885 Müller-Thorgau (12), reported that exposure to a temperature of 0° c. for one month breaks the rest period, and showed that a reduction of respiration and an accumulation of sugars results from the treatment. Appleman (1), has shown the diastatic activity in potatoes to be higher at 0° c. than at ordinary temperatures. Babcock, (2) has initiated growth in dormant seeds both by exposing them to warm, dry air and by treating them with hydrogen peroxide. Good results were also obtained

by soaking the seeds in diastase and in glucose solutions. Davis and Rose (3), have shown that the dormant period of certain seeds can be greatly shortened by removing the seed-coats and keeping the seeds moist at 5° - 6° c.

The purpose of this investigation is to endeavour to throw some light upon the actual mechanism of the changes that take place in this breaking of dormancy, and up to date the work has been done wholly from the point of view of amylase activity. Supposing this activity to be increased by the various treatments, the result would be increased hydrolysis of starch, and hence a greater food supply for the embryo.

OUTLINE OF EXPERIMENTS

Thus the first experiments carried out were intended to prove whether or not increased food supply would hasten the germination of potatoes. Work was begun early in November, 1930, using U.B.C. potatoes, furnished by the Department of Agronomy of the University. The seed was cut with a cork-borer, diameter 2 cm., each seed-piece having one eye at the top, and being approximately 5 cm. long. The treatments were of four types:-

(1) Injections of maltose solutions - 0.5 - 5%

(2) " " diastase " - 1%

(3) Soaking 24 hours in maltose solution - 1%

(4) Dipping in concentrated diastase solution.

The injection method was abandoned, owing to the difficulty of injecting sufficient volumes of solution.

The seed was planted in flats, with the tops level with the surface of the soil, and kept in the dark at about 20° c.

The sprouting responses were disappointing, in that the untreated control seed gave better results than the treated seed. It was concluded that the stimulus given in cutting the seed was the optimum for breaking of dormancy; hence the next set of experiments was carried out on whole tubers. In this case the maltose treatments produced definitely better results than the control. However, the latter sprouted fairly readily, and germination experiments were abandoned early in January, since it was concluded that the tubers were no longer sufficiently dormant to give conclusive results.

Before this time, some experiments were carried out on the growth of the cut seed described above, with the storage portion surrounded by different gases. It was thought that the favourable effect of the cutting stimulus might be due to increased permeability to oxygen, and hence to an increase in respiration. These experiments were therefore performed in the hope that they would give some information as to whether respiration in the storage portion was a vital factor in breaking dormancy.

Experiments were tried in air, oxygen, nitrogen, and carbon dioxide. The apparatus used was a quarter-pint cream

bottle, with moist cotton in the bottom, and a tight-fitting cork. In the cork were three holes, one large one for the insertion of the seed-piece, two smaller ones for gas inlet and outlet. The seed-piece was pushed in until its surface was level with that of the cork. Melted wax was then run round the edge. For each experiment ten of these bottles were connected in series. The gas was run through slowly, and the whole kept in an incubator at 25° c.

The experiment was started on January 14th. On January 17th there were eight large, strong sprouts in the oxygen set, nine small ones in the air set, and one very small one in the CO_2 set. Unfortunately the supply of nitrogen was exhausted before sprouting took place, and could not be replenished. The results with CO_2 are not reliable, due to the possibility of a change in p H caused by the gas.

For the reasons given above these experiments were not carried further, but we believe that interesting results could be obtained from them under favourable conditions of dormancy etc.

Starting from the beginning of February, attention has been centred entirely on amylase itself. Denny (11), states that he has been unable to increase the amylase activity of press-juices by treating directly with any of the chemicals that break the dormant period. We have, however, been able to increase the activity of malt diastase acting on pure starch, in vitro, by means of ethylene chlorhydrin, thiourea,

and potassium thiocyanate. This increase may not be a case of "activation", though we have applied this term for lack of a more specific one. At present we are unable to say what the mechanism is.

The rate of hydrolysis of starch by amylase has been followed by means of a modified form of the Wohlgemuth method (13). For each run a set of twelve tubes of starch solution was used, the starch concentrations being:- 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 percent. These solutions were made up as follows:-

A stock solution was first made, 5% for concentrations 0.1 - 4.0%, and 10% for concentration 5%. The soluble starch of the J. T. Baker Company was used. It was weighed, shaken with water in a volumetric flask, boiled, allowed to cool, and made up to volume. Portions of the fresh stock solution were then run into 100 c.c. volumetric flasks from a burette. Water was added, and the solution brought to the boil again. After cooling the required amount of "activating" reagent was added, and the solution made up to volume. 10 c.c. portions of the solutions were then withdrawn with a pipette and placed in test-tubes.

The enzyme used was the "Malt Diastase, U.S.P." of Eimer and Amend. 0.5 gm. of the dry material was weighed out and made up to the mark in a 500 c.c. volumetric flask. The solution was shaken at intervals for fifteen minutes and filtered. 0.05 c.c. of the filtrate was used for every 10 c,c.

of starch solution.

After the addition of the enzyme solution, toluene was added to the tubes, after which they were shaken thoroughly and placed in the incubator at 25° c.

At intervals (every few hours near the beginning of the experiment, every two or three days in the later stages) five drops of each solution were withdrawn and tested on a spot plate with 0.01 c.c. of $\frac{N}{200}$ iodine solution. * If no purple or red colour appeared, the starch in the tube tested was considered to have been hydrolyzed, and its concentration was plotted as an ordinate against time. See figs. 1 - 5.

As a check on this method portions of one of the solutions (4%) in each set were withdrawn at intervals, and the reducing substances, (sugars and otherwise) were determined in the sample. This determination was made by means of copper reduction and titration with thiosulphate (13), after precipitation of starch by boiling alcohol (9). The results of these analyses gave excellent checks on the iodine method.

The "activators" used were as follows:

1. Commercial Ethylene Chlorhydrin (40%) from the Carbide and Carbon Chemicals Corp., hereinafter designated as Chlorhydrin I.

* Larger amounts of iodine were used in the case of KSCN and thiourea.

2. Anhydrous Ethylene Chlorhydrin, from the Eastman Kodak Co., diluted to 40%, designated as Chlorhydrin II.
3. Potassium Thiocyanate, 48.2% (equimolar with 40% ethylene chlorhydrin).
4. Thiourea 7.6% ($\frac{1}{5}$ molar concentration of ethylene chlorhydrin.) This solution could not be made equimolar, owing to the low solubility of thiourea.

The concentrations of "activators" in 100 c.c. of starch solution were varied as follows:

Chlorhydrin I.

0.5, 0.75, 1.0, 1.5, and 2.5 c.c.

Chlorhydrin II.

0.75, 1.0, 1.5, and 2.5 c.c.

KSCN.

1.0 and 5.0 c.c.

Thiourea

0.5, 2.5, 5.0 and 10 c.c.

At the time when incubation was started the pH was determined on several of the solutions, chosen at random, and was found to be 6 $\pm$ 0.2 in every case. The pH of potato pulp was found to be 5.95, so that the amylase of the experiments acts at the same pH as in the natural state.

Experiments were also carried out which were intended to show at what point each of the chemicals caused the

starch to go completely to glucose. 100 c.c. samples were used instead of 10 c.c. One starch concentration only was used, namely 1%. The starch solutions contained:-

(a) 2.5 c.c. chlorhydrin I.

(b) 2.5 c.c. chlorhydrin II.

(c) 2.5 c.c. Thiourea

(d) 1 c.c. KSCN.

A control was also run. 0.5 c.c. of enzyme solution was used. At intervals of three or four days 5 c.c. portions were withdrawn and tested for the formation of osazones by heating with phenyl-hydrazine hydrochloride and sodium acetate. At the time of writing no crystals of maltosazone or glucosazone have been formed, probably owing to the fact that dextrins are still present in the solutions.

In order to correlate the observed increase in diastatic activity with the work on potatoes, two samples of potato-scrapings were weighed out, (250 gm. each) and made up to one litre:-

(a) with water

(b) with water plus 25 c.c. Chlorhydrin I.

Toluene was added, and the solutions were shaken and incubated at 25° c. Every two days 5 c.c. samples were withdrawn and the reducing substances determined by the method given above. After twenty days the pH was determined on both samples, the treated having a value of 6.1, the control 6.3, so that any differences observed are not due to any great change in

hydrogen-ion concentration.

DISCUSSION OF RESULTS

The results of the starch-hydrolysis experiments are shown graphically in figs. 1 - 4. It is evident that all four reagents used increase both the rate of starch hydrolysis and the rate of production of reducing substances.

The Chlorhydrin I was the most efficient "activator", its superiority to Chlorhydrin II being perhaps due to impurities. An analysis of its inorganic constituents showed the presence of traces of iron, manganese, calcium, magnesium, sodium and phosphate. Time did not permit of testing thoroughly the effect of these constituents.

Definitely increased rates were also obtained with thiourea and potassium thiocyanate. A fair correlation between activity and concentration of reagent is seen in all cases.

In order to show that the increases were not due to hydrolysis of starch by the reagents themselves, one set was run containing 2.5 c.c. of Chlorhydrin I in 100 c.c., and with the enzyme omitted. After one week there was no hydrolysis of starch, even in the lowest concentration.

These results do not give any insight into the mechanism of the change occurring. The effect of the chemicals may be merely to split the starch into alcohol-soluble dextrins which give no colour with iodine. Some dextrins are also formed which are insoluble in water, but which, when filtered out

and dissolved in hot water give no colour with iodine.

It was hoped that the osazone experiments would help to elucidate the problem of this mechanism, but, as stated above, no definite results have been obtained.

The results of the experiment on potato pulp are shown in fig. 5. Since these curves run almost parallel it would seem at first glance that there is no "activation" of amylase in potato pulp by ethylene chlorhydrin. However, the possibility of "activation" of antagonistic enzymes must not be overlooked. The first portion of the curve would seem to indicate an excess action of amylase over that of zymase, and the second portion of the curve the reverse of this relation. There is then the possibility of equal "activation" of amylase and zymase, the net result being the same as in the control. That there is "activation" of some enzymes is evidenced by the fact that a black colour developed in the treated pulp three days before a similar colour was noticed in the control. This is probably due to the "activation" of tyrosinase, acting on tyrosine with the formation of homogenistic acid. On the other hand there is the possibility that the potato amylase was initially at its maximum activity, due to the lateness of the season.

CONCLUSION

Many other enzymes in the potato and other plants remain to be investigated. The respiratory enzymes in

particular would seem, from the work of Babcock (2), and others, to be interesting in this connection. It is hoped therefore to carry out experiments on other enzymes at a future date.

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SUMMARY

1. The activity of amylase can be increased, in vitro, by the addition of ethylene chlorhydrin, thiourea, and potassium thiocyanate.
2. The correlation between the increase in activity and the strength of the reagent is fair, though not perfect.

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FIGURE 1

Showing the effect of various concentrations of commercial Ethylene Chlor Hydrin on Malt Amylase.

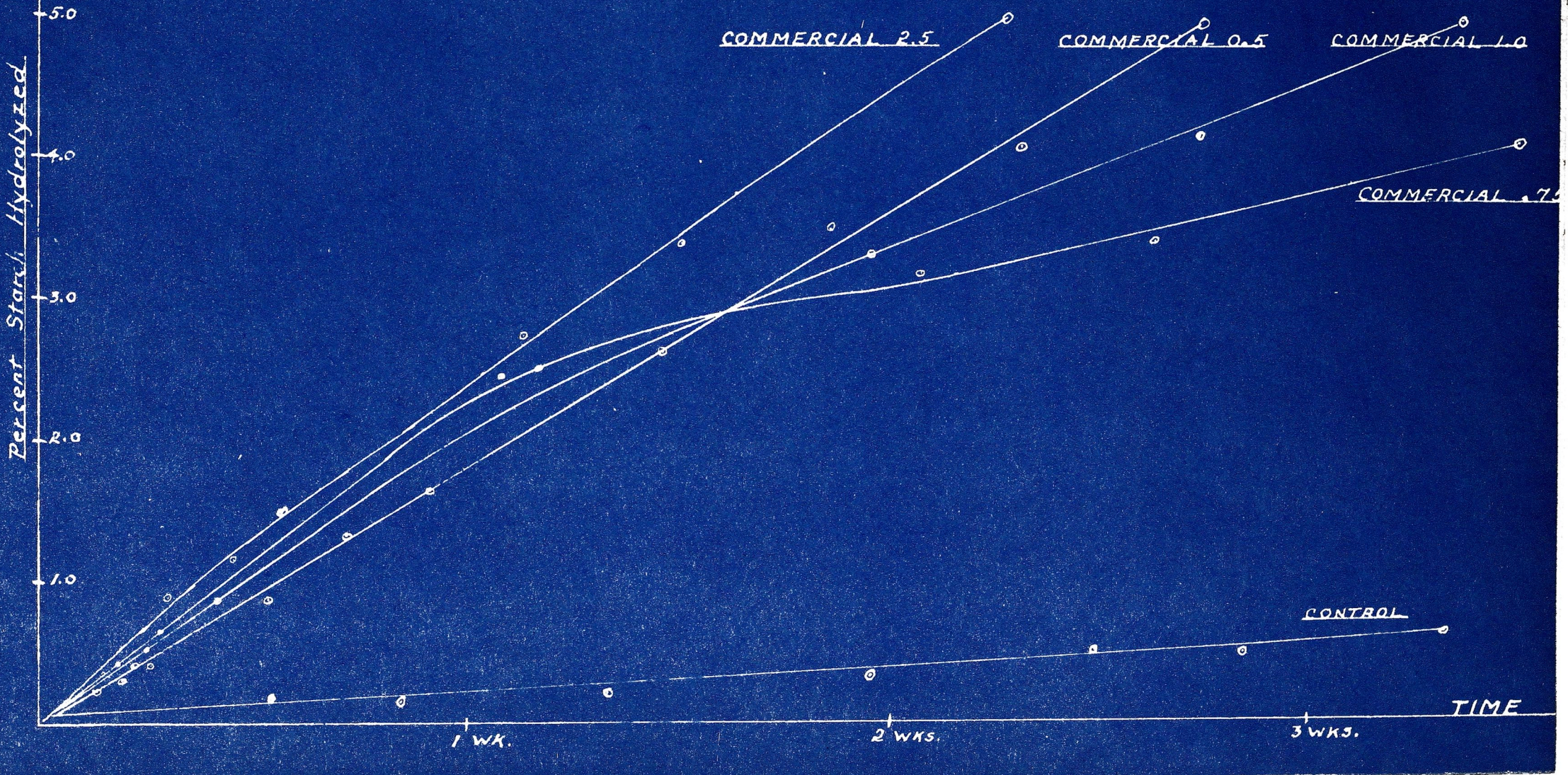

FIGURE 2.

Showing the effect of various concentrations of E. K. Co. Ethylene chlorhydrin on Malt amylase.

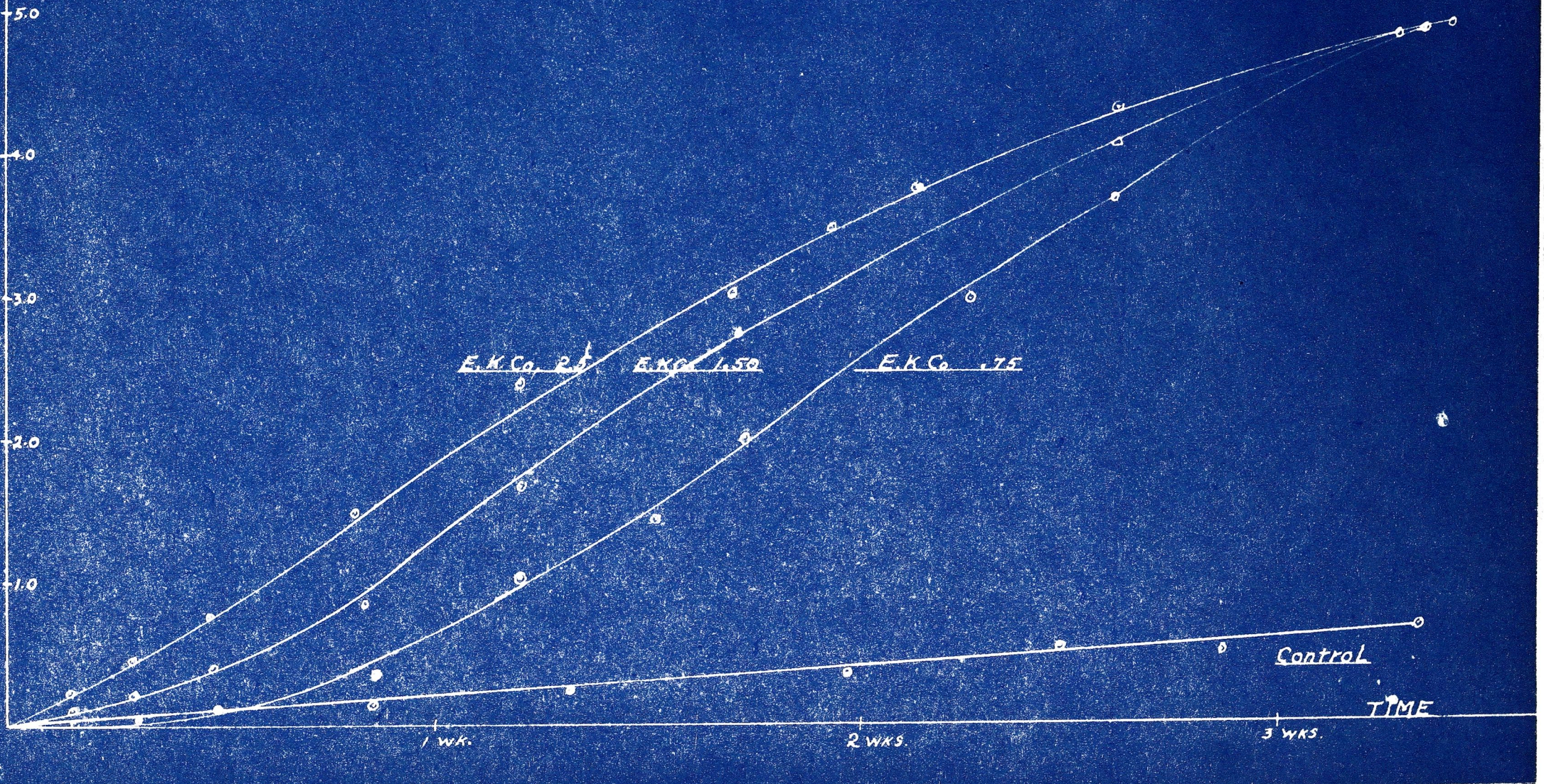

FIGURE 3.

Showing the effect of various concentrations of Thiourea on the activity of Malt Amylase.

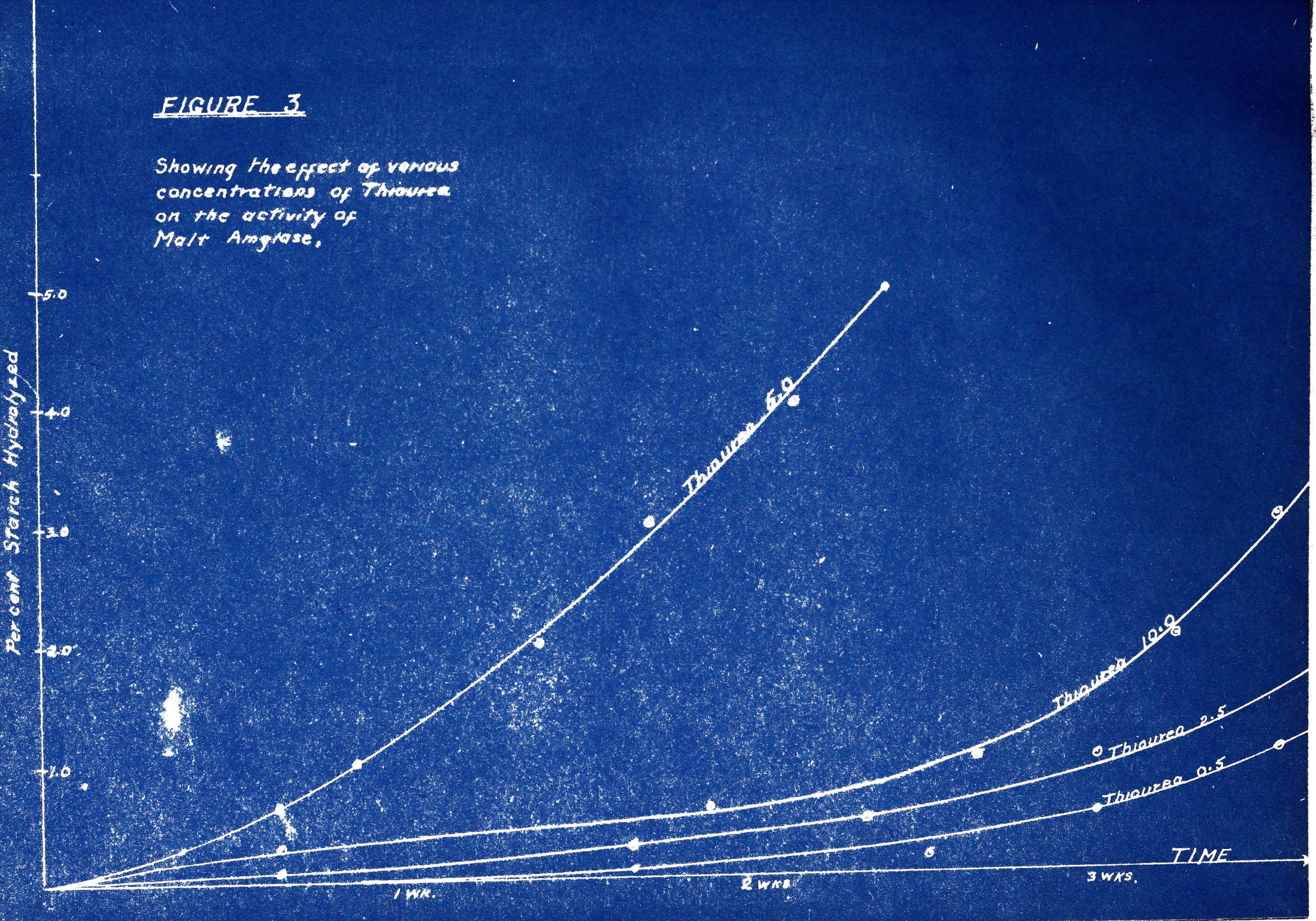

FIGURE 4

Showing the effect of Potassium thiocyanate on the activity of Malt Amylase.