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THE SOURCE OF OXYGEN FOR CHIRONOMUS LARVAE LIVING AT THE PROFUNDAL BOTTOM OF DOUGLAS LAKE

A statement of the problem and the methods to be employed

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STATEMENT OF THE PROBLEM

The larvae of Chironomus spp. are found to inhabit the stagnated oxygenless regions of Douglas Lake and other similar lakes. These animals are facultative anaerobes since they are also found in water which contains an adequate supply of oxygen.

The respiration of Chironomus has been studied by many workers. (Harnish, Leitsch, Fox, Pause, and others) Leitch has shown definitly that it is impossible for enough oxygen to be stored in the hemoglobin of the larvae to last more than a few hours. Pause has demonstrated that Chironomus larvae can live up to 54 hours in an atmospheresof pure Nitrogen. Other work has shown that longer periods of survival are possible. Cole has stated that a possible source of oxygen is a fermentation of the plant detritus of the bottom deposits. His test, that of guaicum with a peroxide is not a specific for oxygen. In this case it probably demonstrated the presence of a plant peroxidase.

Harnish has shown that animals kept in an atmosphere of Nitrogen for a number of hours loose glycogen and gain fat. He states that the transformation is sufficient to supply the necessary oxygen for the animals' respiration. Other workers, working on other anaerobes have also shown this situation experimentally. Von Kemnitz has worked on Gastrophilus equi, Weinland on several endoparasites such as Ascaris and Taenia, and von Brand has demonstrated this transformation in several Annelida. At a later date a comprehensive and critical review of these papers will be prepared, but time does not permit its inclusion in this report. It will suffice to say that many workers have demonstrated the glycogen- fat conversion in a variety of anaerobic animals.

Von Kemnitz writes the equation for the transformation in this manner: Glycogen $\rightarrow$ Fat + CO_2 - H_2O. It is probable that the equation should be in t two steps: 1.) Glycogen $\rightarrow$ Fat + O_2,
2.) Fat + O_2 $\rightarrow$ CO_2 + H_2O.
Obviously no attempt to balance these equations would be justified at present. A hypothetical equation may be written to show the calorific changes involved.

	3 glucose $\rightarrow$	1 stearic acid +	8 O_2.
Equiv. wts.	540g.	284 g.	256 g.
Calories	2160	2556	

Since the stearic acid has a higher calorific content that does the glucose, energy is required to effect the transformation. This point seems to have been overlooked by others who have studied the transformation. The difference in Calorie content is 396 Cal. The oxidation of 99 grams of glucose would furnish this energy. This oxidation would require 105.6 grams of oxygen. If this oxygen is supplied by the transformation, 150.4 grams will remain. This is enough oxygen to oxidize 141 grams of glucose, furnishing 564 Calories. The utilization of some glucose would account for some of the carbon dioxide collected by von Kemnitz and others.

Harnish has shown that during the experimental anaerobiosis of Chironomus thummi larvae glycogen was used up and fat was produced, both at a greater rate than in aerobic animals. Harnish's anaerobiosis was experimental, and was of short duration, and his animals were starving.

This problem will be an attempt to discover whether or not the glycogen- fat transformation can serve as a source for oxygen for feeding animals in natural long-term anaerobiosis.

This report includes a plan of attack on the problem and chemical methods for the determination of glycogen and fat. These methods have been modified slightly from the original and have been written up with the modifications worked in to the description. Both of the methods have been thoroughly tested on rat and mouse tissue and on Chironomus larvae. The methods are presented in their final form as determined by experiments. Experimental data supporting the changes made and the application of the methods to Chironomus are not presented here but are on file.

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PLANS FOR INVESTIGATION

I. A series of collections of Chironomus larvae will be made during the summer from South Fishtail Depression, Douglas Lake.

A. This series shall start before the summer stagnation sets in in South Fishtail Depression, and shall continue as long as possible.

B. Collections shall be made not less than three times a week. More frequent collections will be necessary before and during the stagnation.

C. Physico-chemical date shall be taken at the time of collection of the water as close to the bottom as is practical.

D. Two samples, each weighing a gram or more shall be taken at each collection. The live weight of the samples shall be determined immediatly. The two samples shall be preserved separatly in 95% alcohol. The animals shall be killed in the alcohol immediatly after weighing.

II. A series of control collections will be made from Grapevine Point Depression at the same time and in the same manner as those in I. (This will be done if Grapevine Point Depression does not stratify during the period of collection.)

III. One sample of each collection from both South Fishtail Depression and Grapevine Point Depression will be analysed for glycogen by the modification of the Good-Kramer- Somogyi method, and one will be analysed for fat by the Kumagawa-Suto method.

Note;- The weight of the chitin will be determined when is is separated in the glycogen determination.

IV. A collection shall be made of the bottom mud of the two depressions. This shall be analysed for fat, total Nitrogen, soluble carbohydrates. The collections of bottom deposit will be sun-dried after collection.

V. A third sample of animals will be taken at each collection. Total nitrogen will be determined on this sample. (Under consideration)

VI. The possibilities of obtaining oxygen data on the botton material will be investigated.

VII. The oxygen consumption of the bottom deposits after the removal of the macro-fauna will be investigated,

VIII. The oxygen consumption of Chironomus larvae will be investigated.

THE GOOD-KRAMER-SOMOGYI MODIFICATION OF THE PFLÜGER GLYCOGEN DETERMINATION
AS APPLIED TO CHIRONOMUS LARVAE

A group of larvae, of which the live weight is known, are killed and preserved in 95% alcohol. Before the determination is made the majority of the alcohol is evaporated off over a steam bath, and the larvae are dried for 24 hours at 60°C. (Experiments on rat and mouse livers show that the preservation and drying do not effect the availability of the glycogen. Experiments on Chironomus larvae ~~haxx~~ shows that there is no change in the weight of the residue upon longer heating.)

The residue is then ground to a powder, and the analysis is carried out on the powder. It is essential at this step that the chitinous hull be broken compltely so that it will not act as a trap for the glycogen solution. The powder is placed in a 13X100 mm. test tube. Some residue will remain in the beaker used for the drying. This should be washed out thorroughly with two 1cc. portions of 30% KOH. The washings are added to the powder. (If more than 1gram of tissue is used, KOH should be added so that its volumn will be twice as many cc. as there are gram of tissue.) The tubes are then placed in a boiling water bath for $\frac{1}{2}$-1 hour. At tthe end of this time only the chitin remains undissolved. The solution is then filtered with vacuum through a weighed Gooch crucible. Since glycogen is suluble in hot water but not in cold,it is advisable to have the crucible hot. It may be heated by keeping it over a boiling water bath for a few minutes. The testtube and the crucible should be washe out with several small portions of hot 30% KOH. Since the heating with KOH saponifies the fat in the tissue, there will be a great deal of foaming during the filtration. This may result in the loss of glycogen. it can be prevented by the addition of small amounts of ~~absolute ethyl~~ caprilic alcohol to the wash KOH. The filtrate is collected in an 18X110 mm. testtube. It should not exceed 5cc. After the filtrate is collected the collecting tube should be removed. The washing has not been sufficient to transfer all of the chitin to the crucible; this may be done with water. Since chitin is not affected by KOH, it will be retained on the asbestos in the filter. This is then dried to constant weight. The drying temperature should not exceed 60°C. The filtrete is cooled to room temperature and 1.1 to 1.5 volumes of 95% alcohol are added. This precipitates the glycogen. The mixture is then brought to a boil and cooled to room temperature, and centrifuged. The supernatant liquid is drained off and discarded The glycogen remaining is then hydrolosed by adding 1N H_2SO_4 and placing it in a boiling water bath for two hours. Experiments have shown that the hydrolysis period should be at least twoo hours long, and that 3cc. of the H_2SO_4 is sufficient. The glycogen is hydrolysed to glucose.

The hydrolysate is then analysed for glucose by the method of Shaffer and Somogyi. It is diluted to 25cc. in a volumetric flask, and 5cc. portions are pippetted into 250mm. test tubes.5cc. of the Shaffer-Somogyi Copper Reagent No. 50 (containing 1 gram of KI) are added to each tube. A control of the reagents is set up by adding 5cc. of the reagent to 5cc. of distilled water. The tubes are c covered with a 1-holed rubber stopper and are placed in a boilibg water bath for 15 minutes. After a few minutes cooling in a cold water bath 2cc. of a soultion containing 2.5% each of KI and K-oxalate are added to each tube. This is followed by 5cc. of 1N H_2SO_4. The tubes are again covered and are shaken gently to mix the reagents. The iodine released by this procexdure is titrated with .005N $Na_2S_2O_3$. The difference between the control and the experimental tubes shows the amount of Iodine used in oxidizing the Copper that has been reduced by the glucose. Shaffer and Somogyi have shown that this difference multiplied by the factor .113 gives the milligrams of glucose in the solution withinan error of 3-5%.

Note:- The original methods have been modified to fulfill two requirements. 1.) Work on material that has been preserved in alcohol and dried, and 2.) Work on material that contains a large amount of chitin.

Note: In order to avoid transfer of the filtrates from a filtering flask to a centrifuge tube, a special scheme for vacuum filtration has been devised. This scheme is described in another section.

Ref: Good, C.A., H. Kramer, and M. Somogyi; The Determination of Glycogen.
Journal of Biochemistry, Vol. 100, pp. 485-491, 1933.
Shaffer, P.A. and M. Somogyi; Copper-Iodometric Reagents for Sugar Determinations.
Journal of Biochemistry, Vol. 100, pp.695-713, 1933.

THE KUMAGAWA*SUTO METHOD OF FAT DETERMINATION AS APPLIED TO CHIRONOMUS LARVAE

A group of larvae, of which the live weight is known are killed and preserved in 95% alcohol. Before the determination is made the majority of the alcohol is evaporated off over a steam bath, and the larvae are dried for 24 hours at 60°C.

The residue is ground to a powder and the analysis is carried out on the powder. The chitinous hull should be broken up to prevent it from retaining some of the fat. Since some of the fat will have been in solution in the alcohol it will be deposited in the vessle used for drying. This fat should be washed out carefuly with 20% KOH, and the washings added to the bulk of the material. The powder is placed in 25cc. of 20% KOH(including the washings) and placed on a boiling water bath. This step is best carried out in 50ml. beakers. Alarge funnel is inverted over the beakers on the water bath so as to form a steam chamber. The heat treatment is continued for two hours. At the end of this time all of the fat present is saponified. The material is transferred to a 250ml. separatory funnel and is cooled. 20cc of 20% HCl is added. The contents are shaken and cooled. 10cc more 20% HCl are added, and the material is again shaken and cooled. After cooling 70-100 cc. of ethyl ether are added to and mixed with the acid contents of the funnel. The solid material forms a layer between the aqueous and ether solutions. The aqueous solution is drained off from below and the ether portion is decanted. The solid which remains in the funnel is washed several times with 5cc. portions of ether which are then added to the first ether extract. To the last washing 5 cc. of 20% KOH are added. This is shaken well, and the aqueous solution from the first extraction is added. The aqueous layer separated from this mixture is drained off and discarded. The ether soultion is added to that of the first separation. The ether extract is now evaporated to dryness. This is best done by distilling it off. The use of condensers and water baths heated by electric heaters with enclosed filaments lessens the danger of fire. By this means most of the ether is recovered and may be used again. The residue from this evaporatin should be dried thoroughly at 50°C. The temperatures during the evaporations should never exceed 60°C. since the fat is somewhat volatile at higher temperatus The residue, after drying, is dissolved in absolute ethyl ether and is filtered through asbestos. This is best done by means of a Gooch Crucible and a filter pump. This ether extract is dried as above. While still warm it is dissolved in 20-30cc of petroleum ether (b.p. 30-60 C.). This soultion is cloudy and should be allowed to stand for several hours until the white precipitate has settled to the bottom of the flask. The soultion is then filtered through asbestos into a weighed 50cc. Erlenmeyer flask. The petroleum ether is evapotated off by the method described for the ethyl ether. The residue is dried to constant weight at 50°C.

The final residue of this determination is a mixture of several fatty acids and the unsaponifiable lipids such as cholesterol. These can be separated further but for this work it has not been considered essentail.

Note:- The application of the original method to Chironomus requires no modification except that the animal material be ground up to prevent the chitinous hulls from trapping the fat solution.

Note:- In order to avoid changing the filtrates from a filtering flask to a weighed flask, a scheme of vacuum filtration has been devised. This scheme is described in another section.

Ref. Kumagawa, M. u. K. Suto; Ein Neues Verfahren zur quantitativen Bestimmung des Fettes und der unverseifbaren Substanzen tierischen Material, nebst der Kritik einiger gebrauchlichen Methoden.
Biochemische Zeitschrift, Bd. 8, ss.212-247, 1908.

A DEVICE FOR VACUUM FILTRATION INTO VESSELS OTHER THAN FILTERING FALSKS

In order to filter a meterial under vacuum without having the necessity of transferring it into a centrifuge tube or wieghed flask a chamber was set up in which the recieving vessel could be placed and subjected to vacuum. The receiving vessel was placed on a glass plate. A bell jar with opening at the top and sides was placed over it. A Walter type crucible holder was placed in the top hole, and a tube to the filter pump led from the side hole. Thus the entire chamber could be evacuated, and the filtrate could be collected in the desired type of container, either a flask or centrifuge tube.