ENZYMIC DECOMPOSITION OF THE CEREBROSIDES OF THE BRAIN

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Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 50, No. 12, pp. 44-47, December, 1960
Original article submitted December 12, 1959

The study of the cerebrosides of the brain has so far been confined to the more precise definition of their structure. The participation of the cerebrosides of the brain in the metabolism of that organ is a matter on which still little is known. In a survey paper presented to the First Symposium on Neurochemistry, Klenk [15] reported that in Gaucher's disease, Niemann-Pick's disease and certain other conditions there is an increase in the lipid content, and hence in the cerebroside content of the brain. An increase in the cerebroside content in the diseases mentioned above has also been described by Thannhauser [21]. We have observed a decrease in the quantity of cerebrosides in the brain tissue of rabbits in generalized tetanus, in gas gangrene and in strychnine poisoning[2]. These facts, together with a series of researches into the metabolism of the cerebrosides by means of C^{14}-glucose and C^{14}-galactose [4-7, 17-20, 23] suggest that these compounds possess definite metabolic activity. Meanwhile, in spite of numerous attempts, no research worker has yet succeeded in establishing the enzyme systems responsible for the reaction of decomposition of the cerebrosides in the brain of animals.

The first communication on this question was made in 1920 by B. I. Slovtsov [3]. He observed the breakdown of cerebrosides by emulsin—an aqueous extract of bitter almonds. On the other hand, many workers studying the cerebrosides have found no appreciable decomposition of these compounds by brain tissue. In 1921, for example, A. M. Georgievskaya [1] reported that even after prolonged autolysis, there was no decrease in the cerebroside content of brain tissue. In 1929, Jungmann and Kimmelstiel [13] claimed a slight but perceptible enzymic decomposition of the cerebrosides of the brain during autolysis, byt the results of their experiments are extremely contradictory and give no grounds for reaching a definite conclusion. We were unable to detect any appreciable decrease in the cerebroside content of a homogenate of rabbits' brain after autolysis for 24 hr at 37° [2]. All the data described above were obtained after autolysis and are open to justifiable criticism. In a well-known paper, however, Johnson, McNabb, and Rossiter [12] reported that after incubation of brain slices in buffer solutions of different pH for 10 days, no change in the cerebroside content was observed.

This unusual resistance of the cerebrosides to enzymic breakdown led Lumsden [16] and others to postulate that in ordinary conditions the enzymes which split up the cerebrosides are inactive, for they are surrounded by a monomolecular layer of anti-enzymes, produced by the oligodendroglia. In certain pathological conditions the oligodendrocytes are destroyed, creating the necessary conditions for action of the cerebroside-splitting enzymes. This hypothesis agrees to some extent with the findings of Thannhauser and Thannhauser and Reichel [22], who reported that they had observed the decomposition of cerebrosides by an extract of brain tissue, which took place only in the presence of large amounts of SH-groups. These authors suggest that this decomposition is associated with activation of an enzyme—cerebrosidase—by SH-groups.

The work of these authors just mentioned is, in fact, the only one in which mention is made of observation of the enzymic breakdown of the cerebrosides. We accordingly decided to repeat it in order to investigate the activity of the cerebrosidase in the brain of rabbits during those pathological processes which, as we previously showed [2], are accompanied by a decrease in the cerebroside content of the brain.

TABLE 1

Galactose Content of Cerebrosides in Rabbits' Brain Homogenate after Incubation (as % of dry proteins)

In cerebrosides					
free		combined		total	
with cystein	without cystein	with cystein	without cystein	with cystein	without cystein
2,39	2,29	0,81	0,88	3,20	3,17
2,53	2,60	0,92	1,00	3,45	3,60
2,35	2,36	1,06	0,88	3,41	3,24
2,31	2,51	0,93	1,12	3,24	3,69

TABLE 2

Analysis of the Incubation Mixture

pH of medium	Complete mixture		Without substrate		Without enzyme	
	with cystein	without cystein	with cystein	without cystein	with cystein	without cystein
Estimation of galactose in trichloroacetic filtrate as reducing substances (in ml thiosulfate)						
5.0	0.55	1.80	0.68	1.75	1.07	1.87
7.00	1.00	1.75	1.10	1.78	–	–
Estimation of galactose in trichloroacetic filtrate by anthrone method (in colorimeter readings)						
5.0	0.05	0.06	0.06	0.06	0.01	0.03
7.0	0.05	0.06	0.06	0.05	–	–
Estimation of galactose in hydrolyzate of precipitate by anthrone method (in colorimeter readings)						
5.0	0.15	0.15	0.08	0.04	0.11	0.12
7.0	0.15	0.13	0.05	0.05	–	–

METHOD AND RESULTS

We carried out the first experiments with homogenates of whole brain of rabbits. These were incubated for 48 hours at 37° in phosphate and phosphate-citrate buffers at pH = 7.3, in the presence of 200 mg of cystein. The substrate was the cerebrosides of the homogenate themselves. After incubation, the proteins and lipids were precipitated with trichloroacetic acid, the precipitate was removed by centrifugation, and the lipids were extracted in a Soxhlet apparatus successively for 12 hours each with acetone, ether and a 1:1 mixture of methanol and chloroform. After evaporation of the solvent, the lipids were hydrolyzed with 10% H_2SO_4, the mixture being heated on a boiling water bath for 2 hours. The cooled solution was filtered, and the cerebroside content of the filtrate was estimated as galactose by the anthrone method. Since brain tissue contains free and combined cerebrosides, we deemed it necessary to investigate both fractions.

It may be seen from Table 1 that the galactose content of the free and combined cerebrosides, expressed as a percentage of the dry proteins of the brain, in the samples with cystein was not different from that in samples without cystein. In both cases the content of cerebrosides was the same as in the brain of rabbits when determined immediately after death of the animals.

In the next series we fully reproduced the experiments described by Thannhauser and Reichel. The enzyme preparation was obtained from rabbits' brain. The brain of an animal which had just been killed was frozen in liquid air, ground to a powder and vacuum-dried at a low temperature. The 2 g of fine, dry powder thus obtained was extracted with 25 ml of 0.9% NaCl with constant agitation for 8 hours. The extract was centrifuged and the centrifugate dialyzed against distilled water for 48 hr, and after dialysis was used as the enzyme solution. According to Fujino [8], who repeated the experiments of Thannhauser and Reichel, this enzyme solution can be kept in the cold for several months without losing its activity. As substrate we used a 0.1% aqueous colloidal solution of pure cerebrosides, isolated from ox brain, as described in Fujino's paper [8].

The incubation mixture consisted of 1 ml of enzyme solution, 5 ml of substrate solution and 3 ml of buffer-veronal in the samples of pH 5 and 7, and citrate-phosphate in the samples of pH 8. To the test samples was added 1 ml of a solution of 20 mg of cystein, neutralized to pH 7. Incubation continued for 18-24 hours at 37°. During this time, according to the authors cited above, complete hydrolysis of the quantity of cerebrosides taken must have occurred. It was assumed here that each molecule of cerebrosides splits up into galactose and ceramide. Thannhauser and Fujino traced the rise in the galactose content of the incubation mixture by the increase in the content of reducing substances, which they determined by a modification of the method of Hagedorn and Jensen.

In the first experiments we determined the galactose content of the trichloroacetic filtrate after incubation as anthrone. No increase in the galactose was observed in any of the test samples. In the experimental conditions described, no breakdown of cerebrosides thus took place. This unexpected finding led us to carry out a series of experiments to determine the galactose content by the method of Hagedorn and Jensen. The results of this series of experiments showed that, in the samples containing cystein, the content of reducing substances was higher than in the samples not containing cystein. In Table 2 we give the results of a typical experiment; in this case, as in the experiments of Thannhauser and Reichel, the difference was greater at pH 5 and less at pH 7.

It was apparent that an increase in galactose content had taken place here, which must indicate decomposition of the cerebrosides. It follows from the figures in Table 2, however, that the content of reducing substances in the control samples, containing either enzyme without substrate or substrate without enzyme, in those cases in which cystein was added to them, was higher than in the samples not containing cystein. This difference in the samples containing enzyme alone, without substrate, was almost equal to that in the full test samples.

In the samples containing substrate but no enzyme the difference was significantly smaller.

Besides estimating the reducing substances, in the same experiments we made parallel determinations of galactose as anthrone, and we found no increase during incubation in any of the samples (see Table 2). The readings of the scale of the electrophotocolorimeter, shown in this table, correspond to the color given by the anthrone itself. As a control we determined the galactose in the hydrolyzate of the precipitate obtained by addition of trichloroacetic acid to the samples. In all cases the amount of galactose in the hydrolyzate of the complete samples was equal to that in the control samples not containing enzymes (see Table 2). The results described led us to postulate that the excess of reducing substances which Thannhauser and Reichel, and later Fujino, took to be an increase in galactose as a result of the breakdown of cerebrosides, was in fact due to the presence of cystein in the incubation mixture.

Our experimental results do not, however, suggest that the brain does not contain a cerebroside-splitting enzyme system, for if that were so it would be impossible to account for the fact, established by means of C^{14}-galactose and C^{14}-glucose, that the galactose in the cerebroside molecule may undergo metabolism. In our view, possible ways of investigation of these enzyme systems are shown by recently published work on the elucidation of the structural formula of the cerebrosides. These researchers set out to investigate if α- or β-galactose was a component part of the cerebrosides. For this purpose cerebrosides were subjected to the action of α- and β-galactosidases.

Hamasato [10] observed the breakdown of cerebrosides by β-galactosidase from the snail's liver, whereas α-galactosidase was inactive in his experiments. Kiss and Jurcsik [14] observed the breakdown of cerebrosides by an α-galactosidase obtained from emulsin. In 1956, Fujino and Negishi [9] published work in which they succeeded in splitting cerebrosides with digestive juices. These authors found that pancreatin, pepsin "b" and trypsin split off galactose from galactolipids. In the course of 2 hours 40% of the galactose is split off cerebrosides. All three preparations possessed both α- and β-galactosidase activity. Separation of these enzymes showed that only β-galactosidase possesses the property of splitting the galactolipids.

It will be seen from the papers cited that it is not yet fully clear whether α- or β-galactosidase splits galactose from the cerebroside molecule. On the other hand, nobody has isolated these enzymes from brain tissue, although the experimental results that have been described suggest that the enzymes which split the cerebrosides in the brain of animals belong to the galactosidase type.

SUMMARY

Until now all attempts to find a cerebroside-splitting enzyme system in the brain have remained unsuccessful. Thannhauser and Reichel observed the splitting of cerebrosides by a brain tissue extract in the presence of SH-groups. The reaction was evaluated by the rise in the amount of reducing substances in an incubated mixture. When reproducing the above experiments, the authors obtained negative results when galactose, split from cerebrosides, was determined by the specific reaction for sugar (with anthrone reagent). The cerebroside-splitting enzymes in the brain of animals probably belong to the galactosidase type.

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