INCORPORATION OF D-GLUCOSE-U-C^{14} INTO GANGLIOSIDES IN A CELL-FREE SYSTEM*

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Incorporation of D-glucose *in vivo* into gangliosides has been reported (Burton *et al.*, 1958; Moser & Karnovsky, 1959; Suzuki & Korey, 1963, 1964). Korey *et al.*(1963) presented the evidence of incorporation of glucose into brain gangliosides *in vitro*. Recently, incorporation of NANA-C^{14} (N-acetylneuraminic acid) into gangliosides catalyzed by the enzyme present in rat kidney homogenate was reported (Kanfer *et al.*, 1964). This work describes a cell-free system containing rat brain microsomes and soluble fraction which is capable of incorporating the added D-glucose-U-C^{14} into its ganglioside fraction.

Brain microsomes and soluble fraction were prepared from 12 to 13 day old rats in the identical way as previously reported (Suzuki *et al.*, 1964). Briefly, the brains were homogenized in 3 vols of ice-cold 0.25M sucrose. Cell debris, nuclei, and mitochondria were removed by centrifugation at 15,000g x 10 min. The supernatant was centrifuged in a Spinco L centrifuge at 105,000g x 60 min. The final supernatant was used without further procedures. The microsomal pellet was suspended in the buffered salt media used for incubation.

Brain gangliosides were extracted essentially according to Folch *et al.* (1951). The gangliosides obtained after 6 day dialysis of the upper phase of chloroform-methanol extract were separated by preparative descending thin-layer chromatography (Korey & Gonatas, 1963). First, the pattern of incorporation of labeled glucose into gangliosides was determined. After thin-layer chromato-

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graphy, gangliosides were recovered from the silica gel and recombined. Total NANA was liberated by hydrolysis with 0.1N HCl at 100° for 25 min. The solution was dialyzed, and the free NANA in the solution outside the dialysis bag was further purified by high voltage electrophoresis (Suzuki & Korey, 1964). Another aliquot of the ganglioside mixture was hydrolyzed with 1N HCl at 100° for 16 hr. Then sphingosine and fatty acids were extracted with chloroform. Hexoses and galactosamine were separated on the Dowex 50W x 8 column (Boas, 1953). Galactosamine was further purified by high voltage electrophoresis. Radioactivity was determined on total NANA, hexoses, galactosamine and sphingosine-fatty acid fractions. NANA was determined by the thiobarbituric acid method (Warren, 1959), hexoses by orcinol method (Svennerholm, 1956a), and galactosamine by p-dimethylaminobenzaldehyde method (Svennerholm, 1956b).

In another series of experiments, each ganglioside was obtained separately from the thin-layer plates to determine the rate of incorporation into the individual gangliosides. In this instance, radioactivity was measured on the unhydrolyzed individual gangliosides.

Incorporation of D-glucose into Gangliosides. The radioactivity present in the total NANA, hexoses, galactosamine and ceramide of the mixture of gangliosides purified by thin-layer chromatography is shown in Table I. It is clearly indicated that analysis of this fraction is consistent with the total mixture of gangliosides without gross contamination, and that each moiety contains radioactivity. Thus, in this cell-free system, both conversion of the added glucose to the other moieties of gangliosides and synthesis of gangliosides are occurring. Conversion of glucose to NANA seems to be quite active as indicated by the relatively high specific activity of the latter. Galactosamine had the lowest specific activity, a finding similar to that in the *in vivo* study (Suzuki & Korey, 1963).

Incorporation into Individual Gangliosides. The standard system was incubated for different lengths of time to study the incorporation rate of each ganglioside (Fig. 1). Radioactivity was measured on each ganglioside without hydrolysis. The amount of each ganglioside was estimated by its NANA

Table I --- Incorporation of D-glucose-U-C^{14} into Ganglioside Moieties.

The standard system contained, in the final volume of 2.0 ml, ATP 3.2 μmoles; creatine phosphate 20 μmoles; creatine phosphokinase 40 μg; ribonuclease 200 μg; D-glucose-U-C^{14} 1 μc (specific activity 29 mc/mM); microsomes corresponding to 1 g of fresh brain and soluble fraction 0.2 ml. The final salt concentration of the media was: NaCl 0.06M; KCl 0.06M; $MgCl_2$ 0.01M and tris buffer 0.033M at pH 7.8. Incubation was carried out at 37° in air.

Moiety	Analysis		Radioactivity
	μmoles	molar ratio	total counts/min
Total NANA	0.61	2.0	430
Hexoses	0.95	3.1	570
Galactosamine	0.3	1.0	55
Ceramide	—	—	340

The total radioactivity of the starting mixture was 1430 cpm.

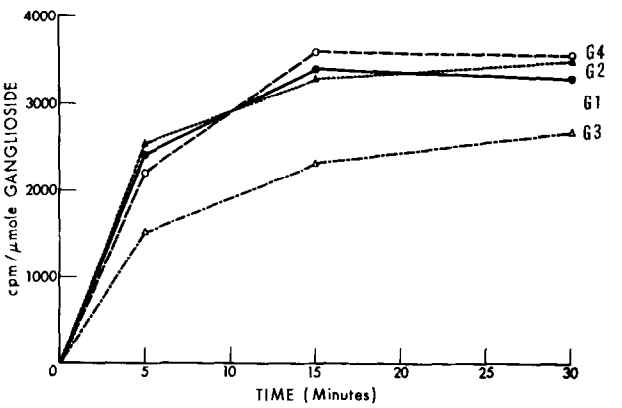

Fig. I --- Incorporation of D-glucose into Individual Gangliosides *in vitro*.

content. Results are then expressed as counts per min per μmole ganglioside with the assumption that G_1 is trisialo, G_2 and G_3 are disialo, and G_4 is monosialoganglioside, respectively (Suzuki & Korey, 1964). All 4 major gangliosides, which are numbered according to Korey and Gonatas (1963), follow similar incorporation curves. Initially, the incorporation is very rapid, but it only

lasts for the first 30 min with rapidly declining rate. G_3 was lower in specific activity than the other 3 gangliosides, all of which showed essentially the same activity.

The total radioactivity of the lower phase also reached the maximum within 15 to 30 min of incubation. The peptides in the ganglioside fraction prepared from this *in vitro* microsomal system were found to be completely free from radioactivity when each amino acid was checked for activity after elution from the two-dimensional chromatogram following 6N HCl hydrolysis. Thus, the chance of radioactive contamination due to peptides has been eliminated.

Discussion. Among the subcellular fractions of the brain, microsomes are known to be relatively rich in gangliosides (Wolfe, 1961). The microsomal fraction was most active in incorporating glucose or galactose into the neutral glycolipid fraction both in *vivo* and *in vitro* (Burton *et al.*, 1958). It seems, therefore, reasonable to search for the enzyme system which catalyzes synthesis of gangliosides in the brain microsomal fraction. Our microsomal fraction has been found to have reasonable morphological homogeneity, relatively free from contamination by the larger subcellular structures (Suzuki *et al.*, 1964). Approximately one-fourth of the total tissue gangliosides was recovered from the microsomes. The cell-free system presented here incorporated the added glucose into ganglioside fraction. As indicated by the radioactivity in all moieties, both synthesis of gangliosides and active conversion of glucose to other forms are taking place.

The primary aim of this investigation was to obtain a well supplemented cell-free system for ganglioside synthesis and no attempt has yet been made to determine the requirement of the system. The soluble fraction was added to give whatever was required as the cofactor. Ribonuclease was added with two reasons: a) to supply enough free nucleotides if any are necessary for ganglioside synthesis, b) to prevent the formation of peptides which may require RNA for their synthesis and are usually associated with the ganglioside fraction.

UDP-galactose was found to be the primary hexose donor in cerebroside formation in microsomes *in vitro* (Burton *et al.*, 1958). CMP-NANA is the primary

sialyl donor for NANA moiety of monosialoganglioside (Kanfer et al., 1964). Besides these two, other nucleotides also may be involved in ganglioside formatio

Incorporation of the added glucose into gangliosides lasts for only 15 to 30 min. An attempt to obtain steadier incorporation has not been successful. It will be noted that the protein synthesis in vitro shows a similar curve in most of the microsomal systems (Raake, 1961), including those which use rat brain microsomes prepared in the identical way (Suzuki et al., 1964). Shortage of any cofactors or inactivation of the enzyme system must be considered as a possibility.

Previously, we have demonstrated that none of the major gangliosides including G_4 (N-acylsphingosine-N-tetrose-N-acetylneuraminic acid) was the precursor of the other ganglioside species when ganglioside formation was studied in vivo (Suzuki & Korey, 1963). Because of the inherent difficulty of the study in vivo, we could not determine the glucose incorporation for a very short period after injection. The radioactivity of the precursor pool also varied greatly according to the time since injection. However, the in vitro cell-free system permitted us to study the incorporation after a very short time interval during which the precursor pool may be considered constant in activity. The resul confirmed our previous data that all 4 major gangliosides are formed simultaneous· ly without any one of them being the precursor of the others.

In our previous in vivo study, all 4 gangliosides showed approximately the same specific activity. In this cell-free system, however, G_3 was noted to have lower specific activity than the others. The reason for this discrepancy is not clear. G_3 is present in the largest amount in rat brain, and if all gangliosides are synthesized at the same rate in vitro because of the disturbed regulatory mechanism which operates in vivo, G_3 would give the lowest activity. But this alone will not explain the whole phenomenon because of the similarly different yields among the other 3 ganglioside species. The possible difference due to age has to be considered also.

Although further studies are needed of the optimal conditions of the system, it is hoped that this cell-free system can serve as a useful tool for investigating *in vitro* ganglioside formation.

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