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EFFECT OF ALPHA-PARTICLE IRRADIATION ON BRAIN GLYCOGEN IN THE RAT

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THE STUDIES of KLATZO, MIQUEL, TOBIAS and HAYMAKER (1961) have shown that one of the earliest and most sensitive indications of the effects of alpha-particle irradiation on rat brain is the appearance of glycogen granules mainly in the neuroglia of the exposed area of the brain. Periodic acid–Schiff (PAS) positive, α-amylase soluble granules were demonstrated within 12 hr after irradiation, preceding by approximately ½ hr the first microscopically detectable vascular permeability disturbances, as shown by the fluorescein labelled serum protein technique. These studies suggested that the injurious effects of alpha-particle energy were on cellular elements primarily, according to the physical properties and distribution of the radiation in the tissue, and that the vascular permeability disturbances played a secondary role in pathogenesis.

The purpose of this study was to correlate the histochemical observations on glycogen with a quantitative assessment of the glycogen in the irradiated brain tissue. It is felt that such a study may contribute to the understanding of radiation injury at the molecular level. A practical aspect of this problem is that the information on biological radiation effects due to accelerated particles from the cyclotron source, as employed in this study, is applicable to radiation from cosmic particles both in free space and entrapped in the Van Allen belts.

METHODS

A total of 90 rats of both sexes (Long–Evans strain) 3 weeks of age, weighing approximately 60 g, and fed on standard chow were exposed to alpha particles from the 60-inch cyclotron in Berkeley, Calif. The energy of the particles was 12 MeV per nucleon and the dose to the surface of the brain, 4000 rad. One rad is defined as the dose of radiation received when energy is absorbed at the rate of 100 ergs/g matter. The surface dose rate during exposure was approximately 10,000 rad/min, which corresponded to about 4×10^9 particles/cm²/sec. The cyclotron aperture through which the particle beam passed was 14·3 mm in diameter and allowed irradiation of most of the dorsal surface of the cerebrum and the cerebellum. The rats were anaesthetized with Nembutal, the scalp was incised in the midline and reflected, and the animals were mounted on a holder and so placed that their heads were 5 mm from the end window of the ionization chamber. The homogeneity of particle density was determined by the method of JANSSEN *et al.*, 1961.

At various time intervals following irradiation the animals were killed by immersion in liquid air. Control animals from the same litters were killed at the same time. With a chisel cooled in liquid air, the cerebral hemispheres were exposed, and pieces 0·8–1·0 g in weight were removed and weighed rapidly on a cooled pan of a torsion balance. For total glycogen the classical alkaline digestion procedure of PFLÜGER (1905) as modified by KERR (1936) was used. The weighed frozen chips of tissue were transferred to a tube containing 2·5 ml of 30% KOH and digested at 100° in a boiling water bath for 30–45 min. The tubes were covered with a filter funnel with the stem sealed to act as a cold

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finger. Following digestion 1·2 vol of 95% ethanol (i.e., 3 ml) were added to the tubes while still warm. After standing overnight in the refrigerator the tubes were centrifuged, and the supernatant fluid was decanted. The residue was suspended in 5 ml of chloroform–methanol (1:4, v/v), warmed to 50° for 5 min, and centrifuged again. The chloroform–methanol extraction was repeated three times. The glycogen in the residue was dissolved in 5 ml of N-HCl and hydrolysed in a loosely stoppered tube at 100° for 3 hr. The solution was neutralized by the addition of N-NaOH and made up to standard volume (usually 20 ml). The glucose was determined in triplicate in 1 ml portions by the copper reduction method of NELSON (1944) and SOMOGYI (1945).

The so-called 'free' or 'trichloroacetic acid soluble' glycogen was determined by transferring the weighed frozen cerebral hemispheres of control and irradiated animals to glass homogenizers, each having a close fitting polythene plunger and containing 2 ml of cold 10% trichloroacetic acid. The tissue was homogenized at 1,200 rev/min for 3 min and centrifuged at 10,000 rev/min (12,000 g) for 10 min in a Servall angle centrifuge. The supernatant fluid was decanted, and the residue was homogenized, as described above, three more times in 2 ml portions of cold 5% trichloroacetic acid and centrifuged; the supernatant fluids were combined and to them were added 5 vol of absolute ethanol. After mixing they were allowed to stand overnight at room temperature. The tubes were centrifuged, the fluid was decanted, and the glycogen was dissolved in 1–2 ml of N-HCl and hydrolysed. The glucose content was determined as above by copper reduction. The 'fixed' or 'residual' glycogen was determined in the residue after trichloroacetic acid extraction by digesting in 30% KOH and proceeding as described for total glycogen.

Irradiated animals were also decapitated for histochemical study at the same time as the glycogen determinations. After removal of the brain, blocks of tissue approximately 2–3 cm in thickness were taken from the more posterior part of the cerebrum, fixed in Rossman's fluid, and stained by the PAS procedure for histochemical demonstration of glycogen granules.

TABLE 1.—EFFECT OF DIFFERENT PROCEDURES ON THE EXTRACTION OF GLYCOGEN FROM RAT BRAIN

Group	Number of rats	Procedure	Apparent glycogen expressed as mg glucose/100 g tissue ± S.D.
1	3	Chloroform–methanol treatment, KOH digestion	7·7 ± 4·1
2	25	Same as for group 1	52·1 ± 5·8
3	3	Same as for group 1 with omission of chloroform–methanol treatment	132·0 ± 12·8
4	30	Homogenization 4 times with trichloroacetic acid	32·5 ± 5·7
5	27	Residue from group 4 treated as for group 1	26·5 ± 5·9

In group 1, the rats were decapitated, and the cerebral hemispheres were placed in KOH within 1 min. In groups 2–5, the rats were killed by immersion in liquid air.

RESULTS

Extraction of brain glycogen

Unless animals are killed by immersion in liquid air very low amounts of brain glycogen are obtained (Table 1). This illustrates that brain glycogen is much more labile than liver glycogen as was shown by KERR (1936). Further, unless the glycolipids are thoroughly extracted after ethanol precipitation of the alkaline digest of brain tissue, high glycogen values are obtained. Inadequate removal of glycolipids probably accounts for the high values for rat brain glycogen reported by some workers, e.g. KŘIVÁNEK (1958). The values for total glycogen (Table 1) agree with those obtained by CARTER and STONE (1961) for mouse brain but are lower than those obtained in rat brain (Sprague–Dawley strain, 160–170 g weight). This may be due to the different strain of rats used in this study (Long–Evans strain, 60 g weight).

Acid extractable glycogen represents 62·4 per cent of the total glycogen (Table 1). ...asing the number of extractions to six did not increase the amount of glycogen ...ned. In this respect brain glycogen may differ from liver glycogen in which most ...e glycogen can be extracted by cold trichloroacetic acid by repeated and efficient ...genization (CARROLL, LONGLEY and ROE, 1956; ROE, BAILEY, GRAY and ...SON, 1961). However, brain glycogen may be physically entrapped to an extent ...ring a much more rigorous homogenizing procedure than that used in this study. ...sum of the 'trichloroacetic acid extractable' glycogen and the residual glycogen is ...per cent higher than the total glycogen. There are two possible explanations ...this. Some degradation of brain glycogen by 30% KOH at 100° may lower the ...l glycogen values, or less effective removal of contaminating glycolipids by ...roform–methanol from the trichloroacetic acid precipitate may give higher ...dual' glycogen values.

TABLE 2.—EFFECT OF α-PARTICLE IRRADIATION ON TOTAL GLYCOGEN CONTENT OF RAT BRAIN

Time after irradiation (hr)	Glycogen content* (mg glucose/100 g brain)		Statistical significance† (*P*)
	Control	Irradiated	
39	52·2 ± 6·6 (3)	58·5 ± 3·4 (3)	0·02 < 0·05
61	51·6 ± 5·2 (3)	63·5 ± 7·8 (3)	0·02 < 0·05
133	54·2 ± 9·3 (4)	70·7 ± 5·8 (5)	< 0·01
206	50·5 ± 6·6 (6)	56·8 ± 4·2 (6)	0·2 < 0·1
380	49·5 ± 2·5 (6)	45·2 ± 7·5 (6)	0·02 < 0·05
630	52·9 ± 3·8 (3)	47·2 ± 5·9 (3)	0·1 < 0·2

* Mean values ±S.D., number of animals in parentheses.

† *P* values calculated by comparing irradiated values with the mean for the whole series of controls, 52·1 ± 5·8 (25). There were no significant differences among the various control groups.

Effect of alpha-particle irradiation

A significant increase in the total glycogen of cerebral hemispheres is evident (Table 2) at 39 hr (2nd day after irradiation) and reaches a maximum at 133 hr (5th day). By 380 hr (14th day) the glycogen content is lower than in the control animals. The 'trichloroacetic acid extractable' glycogen also increases significantly, parallel to the increases in total glycogen (Table 3). However, for 'residual glycogen' there was no significant difference between control and irradiated animals (Table 4). The sum of the 'trichloroacetic acid extractable' and 'residual' glycogen of irradiated animals was 11–13 per cent higher than the total glycogen. This difference was also observed in the control animals.

Histochemical appearance of glycogen granules in alpha-particle irradiated brain

Rats killed 39 hr after irradiation revealed numerous PAS-positive granules scattered in the irradiated portions of the cerebral cortex. The 'Bragg peak band'*,

* The energy at the greatest depth of particle penetration in tissue or at the peak of the Bragg curve when ionization and linear energy transfer are maximal, is about five times greater than at the surface of the brain. The term 'Bragg zone' refers to that part of the cerebral cortex in which demonstrable cellular damage has occurred and the term 'Bragg peak band' to the region in the deepest part of the 'Bragg zone' in which destruction of cellular elements was maximal.

in which energy transfer (30,000 rad) and tissue injury are maximal, was conspicuous by the paler PAS staining of the neuropil and the absence of PAS-positive granules. In some animals, the PAS-positive granules were seen also below the 'Bragg peak band', and the demarcation between these two areas was in a horizontal straight line passing approximately through the lower layers of the cortex (Fig. 1). Treatment of the sections with a solution of crystalline α-amylase buffered in phosphate, pH 7·0, produced complete extraction of the PAS-positive granules (Fig. 2), whereas control sections incubated in buffer alone showed no such effect. Examination under the high

TABLE 3.—EFFECT OF α-PARTICLE IRRADIATION ON 'TRICHLOROACETIC ACID EXTRACTABLE' GLYCOGEN CONTENT OF RAT BRAIN

Time after irradiation (hr)	Glycogen content* (mg glucose/100 g brain)		Statistical significance† (*P*)
	Control	Irradiated	
39	30·5 ± 8·4 (6)	39·7 ± 4·8 (6)	<0·01
61	28·7 ± 2·8 (6)	43·7 ± 7·0 (6)	<0·01
133	32·3 ± 6·6 (6)	50·1 ± 15·7 (6)	<0·01
206	34·8 ± 1·8 (6)	49·2 ± 8·2 (6)	<0·01
380	36·2 ± 4·1 (6)	36·4 ± 5·3 (6)	none

* Mean values ±S.D., number of animals in parentheses.

† *P* values calculated by comparing irradiated values with the mean for the whole series of controls, 32·5 ± 5·7 (30). There were no significant differences among the various control groups.

TABLE 4.—EFFECT OF α-PARTICLE IRRADIATION ON 'RESIDUAL' GLYCOGEN CONTENT OF RAT BRAIN

Time after irradiation (hr)	Glycogen content* (mg glucose/100 g brain)	
	Control	Irradiated
39	22·8 ± 7·1 (6)	30·5 ± 13·2 (6)
61	26·2 ± 7·7 (6)	33·9 ± 5·5 (6)
133	33·1 ± 3·1 (3)	27·9 ± 5·2 (4)
206	26·2 ± 4·5 (6)	26·0 ± 1·9 (6)
380	26·7 ± 2·5 (6)	28·1 ± 7·7 (6)

* Mean values ±S.D., number of animals in parentheses. None of the averages from the irradiated groups differed significantly from the mean of the whole series of controls.

power of the light microscope frequently showed a relationship of the glycogen granules to the neuroglial cells. In such instances glycogen granules were demonstrable as intra-cytoplasmic inclusions in the processes and around the nucleus (Fig. 3).

In rats killed 61 hr after irradiation, the glycogen granules showed a similar distribution, although they were less numerous than at the 39 hr stage. A progressive reduction in the number of glycogen granules was evident in rats killed at 133 and 206 hr. At these time intervals, however, occasional microglial cells in the vicinity of the 'Bragg peak band' contained abundant intracytoplasmic glycogen inclusions. Rats killed at 380 hr showed only a few glycogen granules located in the cortex close to the

FIG. 1.—Abundant glycogen granules are visible on both sides of the 'Bragg peak band'. The 'Bragg peak band' (outlined by the interrupted lines) is located in the middle layers of the cerebral cortex. (39 hr after irradiation. PAS stain, counterstained with haematoxylin) ×240.

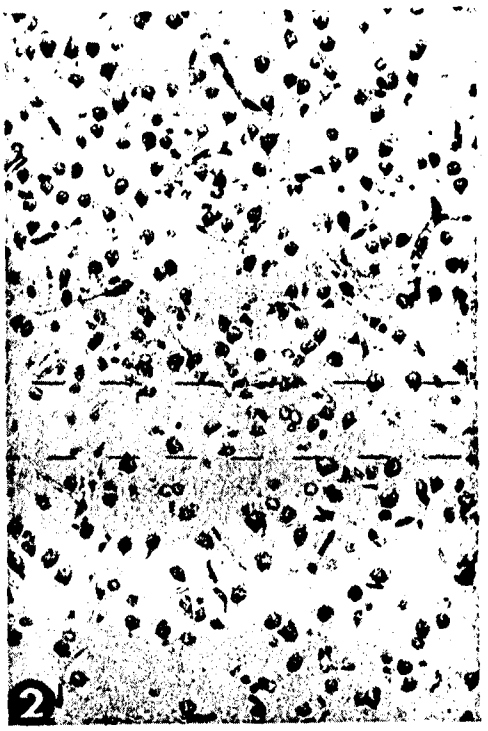

FIG. 2.—Adjacent section after treatment with α-amylase. The PAS-positive granules are not visible. The 'Bragg peak band' is outlined by the interrupted lines. (PAS stain, counterstained with haematoxylin) ×210.

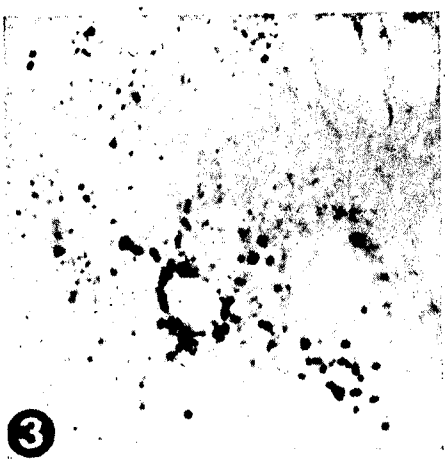

FIG. 3.—An astrocyte in the irradiated cerebral cortex outlined by the presence of PAS inclusions in the processes and around the nucleus 39 hr after irradiation. (PAS stain) ×980.

interhemispheric fissure. In rats killed at 630 hr, occasional PAS-positive granules were visible, but after α-amylase treatment they were still recognizable though paler in colour. The irradiated portions of the cortex also showed occasional coarser PAS-positive granules which were resistant to α-amylase treatment and stained darkly with Sudan Black B.

DISCUSSION

To our knowledge this is the first chemical study of glycogen in brain following exposure of animals to particle radiation. During periods up to 8 days after the irradiation a significant increase in the total and acid extractable glycogen is correlated with the histochemical appearance of glycogen granules in neuroglial cells and possibly reactive amoeboid microglial cells first demonstrated by KLATZO, MIQUEL, TOBIAS and HAYMAKER (1961). Only suggestions can be given at present as to the mechanism of this increase of brain glycogen. The passage of alpha particles through the tissue is associated with energy loss and ionization. The ionization is greatest at the end of the particle track and results in the cell damage demonstrable in the 'Bragg peak band' (JANSSEN *et al.*, 1961). Glycogen granules are not present in the 'Bragg peak band' but appear in aggregates above and below the band. In these locations it is unlikely that their appearance represents a direct ionization effect. Pertinent here are the histochemical observations of FRIEDE (1954) and SHIMIZU and HAMURO (1958), who found that in certain types of brain injury the severely damaged areas were devoid of glycogen while at the periphery of the lesions and beyond, glycogen was abundant in glial cells. The increase in glycogen around areas of damaged cells in the Bragg peak band may be derived from carbohydrate substances liberated in the area of injured tissue. It is suggested that the uptake of such substances, their concentration in neuroglia by pinocytosis (a mechanism studied in the nervous tissue with regard to proteins by KLATZO and MIQUEL (1960), and their conversion to glycogen may provide an explanation of the histochemical findings.

There is a difference in the times of maximal appearance of glycogen following irradiation as seen histochemically and as chemically determined. The greatest amount of small PAS-positive, α-amylase soluble granules appears at 39 hr whereas the greatest increase in chemically determined glycogen is at 133 hr following irradiation. This discrepancy is due probably to an inability of the histochemical method to evaluate the amount of glycogen present. There is no evidence to suggest a direct relationship between the number of PAS-positive granules visible under the microscope and their glycogen content. Consequently 133 hr after irradiation, even though there is more glycogen present, the PAS reaction is less, possibly because there is a smaller number of larger glycogen accumulations or because complexes between glycogen and tissue proteins give a weaker PAS reaction.

The results of this investigation also show that a constant and reproducible proportion of the total glycogen of rat brain is extractable with trichloroacetic acid. The division of tissue glycogen into two fractions based on its solubility in trichloroacetic acid is of doubtful physiological significance. Recent studies of liver and muscle glycogen (HANSON, SCHWARTZ and BARKER, 1960; ROE, BAILEY, GRAY and ROBINSON, 1961) have shown that variations in the extraction and isolation procedures alter the quantities of the two glycogen fractions and that the so-called 'bound' or 'residual' glycogen is probably held in tissue by physical entrapment. In the present study, although the homogenizing procedure was not as rigorous as that of ROE *et al.* (1961), further

homogenizing did not increase the amount of acid-extractable glycogen from brain tissue. The increase in brain glycogen following irradiation occurred only in the acid extractable fraction. This finding suggests either that the increase in total glycogen occurs in a compartment readily extractable by trichloroacetic acid, or that most of the carbohydrate present in the trichloroacetic acid insoluble residue is present as non-glycogen carbohydrate complexes, as was shown by CARROLL, LONGLEY and ROE (1956) in studies of liver glycogen.

SUMMARY

Glycogen determinations were made on the cerebral hemispheres of rats after irradiation with alpha particles from a cyclotron source. A significant increase in the level of total and trichloroacetic acid extractable glycogen occurred for 8 days after exposure. This increase was correlated with the appearance of histochemically defined glycogen depositions mainly in glial cells around the 'Bragg peak band' of radiation injury. In the interpretation of these findings, the possibility is suggested that carbohydrate substances are liberated in the area of tissue damaged by radiation and are converted to glycogen by neuroglial cells.

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