EMBRYOGENESIS AND GLUCOSE METABOLISM IN CARROT CELL SUSPENSION CULTURED *IN VITRO*

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Introduction

One of the most dramatical changes of events in cells observed in experimental and developmental botany is the regeneration of plantlets following the induction of embryos in somatic cell suspension cultured *in vitro*. Since the first observation of embryo formation reported from carrot cell suspension cultures,[11] much information concerning somatic embryo initiation and development has been obtained from the system[5,6,9].

It is known that the process of embryogenesis in carrot cell suspension involves separate two phases of cellular development — *i. e.*, firstly formation of masses of proembryogenic cell aggregates and secondly production of embryos from organized superficial cells followed by further development of embryos to plantlets[11,12]. Not only cytological but also physiological and biochemical changes occur both in cells becoming organized as embryos and in those that are not[10]. The relation between the process of embryogenic development and the physiological changes and other materially important processes has been considered in extent[2]. The work is undertaken in first to obtain the information on changes in cellular constituents during embryogenesis and secondly to study on possible roles of 2, 4-D on glucose metabolism and respiration during initiation and subsequent progress of embryogenesis. An attempt has been made to evaluate the synthetic efficiency of culture metabolism on growth and development of cell suspension cultures of carrot described by the formulae of LAMPORT[5].

Materials and Methods

Cell suspension culture:

The establishment of a line of cell suspension of carrot (*Daucus carota*

L., cv. Red core chantenay), the selection of the line of proembryogenic masses, its growth through several subcultures over a period of years and medium composition under which embryogenesis occurs have been described elsewhere[7].

Dry weight determination:

Dry weight was measured by filtering 2×10 ml samples of cell suspension through weighed 2.3 cm circle glass fiber filter (Whatman GF/C Type) held in a membrane filter holder, drying 110°C for 2 hrs followed by 80°C overnight, and weighing. The growth curves were expressed as mg dry weight per culture flask containing 80 ml of the defined liquid medium.

Respiratory measurements:

The oxygen uptake curve during growth plotted as $\mu 10_2$/mg dry weight/hr was obtained using the apparatus of a Gilson differential respirometer. The reaction vessel contained 3 ml cell suspension whose dry weight was determined after the incubation period of 2 hrs.

Experiments for glucose metabolism:

Experiments for the metabolism of specifically labeled glucose by carrot cell suspension were carried out in Gilson differential respirometer at 25°C. The cell suspension after harvest was washed with basal medium excluding glucose or sucrose in 2 of 50 ml glass centrifuge tube and combined them into 40 ml cell suspension with basal medium. An aliquot of cell suspension was placed in a weighing filter holder for determination of fresh and dry weight. Each reaction vessel contained 3 ml of cell suspension with basal medium of 50 mM MES buffer, pH 6.0, in the main compartment with 50 μl of labeled glucose in equal specific activities (5 μCi/5 μ mole/ml) of glucose-1-^{14}C and glucose-6-^{14}C in the side arm and 0.2 ml of 5 M NaOH in the center well. The reaction was initiated by mixing cell suspension with labeled glucose after thermal equilibriation. Three pairs of reaction vessels were prepared for a line experiment. The labeled glucose was purchased from the Radiochemical Centre, Amersham, England. After an incubation period of 2 hrs, cell suspension and $NaH^{14}CO_3$ were collected, and analyzed for glucose utilization.

Fractionation of cellular constituents:

An aliquot of cell suspension was collected in a 15 ml centrifuge tube, immediately frozen and crushed in a chilled mortar. The samples were further homogenized in a motor driven glass homogenizer for 10 strokes.
Soluble solids: The soluble materials were extracted with 70% methanol

and further separated by means of ion exchange resins into neutral, basic and acidic fractions.

Lipids: The slurry was centrifuged and the precipitate was again extracted by successive suspension and decantation for removal of the materials soluble in absolute methanol followed by diethylether: acetone (2 : 1, by volume) and finally absolute acetone. Methanol-diethylether-acetone was evaporated to dryness. Lipids were determined by gravimetric method.

Starch: The resulting dry powder was suspended in 2 ml of 50 mM acetate buffer, pH 5.2, and subjected to α-amylase hydrolysis for 2 hrs at 37°C. After the extraction of sugar with 2 volume of alcohol, and repeared the treatment, and dryed.

Protein: The residue was treated with pronase for 2 hrs at 37°C. Alcohol extracts of these were protein-amino acids.

Cell-wall polysaccharides: The residues were incubated with 1 M NaOH at 37°C overnight. In this fraction hemicellulose was removed from cellulose.

An aliquot of these fractions was assayed for radioactivity, sugar and amino acid analyses.

Results

Growth and respiration of carrot cell suspension:

The effect of the application of 2, 4-D (0.9×10^{-6} M, 2, 4-Dichlorophenoxyacetic acid) on the course of cell proliferation in terms of dry weight increase in culture flasks is shown by the data of Fig. 1. in which the dry weight per flask is plotted against time. It is significant that an isolated carrot cell line in a liquid medium with 2, 4-D showed an active growth and a remarkable yield in cell volume of suspension was obtained at the end of a passage of 14 days' culture. The cells were subcultured routine in every 14 days. The growth curve in the figure, when cells were cultured in medium with 2, 4-D, indicates an exponential growth phase of 8 days after inoculation followed by a stationary increase in dry weight. While the oxygen uptake curve during the subculture plotted as $\mu 10_2$/mg dry weight/hr obtained using the apparatus of Gilson differential respirometer reveals a relatively high respiratory rate during the exponential growth phase but somehow decrease in the later period, where proembryogenic masses of cell aggregates could be formed.

The respiratory rate of inoculum was not accessible to measure the real oxygen consumption. Changes in number of cell mass by dilution and nutritional conditions upon the transfer of inoculum affected greatly to the net oxygen uptake in the inoculum stage.

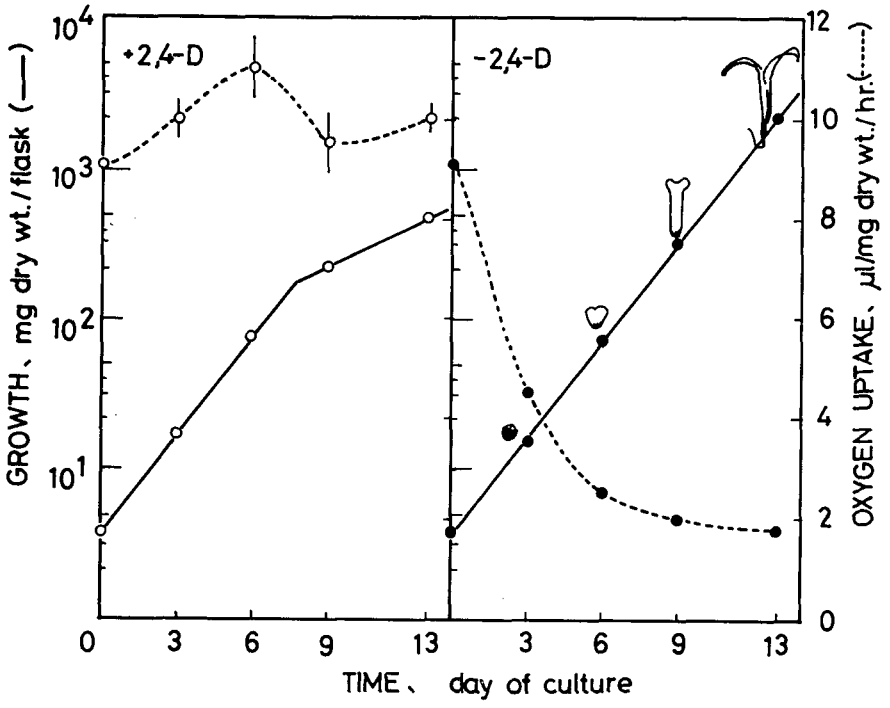

Fig. 1. Growth and respiration of carrot cell suspension cultured in medium with 2, 4-D and without 2, 4-D. Although net oxygen uptake by embryogenic culture increases with age, expressed on a per dry weight basis, the activity is highest in globular embroys. On the growth curve of the graph, developmental stages of shapes of embryos are shown.

Somatic embryogenesis in this cell line of carrot cell suspension was normally suppressed under the conditions of subcultures. The withdrawal of 2, 4-D allowed to develop the embryogenic potentials in carrot cells. The studies were also carried out with four arbitrarily recognized structures that were globular, heart- and torpedo-shaped embryos as well as the proembryogenic cell masses of inoculum from which the somatic embryos were initiated.

Under the conditions favourable to embryogenesis described, the cells grow vigorously as shown in the same figure for growth as dry weight increase which extends to about 14 days. The cells are in an exponential phase for about 12 days and during this time the mean generation time is approximately 33 hours. An inoculum of 4 mg (dry weight) of cells increases 500 folds masses of somatic embryos in dry weight basis during 13 days'

culture. Although the net oxygen uptake by carrot embryos increases with age, express on a per embryo basis, the activity is the highest in young embryos, and the lowest in plantlets which having an axis with cotyledons and radicle. On the growth curve of the graph, the developmental stages of embryo with arbitrary shapes are shown.

Changes in cellular constituents of carrot cell suspension:

An attempt to estimate the amounts of cellular constituents on which the dry weight increase is dependent has been made. Since the inoculum of proembryogenic masses of cells prepared from carrot cell suspension subcultured in medium with 2, 4-D are rich in protein bodies and oils, the accumulation and degradation of storage materials is one of the main interests in the study of biochemical changes during embryogenesis. Nevertheless, the great increase in dry weight was observed in embryogenic culture, while the moisture was continuously decreased with increasing the stage of embryogenic development (ca. 89% in globular to 87% in plantlets). In cell suspension cultured in medium with 2, 4-D, the moisture content was increased due to the occurrence of large vacuolated cells in the stationary phase. The major cellular constituents of carrot cell suspension and embryo

TABLE 1. Major cellular constituents of carrot cell suspension cultured in medium with and without 2, 4-D

Days in culture		Per cent of dry matters			
		Protein (%)	Lipid (%)	Starch (%)	Cellwall (%)
	Cells with 2, 4-D Subculture				
0	Inoculum	23.1	22.3	8.8	5.5
3		17.1	14.5	7.3	3.8
6		19.2	15.5	13.0	3.1
9		20.1	14.0	9.9	4.1
13		22.3	31.7	16.8	5.1
	Cells without 2, 4-D Embryogenesis				
0	Inoculum	27.6	24.4	11.5	11.9
3	Globular embryo	36.5	29.9	8.9	18.3
6	Heart-shaped	32.9	30.5	9.7	13.4
9	Torpedo-shaped	28.5	26.9	26.9	11.3
13	Plantlet	25.6	21.9	12.4	10.9

The others are soluble solids.

types are shown in Table 1. In the table are realized the accumulation of protein in globular stage, lipid bodies in heart-shaped embryos and starch grains in torpedo-shaped embryos and the degradation reserve materials, namely protein, into soluble fraction during plantlet formation. The relatively greater deposition of hemicellulose and cellulose was observed in embryogenic culture, but slightly lesser extent in cell suspension subcultured in medium with 2, 4-D.

Glucose uptake and oxidation:

Glucose uptake was measured by summing up the radioactivity each of isolated fractions from cell suspension after respiratory measurement of 2 hours. Cell suspension cultured in medium with 2, 4-D took up glucose from medium at about the same rate throughout the passage of subculture, while on the course of embryogenesis cells in medium without 2, 4-D utilized more glucose from medium than those of subculture. The drastic change of glucose uptake in embryogenic culture is shown in Fig. 2.

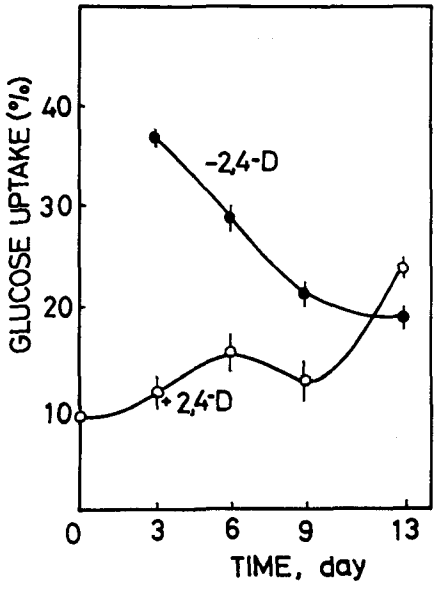

Fig. 2. Glucose uptake of carrot cell suspension cultured in medium with and without 2, 4-D. Embryogenic culture signs −2, 4-D, and the addition of 2, 4-D to medium suppressed embryogenesis.

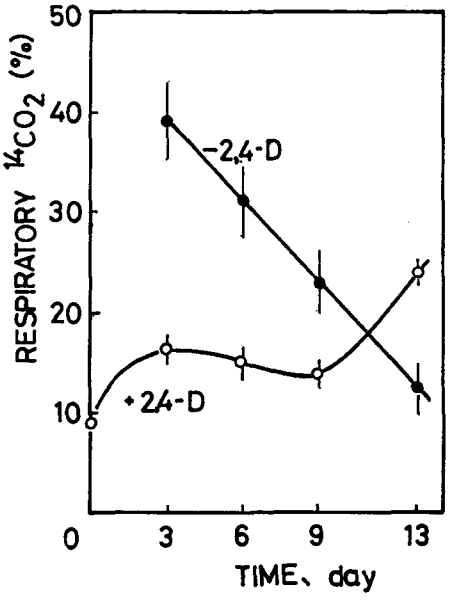

Fig. 3. Percentage utilization of glucose taken up for respiratory CO_2 by carrot cell suspension cultured in medium with and without 2, 4-D.

The metabolic activation of carrot cell suspension by the removal of suppression of embryogenic potentials after inoculation of proembryogenic masses of cells into nutrient medium without 2, 4-D obviously has a marked effect on the rate of increased glucose uptake, since there appears to be a great difference of glucose utilization between the cells in medium with or without 2, 4-D during the initial exponential growth phase of cultures. Approximately 50% of the total labeled glucose taken up was remained in the neutral fraction after extraction by aqueous alcohol followed by the separation of acidic and basic fractionation by means of ion exchange resins.

The recovery of $^{14}CO_2$ from labeled glucose incorporated in cells is illustrated in Fig. 3. In cell suspension cultured in medium with 2, 4-D the production of respired $^{14}CO_2$ was 10 to 20% of total glucose taken up, while the surprising amount of $^{14}CO_2$ from labeled glucose was recovered from the globular embryos, *i. e.*, the 3rd day culture after inoculation into medium without 2, 4-D. This is due to relatively small pool size present in globular embryos when compared it with cell suspension.

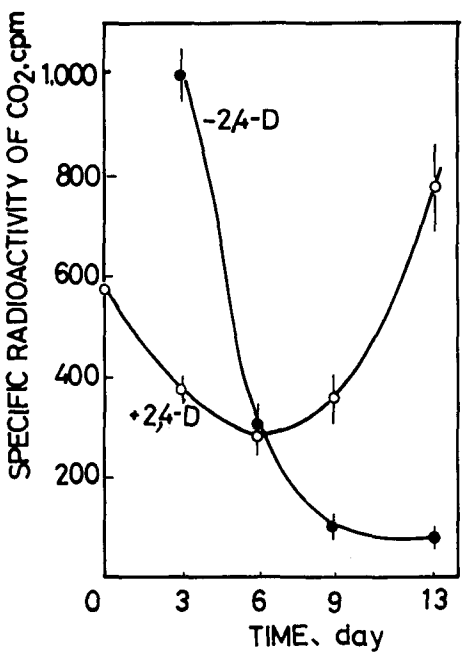

Fig. 4. Specific radioactivity of respired CO_2 in carrot cell suspension cultured in medium with and without 2, 4-D. The data obtained from the ratio of CO_2/O_2 (cpm/μl), assuming RQ approximates unity.

Respiration and pathway participation:

In Fig. 4., the specific utilization of glucose for respiratory substrate is illustrated in which the removal of 2, 4-D from cell suspension indicated the remarkable induction of metabolic activation in carrot cells. Also is it significant that the specific activity of released $^{14}CO_2$ was successively decreased in concomitant increase with the stage of embryogenic development in carrot cells.

The release of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C of specifically labeled glucose was studied as parameters of respiratory activity and pathway participation as the C 6/C 1 ratio in Fig. 5. In cell suspension cultured in medium with 2, 4-D, C 6/C 1 ratios were constant during subculture period, while embryogenic culture had increased glucose oxidation immediately after inoculation in medium suitable for embryogenesis, indicating the C 6/C 1 ratio of 0.85 with respect to globular embryos and the ratio of 0.6 to torpedo shaped embryos. On the stage of plantlet formation where bipolar embryos were established, the ratio was suddenly decreased to the level as low as 0.35, the value of which was equivalent to those of cell suspension cultured in medium with 2, 4-D in which the proembryogenic masses of cell aggregates were found.

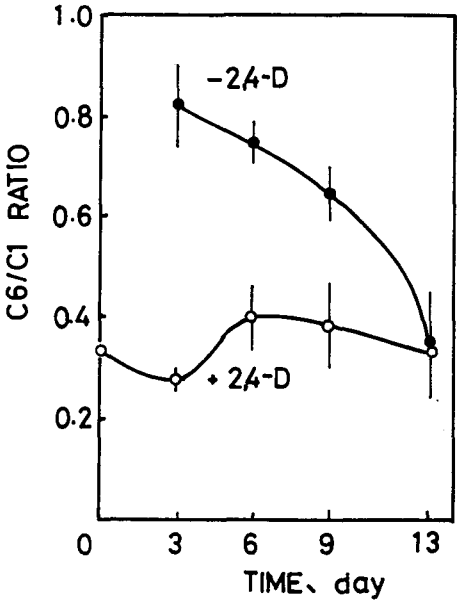

Fig. 5. The C6/C1 ratios in carrot cell suspension cultured in medium with 2, 4-D and without 2, 4-D.

Glucose incorporation into cellular constituents:

To assess possible differences between the patterns of glucose metabolism of carrot cell suspension with embryogenic potentials from those of embryos developing to plantlets, the relative incorporation of glucose into cellular constituents were determined. The capacity of carrot cell suspension to metabolize glucose to cellular constituents does not change markedly with age of culture if comparisons are based upon the dry weight of cells. The increase in glucose uptake was mainly dependent upon the activation of respiratory metabolism by the withdrawal of 2, 4-D and probably upon the capacity of the induced protein synthesis. In Table 2 are listed the percentages of glucose label that was incorporated into lipids, starch and proteins and cell-wall by cell suspension culture. The differences in the amount of glucose utilized between two cell suspension cultures in medium with and without 2, 4-D were not significant.

In general, cell suspention of subculture utilized more glucose for the synthesis of lipids than did the embryogenic culture. The amounts of glucose incorporated into starch and cell-wall polysaccharides (cellulose, hemicellulose,

TABLE 2. The Utilization of glucose by carrot cell suspension cultured in medium with and without 2. 4-D

Days in culture		Percentage of glucose incorporated into				
		Protein (%)	Lipid (%)	Starch (%)	Cellwall (%)	CO_2 (%)
	Cells with 2, 4-D Subculture					
0	Inoculum	0.3	3.5	7.2	8.6	8.8
3		0.4	8.2	6.0	7.8	18.2
6		0.8	6.2	6.8	9.4	16.7
9		0.4	4.0	6.0	8.2	11.5
13		0.9	4.1	10.5	13.2	23.4
	Cells without2, 4-D Embryogenesis					
0	Inoculum	0.2	4.0	6.7	4.9	8.8
3	Globular	1.6	3.2	7.1	8.7	33.7
6	Heart-shaped	2.8	2.7	8.2	8.3	29.2
9	Torpedo-shaped	2.7	5.1	5.9	9.6	19.1
13	Plantlet	2.6	3.4	6.0	7.5	14.3

The other components are alcohol soluble materials.

and pectic substance) were roughly comparable to the amounts of the two fractions of both cultures with the greatest incorporation into starch occurring in globular and heart shaped embryos. Note that the greatest incorporation of glucose into respiratory CO_2 also occurred in embryos of globular stage.

Discussion

Under the conditions described, carrot cells grow vigorously as shown in Fig. 1., for growth as dry weight increase which for carrot cells extends to about 14 days when subcultured routine way. The cells are in exponential phase for about 8 days and during this time the mean generation time is approximately 36 hours. When the QO_2 of exponentially growing carrot cell suspention determined by standard manometric method is about 10 $\mu l O_2$/hr/dry weight basis, the total amount of oxygen uptake for a mean generation time, while the cells are growing exponentially, is given by an approximation of:

$$3/2tQO_2\,dwt$$

where t: mean generation time (hr)
QO_2: $\mu l O_2$ uptake/hr/mg dry weight
dwt: initial dry weight in mg.

Substituting into the above equation, one calculates that 5.4 ml O_2 are consumed while culture increase by 10 mg dry weight. According to LAMPORT[5], the efficiency of glucose metabolism during exponential growth of cell suspension culture could be calculated from the following assumptions. Assuming an average weight of residual moiety of carbohydrate as 162 (180-18) and RQ would be unity, then 5.4 ml O_2 uptake/10 mg cell stuff synthesized corresponds to : 225 μmoles of O_2/61.7 μmoles synthesized, or 225/6=37.5 μmoles of hexose oxidized/61.7 μmoles synthesized. Thus one mole of hexose is oxidized for the addition of 1.65 moles of cell stuff, giving an apparent synthetic efficiency of 1.65. This figure is calculated on the basis of including the intracellular sugar (approximately one third of the cellular dry weight). While, the synthetic efficiency of the respiratory metabolism in embryo increases upto 16.0 in late torpedo-shaped embryos and plantlets whose the respiratory rates were minimum of 2.0 $\mu l O_2$/hr/mg dry weight.

Carrot cell suspension of high embryogenic potentials contains such large amounts of extracellular reserve stuff of protein, lipid and starch that it is difficult to make comparisons between the metabolic activities of subcultured cells and developing embryos when the activities of growing cells are expressed either in terms of its fresh weight, its protein content or its

nitrogen content. It has been pointed out the importance of basing comparisons of the activities of cells upon the metabolically active component of cells, the number of cells present of which the DNA content is direct measure. When DNA is used as the basis for comparisons, there are in general differences between the metabolic capacity of cells and DNA content of the cells in which DNA be an intrinsic capacity of metabolic activity. In contrast to comparisons based on dry weight which suggests that cell suspension cultured in medium with 2, 4-D shows an increase capacity to synthesize lipid and starch, the globular embryos show an increase in the capacity to synthesize protein and starch, and to metabolize more glucose to CO_2 together it with to cell-wall polysaccharides.

The effects of the withdrawal of 2, 4-D upon glucose metabolism of carrot cell suspension, *i. e.*, the rate of increased glucose uptake and $^{14}CO_2$ production from glucose-6-^{14}C and reduced incorporation of labeled glucose into lipids are characteristic of the changes produced by the release of suppression of embryogenic potentials.

The increase in glucose uptake and CO_2 release may well be another instance of stimulated embryogenesis by cyanide[4]. There was a relationship between the change in the cyanide sensitivity of respiration, the cellular ATP concentration and somatic embryogenesis. When the accumulation of ATP were required for embryogenesis, the metabolic activation of globular embryos would be in accordance with the most distinctive utilization of glucose catabolized via the glycolytic-TCA cycle pathway to respiratoty CO_2 and to generate ATP.

The operation of the pentose phosphate pathway during cell multiplication and differentiation in cell suspension cultured in medium with 2, 4-D was considerably augmented not only by tracing the simultaneous anabolism of the increased lipid synthesis in these cells but also by examining the state of cell growth when the cells regenerated their masses rapidly. The significant increase of the participation of the pentose phosphate pathway in torpedo-shaped embryos and more likely in plantlets was also recognized where cells predominantly differentiated to organize a bipolar axis with apical meristems. These changes of respiratory metabolism during the embryogenic development following the initiation of proembryogenic masses of cell aggregates are interpreted as a series of developmental activations such as induced respiration[1] that involve synthesis of ribosomal RNA and protein *de novo*[2,8].

Summary

1. Somatic embryos are initiated from carrot cell suspension by the removal of 2, 4-D from the nutrient medium.

2. Synthetic efficiency of cell suspension is low as 1.65., while the efficiency increases with age of embryogenic development to 16.

3. The metabolic activation was observed in embryogenic culture whose metabolic pathway was predominantly the glycolytic TCA cycle to respire CO_2 in globular embryos, while the participation of the pentose phosphate pathway predominates in late torpedo-shaped embryos and plantlets. The low value of 0.35 was recorded for cell suspension cultured in medium with 2, 4-D in which the proembryogenic masses of cell aggregates were apparantly found.

4. The changes of cellular constituents of storage stuff were discussed in relation to the glucose assimilation to these reserve materials of lipids and starch and of protein.

Acknowledgments

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