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Turnover of Galactosylglycerol and Osmotic Balance in *Ochromonas*[1]

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ABSTRACT

Osmotic balance in *Ochromonas malhamensis* is mediated directly by fluctuations in the pool size of α-galactosylglycerol (isofloridoside). Chase experiments with glucose-^{14}C indicate that the pool is in rapid turnover even at constant size. The turnover rate is related to the pool size. Regulation of the pool size seems to occur at enzymic steps involved in the formation, as well as those involved in the degradation of isofloridoside.

The osmotic balance in the golden brown flagellate *Ochromonas malhamensis* is mediated by IF.[2] When substances raising the osmotic pressure, such as salts, glucose, mannitol, or polyethyleneglycol, are added to a flagellate suspension, the cells first shrink and then regain their volume because of the accumulation of IF in a concentration high enough to compensate directly for the outside stress. Any organic material such as exogenous glucose, photosynthesis products, or endogenous reserve polysaccharides can be used to produce IF. If the outside osmotic pressure is decreased, the osmotic excess of IF is converted to reserve β-1 → 3-glucans. Thus, the two carbohydrates seem to be readily interconvertible, and the direction of the conversion is determined by the osmotic value of the surrounding fluid (3, 7). This offers a simple model system for studying the biophysical and biochemical steps involved in the regulation of osmotic balance, since the biochemistry and regulation of carbohydrate metabolism seems fairly well understood.

The formation and degradation of IF appears to involve the classical glycolytic reactions (4) including a β-1 → 3-glucan phosphorylase (1). The key enzyme leading to IF formation is an UDP-gal:*sn*-glycero-3-phosphoric acid 1-α-galatosyl-transferase (5). Physiological experiments suggest that the regulation involves activation of pre-existing enzyme molecules rather than *de novo* synthesis of enzymes (7).

To evaluate enzyme studies, information on the flow of carbohydrate into and out of the IF pool is needed. In this connection, the results of chase experiments with glucose-^{14}C in suspensions of *Ochromonas* under various external osmotic conditions are reported here, and the possible sites of regulation of the IF pathway are discussed.

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[2] Abbreviation: IF: isofloridoside or α-galactopyranosyl(1 → 1)glycerol.

MATERIALS AND METHODS

Ochromonas malhamensis var. Pringsheim was grown on a defined medium as described elsewhere (7) and suspended so that a 1:50 dilution gave an o.d. of 0.4 to 0.8 (510 nm, 1-cm light path). The suspension medium had the same composition as the growth medium but only at one-fifth the concentration and 2 to 4 mg/ml of glucose. The suspension was aerated at 27 C and illuminated with one daylight fluorescent tube at about 35 cm distance (2 klux) for about 1 hr to reach steady state conditions. A solution of 0.75 M NaCl was added when the osmolality had to be raised and a decrease in osmotic pressure was effected by appropriate dilution with water.

As other experimental necessities caused the osmotic value to vary slightly with different experiments, it was measured routinely using the supernatant fraction from a 1-ml aliquot and an electronic semimicroosmometer (Knauer and Co., Berlin), calibrated against a 0.4 osmolal solution of NaCl (12.687 g/kg water).

At the times indicated by the arrows in the figures, 1 μc/ml glucose-U-^{14}C (300 mC/mmole) was added, and samples equivalent to 1 ml of the original suspension were pipetted into centrifuge tubes held at 90 C, heated for a further 10 min, freeze-dried, reconstituted with 1 ml of water, and centrifuged. The supernatant from this centrifugation contained the water-soluble material out of the cells originally present in 1 ml of algal suspension. All data and calculations are given, therefore, per 1 ml of the original cell suspension. In some cases, the algae were inactivated in 4 volumes of hot 99% (v/v) ethanol and held for 5 min at 75 C. The ethanol was removed with a stream of air, and the sample was treated as above.

For the determination of IF, 0.2 ml of the cell extract was mixed with 0.2 ml of 0.1 M sodium acetate at pH 5.6 and 0.1 ml of toluene to prevent bacterial growth. After incubation with 0.5 mg α-galactosidase (6) for about 50 hr at 30 C, the liberated glycerol was measured enzymically with glycerokinase and pyruvate kinase (2). With this method, 90 to 95% of authentic α-galactopyranosyl-(1 → 2)-glycerol was found when added to the algal cell extracts.

To separate the radioactive glucose and IF, 40 μl of the cell extract were applied as 5-cm streaks on Whatman No. 1 filter paper and chromatographed for 45 hr in 1-butanol-pyridine-water-concentrated acetic acid (60:40:30:3[by volume]). The spots of glucose and IF were located by radioautography and counted on the filter paper directly in a solution of 5 g PPO/l of toluene in a Beckman LS 100. The radioactivity remaining at the start line was counted as above to obtain a crude measure of the amount of glucose-^{14}C incorporated into the reserve β-1-3-glucan (polysaccharide in Table I). The amount of radioactivity in IF of the sample taken about 5 sec after the addition of glucose-^{14}C was subtracted from all values. This

was necessary because the added glucose-^{14}C was contaminated with traces of galactose-^{14}C which moved together with IF in the above solvent mixture.

After counting the ^{14}C-glucose as above, the spot was eluted from the chromatogram, the amount of substance was determined with the glucose oxidase method using a test kit of the Boehringer-Mannheim Co., and the specific radioactivity was calculated.

RESULTS AND DISCUSSION

To demonstrate turnover of the IF pool in *Ochromonas* the algae were suspended in dilute nutrient solution containing glucose-^{12}C, and glucose-^{14}C of high specific radioactivity was added under various osmotic conditions. Suspended in a medium of low osmotic value (Fig. 1A) the cells contained a low but constant amount of IF. When the external osmotic pressure was increased, the IF pool size increased and reached a high level at which it remained constant as long as the osmotic stress was not diminished (Fig. 1, B and C). A decrease in osmotic stress was followed rapidly by a sharp decrease in the IF present in the cells (Fig. 1, D and E).

At any level of osmotic value and pool size, radioactive glucose was incorporated into IF. The amount of IF-^{14}C formed from the exogenous glucose-^{14}C can be calculated from the specific radioactivity of the latter and is included as μmoles/ml·hr in Table I. There was a 10-fold increase shortly after the increase in osmotic pressure when a net increase in pool size occurred (Table I, A and B). This indicates that the amount of IF in the cells is increased by enhanced production rather than by reduced degradation.

The amount of IF-^{14}C formed from the exogenous glucose-^{14}C shortly after the increase of the osmotic value (Table I, B) accounts for about 83% of the net change in pool size determined by direct measurement. This indicates that in the presence of exogenous glucose the IF is produced mainly with carbon material from this source as only minor amounts of unlabeled endogenous material seem to enter the pool. This conclusion is in agreement with the results of unpublished experiments similar to those reported in Figure 1 and Table I, but with cells in which the cell reserve materials were labeled by preincubation with glucose-^{3}H. When glucose was not supplied in the suspension medium, the IF was formed at about the same rate from endogenous material alone.

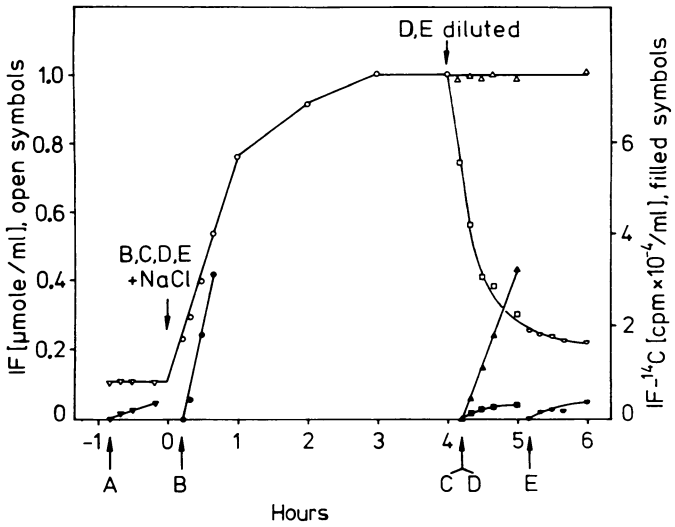

FIG. 1. Pool size and labeling of IF under various osmotic conditions. Five identical suspensions: A, ▽—▽ and ▼—▼; B, ○—○ and ●—●; C, △—△ and ▲—▲; D, □—□ and ■—■; E, ◡—◡ and ◒—◒ of *Ochromonas* in dilute nutrient solution containing 4 mg/ml glucose were aerated. At the times indicated by the arrow, NaCl was added to increase the osmotic value in samples B, C, D, and E from 30 to 185 mOsmole. Samples D and E were diluted 4 hr later to bring the osmotic value from 185 down to 30. Glucose-^{14}C (1 μc/ml) was added at the times indicated by the lower arrows to the respective samples; aliquots drawn and inactivated by heating in water.

Table I. *Products Formed from Glucose-^{14}C under Conditions of Low and Increased External Osmotic Value*

Same experiments and sample treatment as Figure 1.

Sample in Fig. 1	Osmotic Condition	Glucose-^{14}C Converted			IF-^{14}C Formed from Glucose ^{14}C[3]	IF Net Change[4]
		Totally[1]	To polysaccharide	To IF[2]		
		μmoles/ml·hr				
A	Constant, 30 mOsmole	2.30	0.68	0.09	0.06	0.0
B	10 min after increase	2.40	0.62	0.82	0.54	+0.65
C	Constant, 185 mOsmole	2.57	1.55	0.45	0.30	0.0
D	10 min after decrease	2.20	0.34	0.05	0.03	−0.81
E	70 min after decrease	2.60	0.51	0.05	0.03	−0.04

[1] Calculated from the specific radioactivity of glucose-^{14}C from the amount which disappeared from the suspension fluid.

[2] Calculated from the initial slope of the curves with the filled symbols in Fig. 1 using the specific radioactivity of glucose-^{14}C.

[3] Calculated from the amount of IF-^{14}C formed from glucose-^{14}C assuming that 1 molecule of IF will be generated from 1.5 molecules of glucose.

[4] Calculated from the curves with open symbols of Figure 1.

It is also evident from two other findings that the regulation occurs at least in part at the site of production. When the constant high pool size was reached the inflow of material into IF fell to 55% of the maximal value (Table I, sample C compared with B). The values in other experiments not documented here were 47, 63, 63, and 69%, respectively. In addition, 10 min after a decrease of the osmotic value (Table I, D) the incorporation rate was diminished again to about the value measured before the addition of the osmotic agent (Table I, A).

Obviously, reactions leading to the formation of IF are strongly stimulated when an osmotic stress is applied to the algal cells. This stimulation is diminished but not nullified when the osmotic balance is reached by the production of IF. Only under conditions which do not require the build up or maintenance of a high pool size will the rate of IF formation fall to the basic level. The differences in the rates of IF production can be explained only on the basis of the activity of the respective enzymes. Obviously, these enzyme activities can be modified depending on the requirement of the cells for osmotic potential.

One point possibly not directly related to the formation of IF should be stressed. When the cells under high osmotic pressure had reached a constant high IF content then the assimilation of glucose-^{14}C into polysaccharide was found to be increased, although the total amount of glucose-^{14}C metabolized was not altered significantly (Table I, C). This result is not easily explained, but may indicate that under this condition metabolic reactions other than those directly involved in the biosynthesis of IF are influenced also.

The observed flow of material into pools of constant size re-

quires that the same amount of IF disappears per unit of time. This would mean that under the conditions resulting in high pool size (Table I, C) at least 0.30 μmole and under conditions resulting in low pool size (Table I, A) 0.06 μmole of IF are degraded per hr per ml suspension. It was shown before (3) that labeled IF which disappears from the cell upon a decrease of osmotic value is fully transformed to the reserve polysaccharide chrysolaminarin, a β-1-3-glucan. The degradation products of the IF from the turnover at constant pool size are, therefore, very likely converted also to reserve polysaccharides.

One might speculate that the higher degradation rate of IF observed at a high and constant pool size (Table I, C) as compared to a low and constant pool size (Table I, A) is a direct consequence of the higher concentration of IF as the responsible enzymes may now function closer to substrate saturation. The net change in pool size of the IF after dilution (Table I, D), however, is almost three times higher than the degradation rate deduced from the turnover rate at the high osmotic value (Table I, C). The degradation capacity, therefore, clearly appeared enhanced shortly after the decrease in osmotic stress.

It was first thought that this observation could possibly be due to an artifact as the rates in two parallel suspensions were compared and after the dilution a greater volume of the algal

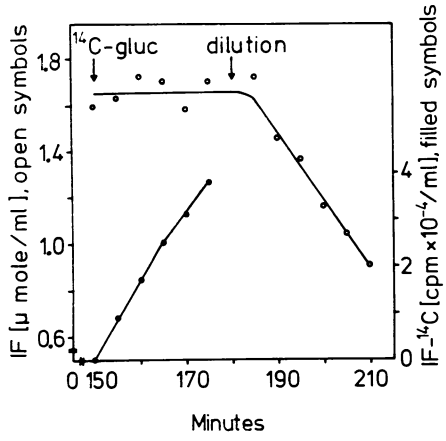

FIG. 2. Incorporation of glucose-^{14}C into IF at constant high pool size and decrease of the IF pool after a decrease in osmotic value. A suspension of *Ochromonas* was aerated for 2.5 hr in dilute nutrient solution containing 4 mg/ml glucose and NaCl (207 mOsmole), 1 μc/ml glucose-^{14}C was then added and samples taken. Three hours after starting, the osmotic value was decreased by dilution with 4 volumes of water to 38 mOsmole. All samples were inactivated with hot ethanol. From the slope of the curve with filled symbols, it can be calculated in a way similar to Table I, that 0.39 μmole/ml·hr of IF were formed from the glucose-^{14}C at high osmotic value whereas the net degradation after dilution was 1.71 μmole/ml·hr. Mean values are from four determinations on each point.

suspension had to be inactivated by heating from 27 to 90 C. The experiments were carefully repeated with a single suspension and inactivation of the algae in hot ethanol (Fig. 2). In this experiment also, the net degradation rate after dilution was found to be 4.4 times higher than at high osmotic value. Experiments not documented here gave values of 4.5-, 7.2- and 7.5-fold, respectively.

Obviously, the degrading enzyme system is under control also and can be appropriately regulated if necessary to modulate the pool size. The net degradation reaches a constant rate after about 5 min. It was found before (7) that the time necessary to reach a constant net formation rate after an increase in osmotic value, in contrast, is about 1 to 2 min. Thus the response times of the osmoregulation system to the osmotic stimulus appears to be different for the two events.

The IF content of the cells of *Ochromonas* which in turn leads to a regulation of the osmotic balance seems to be controlled at the site of production as well as at that of degradation. The mechanism by which changes in the osmotic value of the surrounding fluid are sensed and converted to information capable of regulating such biochemical reactions is unknown. Some indirect evidence, however, in regard to the type of regulation at the biochemical level is available. The stimulation of IF formation is insensitive to inhibitors of protein synthesis (7). This together with the very short lag time (ref. 2, see also Fig. 1,B) allows the conclusion that the regulation involves activation of preformed enzymes either by allosteric mechanisms or enzymatic modification. At least one of the regulated enzymes appears to be the transferase responsible for the formation of the phosphorylated precursor of IF, as the apparent activity of this enzyme is higher in cells treated osmotically (5). The turnover data reported here suggest that, in addition, enzymes of IF degradation may have a regulatory function.

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