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A BIOCHEMICAL INVESTIGATION INTO THE INHIBITORY EFFECTS OF GLUCOSAMINE AND *N*-ACETYLGLUCOSAMINE ON THE AGGREGATION *IN VITRO* OF EMBRYONIC CHICK MUSCLE CELLS

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SUMMARY

The effect of glucosamine and *N*-acetylglucosamine on aggregation and energy metabolism was investigated over an 8-h period in cells dissociated by 0·25 % (w/v) trypsin from the skeletal muscle of 9-day-old chick embryos. At 8 h, 0·023 M glucosamine and *N*-acetylglucosamine inhibited the aggregation of cells suspended in Eagle's minimal essential medium by 18·9 % and 16·4 % respectively, as judged on a basis of aggregate size.

Glucosamine and *N*-acetylglucosamine reduced the cellular ATP level by a mean of 36 % and 27 % respectively; values reflected in the 32 % and 19 % loss of total adenine nucleotides caused by these sugars. The adenine nucleotide balance was also changed from a mean control ATP/AMP ratio of 13·7 to 8·75 by *N*-acetylglucosamine and to 8·0 by glucosamine.

Intracellular lactate/pyruvate ratios were similarly disrupted in cells incubated with 0·023 M hexosamine. Although the hourly values fluctuated, it was seen that the amount of lactic acid relative to pyruvic acid, considered as an average for the 8-h period, was raised from 6:1 in controls to 8:1 in *N*-acetylglucosamine-treated and to 10:1 in glucosamine-treated cell preparations. Compared to controls at 8 h, glucosamine enhanced the production of lactate into the suspension medium by 99 %. The *N*-acetyl analogue caused cells to produce more lactic acid than did controls for 4–5 h only, for by 8 h 25 % less of this metabolite was assayed in the culture medium.

The incorporation of D-[U-^{14}C]glucose into glycogen paralleled the results of extracellular lactic acid assays. *N*-acetylglucosamine inhibited the incorporation by 30 % at 4 h, although by 6 h, and for the remainder of the experimental period, there was more ^{14}C-labelled glycogen in these cells than in controls. By contrast, glucosamine inhibited the incorporation of radioactive glucose into glycogen by 42 % at 4 h and, unlike *N*-acetylglucosamine, consistently thereafter.

Glucosamine also enhanced cellular oxygen uptake throughout the experimental period, to the extent of 59 % at 8 h. The oxygen uptake of *N*-acetylglucosamine-treated cells was similar to controls until about the 5th hour, when there was a subsequent inhibition which had accumulated to 13 % by the end of the experiment.

The release of $^{14}CO_2$ by cells was inhibited by glucosamine. This hexosamine depressed production by 19 % at 12 h whereas *N*-acetylglucosamine inhibited this evolution by 9 % at this time.

The metabolic effects of these hexosamines on chick muscle cells *in vitro* are mainly attributed to a central alteration of the adenine nucleotide balance although certain other documented effects of glucosamine are considered to be involved. An inhibition of cell aggregation by glucosamine and *N*-acetylglucosamine is discussed in terms of a depressed cellular metabolic economy.

INTRODUCTION

Sialic acid-containing glycoproteins occur in a number of animal tissues, characteristically as a membrane component (Cook, 1968; Spiro, 1970). Location of sialic acid moieties at the plasma membrane, in particular, had led to their suggested involvement in a number of surface-mediated biological functions, notably cell adhesion (Ambrose, 1966; Curtis, 1967). That sialic acid has a role in this phenomenon has been implied from investigations which showed a decreased adhesiveness of tissue cells to be a consequence of the specific enzymic removal of surface sialic acid (Weiss, 1961; Kemp, 1970) although an increased level of this compound at the cell periphery has also been held to account for the non-adhesiveness of some neoplastic cell strains (Forrester, Ambrose & Stoker, 1964). These findings led to the suggestion that the adhesive process may be impaired by levels of surface sialic acid outside a certain range (Kemp, 1970).

It was considered possible to examine this suggestion by testing the effect of exogenous glucosamine (2-amino-2-deoxy-D-glucose) on the aggregation of tissue cells. This hexosamine, which is incorporated into cell sialic acid (Molnar, Lutes & Winzler, 1965; Kohn, Winzler & Hoffman, 1962), could uncouple glycoprotein synthesis as an extension of its inhibitory effect on other aspects of metabolism (Bekesi & Winzler, 1969; Bekesi, Bekesi & Winzler, 1969) and thereby modify the chemistry of the cell surface. Indeed, Rizki (1961) found that a 5% (w/v) solution of glucosamine reduced the mutual adhesiveness of larval *Drosophila* lamellocytes and Garber (1963), using trypsin-dissociated embryonic chick cells, proposed that a derangement of the cell surface could account for the inhibited aggregation of glucosamine-treated cells. The latter study also reported *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) to be without effect on aggregation and it has therefore been employed in this present investigation as a control for the administration of glucosamine.

Previous investigations into the biochemical effects of exogenous glucosamine have indicated that this glucose analogue may interfere with both glycogen and glucose metabolism (Spiro, 1958; Kono & Quastel, 1962). Similarly, since glucosamine is incorporated into uridine diphosphate-(UDP) *N*-acetylglucosamine (a precursor of sialic acid) after the step by which this amino-sugar nucleotide controls its own formation by feedback inhibition (Kornfeld, Kornfeld, Neufeld & O'Brien, 1964), addition of exogenous glucosamine initiates an uncontrolled synthesis of UDP-*N*-acetylglucosamine which proceeds with a concomitant depletion of the uridine and adenine nucleotide pools (Bekesi & Winzler, 1969). Since these effects could be expected to depress cellular energy levels, which of itself may influence the extent of aggregation, it was decided to examine this facet of the metabolic effects of glucosamine as a necessary prelude to investigating the glycoprotein metabolism of glucosamine-treated cells.

It is the aim of this present study, then, to monitor the effect of glucosamine on some parameters of cellular energy production and to attempt to relate these to the state of cellular aggregation.

MATERIALS AND METHODS

Cell dissociation

Cells were dissociated from the skeletal muscle of 9-day-old chick embryos by treatment for 10 min with a 0·25 % (w/v) solution of crude trypsin (Burroughs Wellcome, 1:300) at 37 °C, according to the standardized method employed in this laboratory (Kemp, Jones, Cunningham & James, 1967). The dissociated cells were washed in Hanks's balanced salts solution and finally resuspended in Eagle's minimal essential medium (MEM). The cell density of cells in the suspension was estimated using a modified Fuchs–Rosenthal haemocytometer. Sterile conditions were maintained throughout the dissociation of the tissue and its subsequent culture.

Aggregation techniques

Aggregation of the dissociated cells was quantitatively estimated by the gyratory shaker method (Moscona, 1961). The cell suspension was placed into 25-ml Erlenmeyer flasks in aliquots of 3 ml at a concentration of $3{\cdot}0 \times 10^6$ cells/ml. D-Glucosamine hydrochloride or *N*-acetylglucosamine (Sigma Chemical Company) in Eagle's MEM was then added to the flask contents to a final concentration of 0·023 M, control flasks receiving an equal volume (0·1 ml) of Eagle's MEM instead of hexosamine. Resulting from the addition of glucosamine as its hydrochloride, the pH of the medium decreased and was therefore corrected with 0·1 ml of 4·4 % (w/v) sodium bicarbonate. This same volume of bicarbonate solution was dispensed to *N*-acetylglucosamine and control cell suspensions. Contents of all the flasks were then adjusted to pH 7·4 by equilibration against a 5 % CO_2/95 % air mixture (Paul, 1965) before being sealed and incubated at 37 °C on a gyratory shaker rotating at 70 rev/min. The process of cellular reaggregation was followed throughout the culture period by determining the dimensions of a given number of aggregates with a calibrated eyepiece micrometer. The resulting data were analysed by linear regression. Viability was examined by estimating the percentage of cells excluding particles of lissamine green dye (Kemp *et al.* 1967) in a 2 % (w/v) aqueous solution.

Analytical methods

Intracellular levels of adenine nucleotides, pyruvic acid and lactic acid were determined in an acid-soluble fraction isolated at given times from the cellular material by a standardized extraction procedure. It was essential for all steps to be performed rapidly and in the cold (4 °C) to minimize breakdown of the labile intermediates.

Contents of each culture flask were poured into a ground-glass homogenizer (Griffiths tube) pre-cooled to −15 °C and the cellular material was then centrifuged for 90 s at 1000*g*. The supernatant was removed and retained for measurement of 'extracellular' lactate. The cell pellet was homogenized with 5 vol of 0·6 N perchloric acid and centrifuged at 2000*g* for 10 min. The acid-soluble extract was removed and the insoluble material triturated with 2·5 vol of 0·3 N perchloric acid. After centrifugation the 2 acid-soluble extracts were combined and brought to pH 6 by the cautious addition of 2 N KOH. These solutions were allowed to stand for at least 30 min to ensure complete precipitation of the insoluble potassium perchlorate. The clear extract was removed after centrifugation and adjusted to a known volume with distilled water before being stored frozen overnight.

After the frozen extracts had been allowed to reach the temperature of ice-water, intermediates were estimated enzymically by ultraviolet spectrophotometry, utilizing test-combinations supplied by C. F. Boehringer und Soehne GmbH (Mannheim, W. Germany). Adenosine triphosphate (ATP) was determined with glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3) by the method of Bücher & Schuart (1964). Pyruvic acid, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were assayed in the same mixture (Adam, 1963) by the successive additions of lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3). Lactic acid in both the deproteinized sample and the suspension medium was assayed after the method of Scholz, Schmitz, Bücher & Lampen (1959) using lactate dehydrogenase. To obtain comparable results, care was taken throughout the experimental series to use a constant cell density since

preliminary observations indicated that the levels of adenine nucleotides per unit number of cells decrease with increasing cell concentration. This phenomenon has also been reported for Ehrlich ascites cells (Bickis & Quastel, 1965).

Radioisotope studies

The effect of exogenous hexosamines on the labelled carbon dioxide ($^{14}CO_2$) released by cells rotated in Eagle's MEM was estimated at intervals after the addition of 3 μCi of D-[U-^{14}C]-glucose (specific activity 16·7 μCi/mg) (Radiochemical Centre, Amersham) to the cell suspensions at zero time. The $^{14}CO_2$ was trapped in hydroxide of Hyamine (Rohm & Haas, Inc.) using a method of Snyder & Godfrey (1961) as modified by Dunn, Owen & Kemp (1970). The radioactivity was measured to 1 % error in a toluene-based scintillation fluid (containing Fluoralloy dry mix TLA, Beckman Instruments Ltd.) with a Beckman LS 100 liquid scintillation spectrometer. The results were expressed as disintegrations per minute (dpm) after correction against an empirically determined quench curve. In view of the suggestion (Martin, 1967) that dilution of labelled glucose may occur on its entrance into a metabolic pool, thus causing a delay in the appearance of label in the carbon dioxide, it was decided to extend the observation period from 8 to 24 h.

Glycogen metabolism of the cells was estimated from the incorporation of labelled glucose into glycogen at given intervals after the addition of 3 μCi of D-[U-^{14}C]glucose to the preparations. Glycogen was isolated and purified using a method developed by Goodridge (1968). Radioactivity accumulated in the glycogen was measured directly rather than, as suggested by Goodridge (1968), after its reduction to glucose. The glycogen was dissolved in 0·5 ml of distilled water and this solution solubilized in 12 ml of scintillation fluid by adding 1 ml of Beckman Bio-Solv Solubiliser, BBS 3 (Beckman Instruments Ltd.). Isotope activity in the samples was then estimated by liquid scintillation spectrometry to an error of 1 %, the counts being converted to dpm after correcting for the experimentally determined efficiency.

Oxygen-uptake studies

Culture flasks were removed hourly from the gyratory shaker and the rate of oxygen uptake of the cell suspensions measured by a polarographic technique (Lessler, Malloy & Schwab, 1965). The linear decrease in the oxygen saturation of the suspension was recorded for 3 min at 37 °C using the oxygen sensor of a Biological Oxygen Monitor (Yellow Springs Instrument Co. Inc., California, U.S.A.) which was calibrated against air-saturated Eagle's MEM.

RESULTS

Effect of glucosamine and N-acetylglucosamine on cell aggregation

Glucosamine at 0·023 M inhibited the aggregation of embryo chick muscle cells over an 8-h period (Fig. 1) for, when compared with the controls (Fig. 7), aggregates from glucosamine-treated cell suspensions were of a smaller size (Fig. 9). As evinced by the statistical analysis (Fig. 1), control aggregates also gained size more rapidly than those treated with this amino sugar, indicating that the inhibitory effect was continuously exerted throughout the 8-h experimental period. The *N*-acetyl analogue in equimolar concentration had a similar effect for muscle cell aggregation although the inhibition was not as pronounced as that exerted by glucosamine. At 8 h, glucosamine had effectively inhibited the size of aggregates by 18·9% and *N*-acetylglucosamine by 16·4%.

The viability of glucosamine-treated single cells at the end of the experimental period was 88% of the controls, whereas that of cells incubated with *N*-acetylglucosamine was not significantly different.

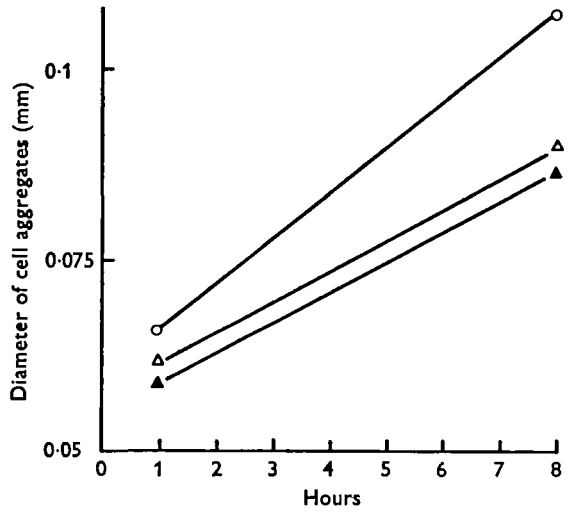

Fig. 1. The effect of 0·023 M glucosamine and *N*-acetylglucosamine on the size attained by aggregates of trypsin-dissociated chick muscle cells rotated in Eagle's MEM at 37 °C. ○, Control; △, *N*-acetylglucosamine- and, ▲, glucosamine-treated cell suspensions. Diameters of 20 aggregates were measured hourly and regression lines fitted to the pooled data of 5 series of experiments.

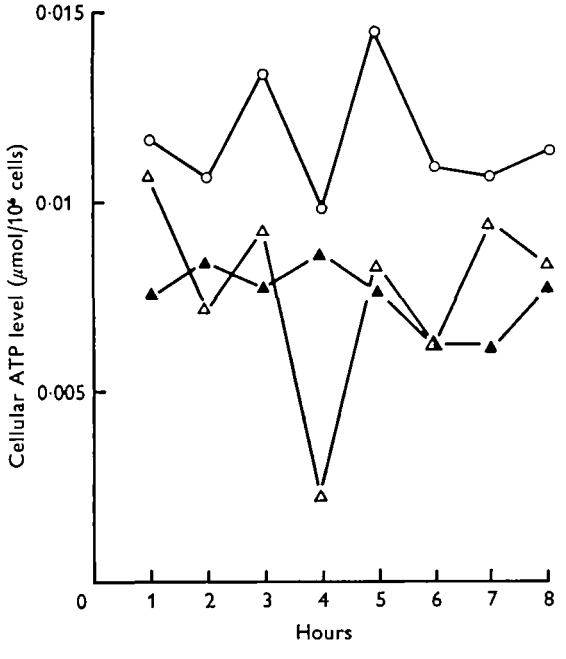

Fig. 2. Effect of 0·023 M hexosamine on the ATP level of embryo-chick muscle cells incubated in Eagle's MEM at 37 °C. These results for control (○), *N*-acetylglucosamine (△) and glucosamine (▲) preparations are typical of 3 series of experiments.

Adenine nucleotide levels in hexosamine-treated cells

Hourly measurements revealed a distinct oscillation in the level of ATP in embryonic muscle cells. Nevertheless, it will be seen (Fig. 2) that there was consistently more ATP in control cells than in cells incubated with either hexosamine. Of the 2 agents, glucosamine induced the greater depression of the level of this nucleotide and limited the range of its variability. Over the 8-h period there was a 36% (mean) loss of the ATP in glucosamine-treated cells as compared to the 27% loss caused by its analogue. The hexosamines not only reduced cellular ATP but also total adenine nucleotides (Tables 1, 2). An average loss of 32% of the total ATP, ADP and AMP occurred during

Table 1. *The effect of* 0·023 M *hexosamine on the adenine nucleotide levels of trypsin-dissociated chick muscle cells rotated in Eagle's MEM*

These results for control (c), *N*-acetylglucosamine (n) and glucosamine (g) preparations are typical of 3 similar series of experiments.

	μmol × 10^{-3} ATP/10^6 cells			μmol × 10^{-3} ADP/10^6 cells			μmol × 10^{-3} AMP/10^6 cells		
Hours	c	n	g	c	n	g	c	n	g
1	11·7	10·7	7·5	1·1	2·7	0·78	0·74	1·0	0·95
2	10·8	7·2	8·4	1·4	3·1	0·9	0·81	0·96	1·0
3	14·0	9·4	7·7	1·1	3·1	1·0	0·76	1·0	1·05
4	9·8	2·3	8·6	0·9	1·5	0·75	0·86	0·86	1·1
5	14·6	8·3	7·6	1·3	2·4	1·2	0·75	0·63	0·97
6	11·0	6·4	6·3	1·5	2·4	0·81	0·92	0·88	0·69
7	10·8	9·5	6·2	1·2	0·3	0·84	0·95	0·89	0·87
8	11·5	8·4	7·8	1·4	2·5	1·15	1·07	0·87	0·82

Table 2. *Total adenine nucleotide and ATP/AMP values for trypsin-dissociated chick muscle cells suspended in Eagle's MEM on a gyratory shaker at* 37 °C

Figures are from experiments typical of a series of 3; control (c), 0·023 M *N*-acetylglucosamine (n) and 0·023 M glucosamine (g) preparations.

	ATP/AMP			Total adenine nucleotide (μmol × 10^{-3}/10^6 cells)		
Hours	c	n	g	c	n	g
1	15·8	10·5	7·9	13·6	14·4	9·2
2	13·3	7·5	8·3	13·0	11·2	10·6
3	17·9	9·3	7·3	15·6	13·5	9·7
4	11·3	2·7	7·7	11·5	4·6	10·0
5	19·5	13·1	7·8	16·7	11·3	9·7
6	11·9	7·3	9·2	13·5	9·6	7·8
7	11·4	10·7	7·2	13·0	13·4	7·9
8	10·7	9·7	9·6	14·0	11·8	9·8
Mean	13·97	8·85	8·12	13·8	11·2	9·3

the 8-h incubation with glucosamine, an effect which is more pronounced than the 19% loss of total nucleotide caused by *N*-acetylglucosamine. The higher control value for cellular ATP was reflected by an ATP/AMP ratio greater than found in hexosamine-treated cells (Table 2). When considered over the 8-h period, the

Table 3. *The effect of* 0·023 M *hexosamine on the intracellular lactic and pyruvic acid levels of trypsin-dissociated chick muscle cells in Eagle's MEM*

The results for control (c), *N*-acetylglucosamine (n) and glucosamine (g) preparations are from experiments typical of a series of 3.

	μmol lactate × $10^{-3}/10^6$ cells			μmol pyruvate × $10^{-3}/10^6$ cells			Lactate/pyruvate		
Hours	c	n	g	c	n	g	c	n	g
1	—	14·0	12·5	1·2	2·6	1·6	—	5·8	7·8
2	19·0	32·0	23·0	1·8	3·3	2·0	10·4	9·7	11·3
3	16·0	53·0	9·0	1·7	3·2	1·75	9·8	16·7	5·5
4	10·0	8·9	26·0	1·36	2·5	2·4	7·45	3·55	10·7
5	5·0	—	29·0	1·65	2·2	2·3	3·4	—	12·8
6	0·9	5·0	27·0	1·2	2·5	1·75	0·77	1·88	15·4
7	11·7	4·0	21·0	2·0	2·3	2·1	5·77	1·82	9·8
8	7·0	21·0	19·0	1·5	2·25	2·8	4·9	9·1	6·9
Mean	—	—	—	—	—	—	6·1	6·9	10·3

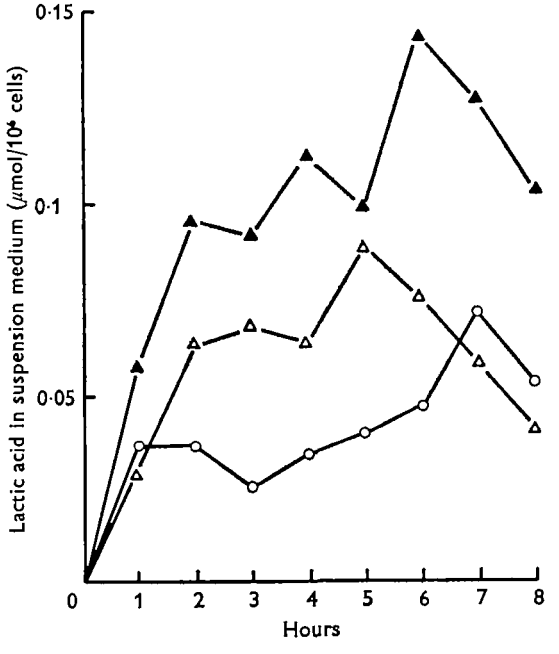

Fig. 3. Lactic acid assayed in the suspension medium of control (○), 0·023 M *N*-acetylglucosamine-(△) and 0·023 M glucosamine-treated (▲) embryo-chick muscle cells rotated in Eagle's MEM. The results are typical of 3 series of experiments.

ATP/AMP ratio of control cells varied about a mean of 13·7. The administration of *N*-acetylglucosamine and glucosamine reduced this ratio to 8·75 and 8·0 respectively, indicative of a greater degradation of ATP.

Changes in intracellular pyruvate and lactate and extracellular lactate

When cells were rotated for 8 h in the presence of *N*-acetylglucosamine or glucosamine, there was a resulting increase of the intracellular lactate/pyruvate ratios above the range determined for controls (Table 3). Although comparison of these data is made difficult by the periodic fluctuation of these metabolites between a considerable range of peak and basal levels, the mean values about which the ratios characteristically

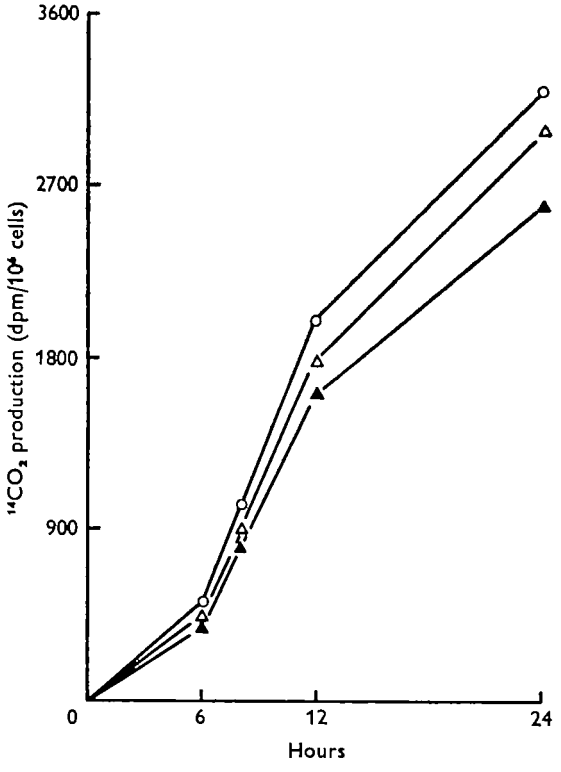

Fig. 4. Effect of hexosamines on $^{14}CO_2$ released by trypsin-dissociated embryonic chick muscle cells rotated in Eagle's MEM containing 3 μCi of D-[U-^{14}C]glucose. Results for controls (○), 0·023 M glucosamine (▲) or *N*-acetylglucosamine (△) preparations are from 3 series of experiments, each treatment performed in triplicate.

oscillated were 6:1, 8:1 and 10:1 for controls, *N*-acetylglucosamine- and glucosamine-treated cell suspensions respectively. Correspondingly, the amount of extracellular lactic acid produced by glucosamine-treated cells (Fig. 3) was consistently higher than that produced by the controls or from cells incubated with *N*-acetylglucosamine. Glucosamine-treated cells secreted 229% more lactate into the medium than did the controls at 4 h and 99% more at 8 h. In contrast, although cells rotated in the presence

of *N*-acetylglucosamine lost 85% more lactic acid than the controls at 4 h, this trend was reversed at 8 h, there being 25% less lactate in the medium of the treated cells than of the controls.

Hexosamine-induced changes in cellular $^{14}CO_2$ production

Both hexosamines inhibited the release of $^{14}CO_2$ from cells rotated in Eagle's MEM containing uniformly labelled glucose and the inhibition was manifest throughout the 24-h period studied (Fig. 4). At 6 h the difference between the inhibitory effects of glucosamine and *N*-acetylglucosamine was slight but by 8 h the inhibition exerted by glucosamine was the greater by 6%. When the amount of radioactive carbon dioxide released by the controls had doubled 4 h later, the disparity between the inhibitory effects of the two amino sugars was more accentuated; glucosamine inhibiting $^{14}CO_2$ output by 18% and *N*-acetylglucosamine by 9%.

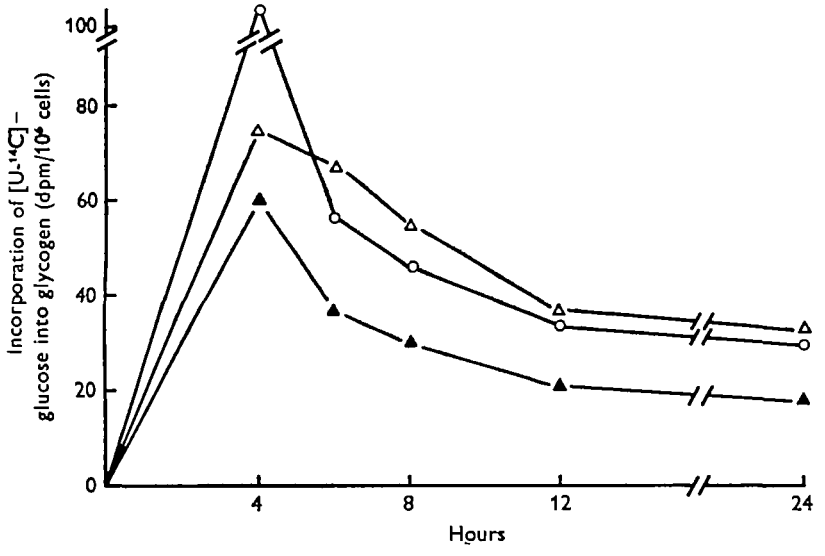

Fig. 5. The effect of 0·023 M hexosamine on the amount of activity measured in glycogen isolated from trypsin-dissociated muscle cells suspended in Eagle's MEM containing 3 μCi of D-[U-^{14}C]glucose. The curves for control (○), *N*-acetylglucosamine (△) and glucosamine (▲) preparations are the average of 3 series of experiments, each given interval from one such experiment being determined in triplicate.

Effect of hexosamines on glycogen metabolism

When cells were rotated *in vitro* with ^{14}C-labelled glucose, the radioactivity subsequently measured in isolated glycogen was maximal for all preparations at 4 h (Fig. 5). After this time, utilization of glycogen resulted in loss of radioactivity from this fraction. The addition of glucosamine to cell suspensions caused 42% less labelled glucose to be incorporated into glycogen at 4 h; *N*-acetylglucosamine exerted a 30% inhibition at this time (Fig. 5). From about the 6-h stage, *N*-acetylglucosamine no longer appeared inhibitory because of the more rapid disappearance of radioactive glycogen from controls. At 12 h this amino sugar showed no apparent inhibitory effect

on cellular glycogen metabolism, although the radioactive glycogen in cells treated with glucosamine was still 45% lower than in the controls.

Effect of hexosamines on oxygen uptake

Glucosamine induced stimulation of oxygen uptake by cells (Fig. 6). This enhanced uptake was observed throughout the test period and by the end of the experiment, glucosamine-treated cells had utilized 59% more oxygen than had the controls. The *N*-acetyl amino sugar did not have a stimulatory effect; moreover, after the 5-h stage, *N*-acetylglucosamine imposed a slight inhibition which by 8 h had depressed the total oxygen uptake by 13%.

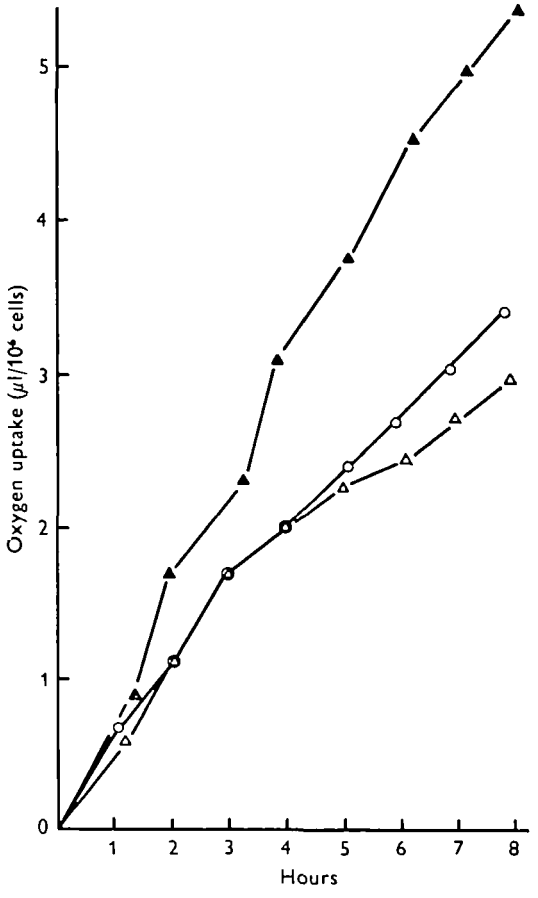

Fig. 6. The effect of 0·023 M hexosamine on the oxygen uptake of trypsin-dissociated chick muscle cells suspended in Eagle's MEM at 37 °C. The results for control (○), *N*-acetylglucosamine (△) and glucosamine (▲) preparations are expressed as an accumulative index of the hourly oxygen uptake and represent one of a series of 5 similar experiments.

DISCUSSION

It may be concluded from this investigation that glucosamine is instrumental in inhibiting the aggregation *in vitro* of embryonic chick muscle cells rotated on a gyratory shaker. This finding endorses the results of a previous inquiry on trypsin-dissociated chick liver and retinal cells (Garber, 1963) which established that the aggregation of those cells was inhibited by glucosamine. The present results are not, however, consonant with Garber's contention (1963) that the *N*-acetyl analogue in equivalent concentration was without effect on aggregation since it had been found that this amino sugar is inhibitory for muscle cells (Fig. 1). A plausible interpretation of this difference is that muscle cells may utilize *N*-acetylglucosamine more efficiently than do liver and retinal cells. Our preliminary observations indicate that this sugar is actively incorporated into macromolecular components of muscle cells whereas it had been shown (McGarrahan & Maley, 1962) that rat liver utilizes *N*-acetylglucosamine poorly compared with the same amount of glucosamine. Furthermore, mannosamine (2-amino-2-deoxy-D-mannose) is rapidly taken up by Sarcoma 180 ascites cells (Raisys & Winzler, 1970) but the acetylated analogue is almost totally excluded. Such variations in the ability of cells to metabolize glucosamine and *N*-acetylglucosamine may well be fundamental in explaining the different effects ascribed to the *N*-acetylhexosamine.

The slight toxicity of glucosamine to cells in the suspension was foreseen as this property of exogenous glucosamine has been noted with a number of tissues (Rubin, Springer & Hogue, 1954; Bekesi, Molnar & Winzler, 1969; Kornfeld & Ginsburg, 1966; Rizki, 1961). Pilot experiments indicated the excessive lethality of 0·035 M ($\simeq$ 0·75%, w/v) glucosamine on muscle cells. Consequently, a 0·023 M ($\simeq$ 0·5, w/v) solution was employed which noticeably inhibited cell aggregation but with a minimized toxicity.

The present results indicate that both glucosamine and *N*-acetylglucosamine inhibit the conversion of radioactive glucose to glycogen. This corroborates the findings of Spiro (1958), who demonstrated a similar effect in rat liver slices, with glucosamine the stronger inhibitor. In contrast, Martin (1967) reported that this amino-sugar does not interfere with the metabolic transfer of glucose to glycogen in rat diaphragm *in vitro*. This contradiction may be resolved by reference to the work of Kono & Quastel (1962), who point towards the ratio of glucose to glucose analogue as having a definite bearing on the degree to which the analogues inhibit several enzymes of glycogen metabolism. This is to be expected since the amount of substrate relative to its inhibitory analogue inevitably circumscribes the extent of enzyme inhibition. In this present study a ratio of hexosamine to glucose of about 4:1 was employed. Spiro (1958) used hexosamines in concentrations up to 8 times greater than glucose but Martin (1967) used the sugars in equimolar concentration. These variations probably modulate the inhibitory effects of the 2 glucose analogues.

The limited formation of labelled glycogen that occurs in the presence of exogenous glucosamine, coupled with the increased production of extracellular lactic acid presumably from stored glycogen, would suggest a 2-fold effect of glucosamine on glycogen metabolism. First, there is an apparent inhibition of the synthesis of glycogen

from glucose and, secondly, an enhancement of glycogenolysis. It should be recalled that the glycogenolytic effect of both glucosamine and *N*-acetylglucosamine has been attributed to their stimulation of glycogen phosphorylase (EC 2.4.1.1) activity (Kono & Quastel, 1962); the larger decrease in the glycogen content caused by glucosamine being due to its additional activity of inhibiting the UDP-dependent enzyme, glycogen synthetase (UDP-glucose-glycogen glucosyl transferase, EC 2.4.1.11). The results of this investigation are consistent, then, with such an interpretation of the effects of glucosamine and *N*-acetylglucosamine on glycogen metabolism.

Since both amino-sugars decreased the ATP/AMP ratios, their stimulatory effect on phosphorylase activity may be functionally explained on the basis that this enzyme of glycogen breakdown is itself directly stimulated by AMP (see Krebs & Fischer, 1962) as well as by a release of inhibition through loss of ATP (Maddaiah & Madsen, 1966). The adenine nucleotide balance that prevails in hexosamine-treated cells would similarly favour the further degradation of the hexose phosphate derived from glycogen catabolism since an increase of AMP relative to ATP may inhibit fructose 1,6-diphosphatase, EC 3.1.3.11 (Newsholme & Underwood, 1965) and overcome the ATP inhibition of phosphofructokinase, EC 2.7.1.11 (Passonneau & Lowry, 1962), thereby activating glycolysis. The increased lactic acid found in the presence of glucosamine and *N*-acetylglucosamine is in accord with this view, though an enhanced production could also be due in part to released inhibition of the pyruvate kinase (EC 2.7.1.40) reaction owing to a decrease in the level of ATP (Tanaka, Sue & Morimura, 1967).

It is noteworthy that since exogenous hexosamine is incorporated into UDP-*N*-acetylglucosamine after the step by which this nucleotide-sugar regulates its own formation by feedback inhibition (Kornfeld *et al.* 1964), administration of glucosamine to muscle cells may cause the uncontrolled synthesis of UDP-*N*-acetylglucosamine with a corresponding depletion of uridine triphosphate (UTP) (Bekesi & Winzler, 1969). It follows that this depletion would moderate glycogen synthesis since UTP is required for transfer of glucosyl moieties to the glycogen chain in the glycogen synthetase reaction. In this connexion, Zancan & Hers (1965) showed that glucosamine in equivalent concentration to glucose inhibited the formation of the glycogen precursor, UDP-glucose (UDPG), by 30–40%, which could also release a UDPG inhibition of phosphorylase (Maddaiah & Madsen, 1966). This interpretation would therefore provide a rationale for glucosamine inhibiting glycogen synthetase (Kono & Quastel, (1962), although an inhibition of hexokinase (EC 2.7.1.1) by the amino-sugars (Spiro, 1958) may also be expected to contribute to the decreased synthesis of glycogen from glucose.

The observed inhibition of $^{14}CO_2$ production from glucose in hexosamine-treated cells could result from competition between the hexosamines and labelled glucose for the hexokinase reaction (Spiro, 1958). Support for this view can be drawn from findings (Kono & Quastel, 1962) that both glucosamine and *N*-acetylglucosamine inhibited the hexokinase of rat liver slices when analogue and glucose were utilized in the 4:1 ratio of this present study. The inhibition of the release of $^{14}CO_2$ is insufficient to explain the more manifest effects that these amino sugars have on other aspects of cellular

metabolism which is therefore in keeping with the proposal that hexokinase is not the site at which glucose analogues inhibit glycogen metabolism (Kono & Quastel, 1962).

The present results show that both glucosamine and *N*-acetylglucosamine depress the cellular ATP level, lending support to earlier reports that cells incubated with glucosamine and low glucose possess a reduced intracellular ATP pool (Creaser & Scholefield, 1960; Bekesi & Winzler, 1969). An explanation for this effect may be found in the postulated excessive production of UDP-*N*-acetylglucosamine in cells incubated with hexosamine. Exogenous hexosamine must be phosphorylated prior to its further metabolism and this could then be expected to cause a coincident fall in the level of ATP. The diminished levels of total adenine nucleotides in hexosamine-treated cells probably also arises from this sequestration of ATP in the formation of hexosamine phosphates – a synthesis which does not contribute to energy metabolism. The decreased ATP/AMP ratio of hexosamine-treated cells may be attributed to the continued phosphorylation of these amino sugars. A resulting excess of AMP would consequently lead to its irreversible deamination (Imai, Riley & Berne, 1964) and thereby effect a net loss from the adenine nucleotide pool which is otherwise too great to have derived from the limited cytotoxicity of glucosamine. The oscillation in the level of cellular ATP may reflect successive activity peaks (see Mitchison, 1969) of certain enzymes in a cell system induced into synchrony by the initial trypsinization procedure (Stubblefield, 1968).

It seems reasonable to assume that the increased glycolytic flux resulting from a glucosamine-stimulated glycogenolysis would, as well as elevating the production of lactic acid, increase the amount of substrate oxidized by way of the Krebs cycle. This view is reinforced by the present finding that glucosamine stimulates oxygen uptake of chick muscle cells. It is significant in this respect that the decreased glycogenolysis of *N*-acetylglucosamine-treated cells, as judged by the diminishing level of lactic acid in the medium after the 5-h stage, coincides with their lower oxygen uptake.

In brief, the metabolic effects of hexosamines in this present investigation may be attributed to their alteration of the adenine nucleotide balance, which is causal in depressing cellular energy metabolism. These results also indicate that the inhibited aggregation of cells rotated with glucosamine or *N*-acetylglucosamine may be correlated with this reduced energy state of the cells. That the adhesive process depends upon the involvement of an active metabolism has been proposed by a number of investigators studying the effects of metabolic inhibitors and low temperature on aggregation (Giudice, 1965; Humphreys & Uehara, 1964; Kemp *et al.* 1967; Steinberg, 1962). The inhibitory effect of exogenous hexosamines, whatever its derivation, would seem to have sequelae for several aspects of cellular intermediary metabolism (Kono & Quastel, 1962; Bekesi *et al.* 1969). On this basis, it is considered that the inhibitory effects of glucosamine and *N*-acetylglucosamine could encompass glycoprotein metabolism at which particular level these hexosamines may impair the adhesive process. The glycoprotein metabolism of hexosamine-treated chick muscle cells is currently under study.

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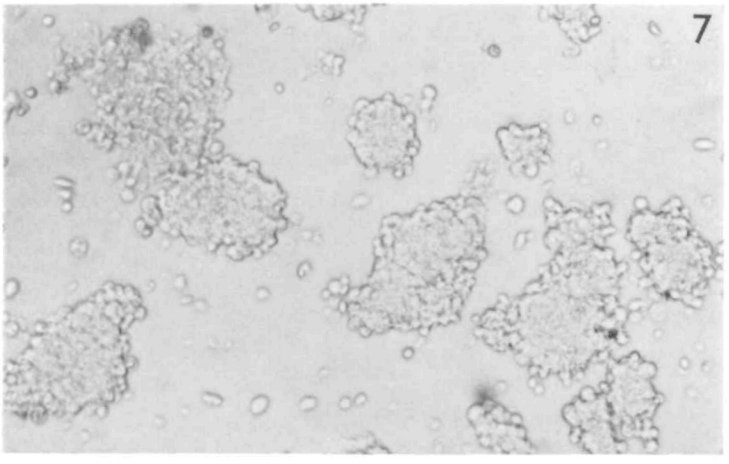
Fig. 7. Aggregates formed by trypsin-dissociated muscle cells after rotation in Eagle's MEM at 37 °C for 8 h. × 125.

Fig. 8. Aggregates produced at 8 h by the rotation of trypsin-dissociated muscle cells in Eagle's MEM containing 0·023 M *N*-acetylglucosamine. × 125.

Fig. 9. Smaller aggregates produced from chick muscle cells incubated in Eagle's MEM with 0·023 M glucosamine at 37 °C for 8 h on a gyratory shaker. × 125.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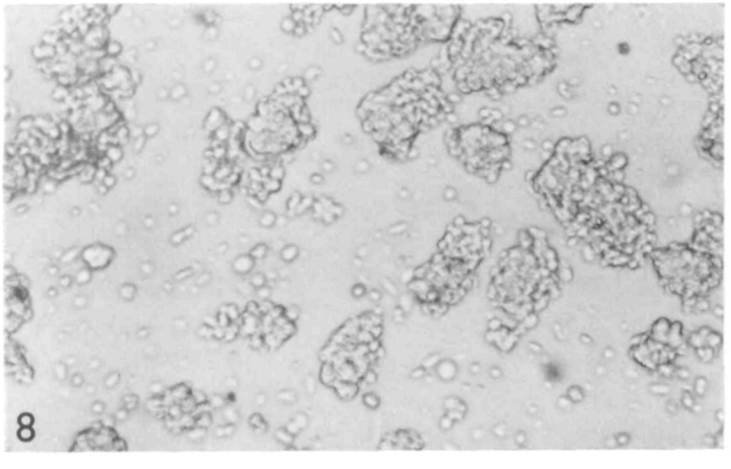
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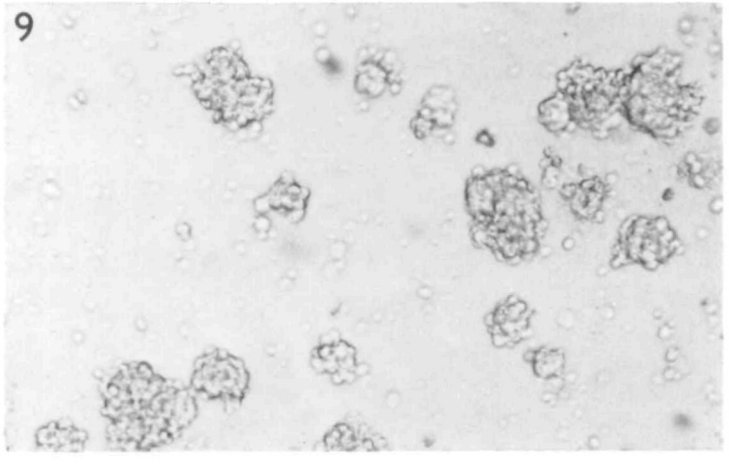
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