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Synthesis of proglycogen and macroglycogen in skeletal muscle of Standardbred trotters after intermittent exercise

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Summary

***Reasons for performing study*: The degradation of glycogen and its two forms, proglycogen (PG) and macroglycogen (MG) has been studied in horses performing different types of exercise, but no information is available about the resynthesis of PG and MG after exercise.**

***Objectives*: To determine the resynthesis of PG and MG in skeletal muscle after intermittent uphill exercise.**

***Methods*: At a training camp 9 well-trained Standardbred trotters performed a training session comprising a warm-up period, 7 repeated 500 m bouts of exercise on an uphill slope and a recovery period. Muscle biopsies (*m. gluteus medius*) for analysis of PG, MG, glucose and glucose-6-phosphate were taken at rest, at the end of exercise and 1, 4, 8, 24, 48 and 72 h post exercise. Blood samples for analysis of glucose, lactate and insulin were collected before exercise, immediately after the last bout of exercise and then as for the muscle biopsies.**

***Results*: The MG and PG concentration pre-exercise was 311 ± 47 and 305 ± 55 mmol/kg dwt respectively. The exercise caused a decrease in PG (Δ 63 ± 26 mmol/kg dwt) and MG (Δ 136 ± 68 mmol/kg dwt). Immediately after the last sprint plasma glucose and lactate increased compared to values pre-exercise. During the first hour post exercise there was a further decrease in MG in 7 out of 9 horses. The rate of glycogen resynthesis during 1–24 h was higher for MG than for PG. The rate of muscle glycogen resynthesis thereafter was slower and did not differ between MG and PG up to 72 h.**

***Conclusion*: After repeated bouts of exercise on a slope, resynthesis of glycogen is a slow process and the resynthesis of proglycogen differs from that of macroglycogen. The fraction most depleted during exercise (MG) had no resynthesis during the first hour of recovery but then had the highest rate of resynthesis during the remainder of the first 24 h period.**

***Potential relevance*: If the time between exercise sessions during training is too short the recovery period will be inadequate for complete restoration of muscle glycogen.**

Introduction

Glycogen is an important energy source for contracting muscle under both aerobic and anaerobic conditions. There is a marked decrease in muscle glycogen concentration in horses after both high-intensity exercise (Lindholm and Saltin 1974; Valberg *et al.* 1989; Hyyppä *et al.* 1997; Schuback *et al.* 2000) and endurance work (Essén-Gustavsson *et al.* 1984; Essén-Gustavsson and Jensen-Waern 2002). Low muscle glycogen concentrations prior to exercise in horses (Lacombe *et al.* 1999; 2001) and man (Bergström *et al.* 1967) are associated with decreased performance in both low- and high-intensity exercise. There are only a few reports on resynthesis of glycogen in skeletal muscle of horses after exercise (Snow *et al.* 1987; Davie *et al.* 1994; Hyyppä *et al.* 1997; Lacombe *et al.* 2004). These studies have shown that muscle glycogen resynthesis is slow and complete repletion of muscle glycogen stores may take up to 72 h. One study also demonstrated that glycogen decreased further 4 h after repeated bouts of intense exercise (Hyyppä *et al.* 1997).

The resynthesis of glycogen in horses has been studied as total glycogen resynthesis and not as separate glycogen fractions. Muscle glycogen can be separated into two fractions, proglycogen (PG) and macroglycogen (MG), on the basis of solubility in acid (Lomako *et al.* 1991; Adamo and Graham 1998; Bröjer *et al.* 2002a). These 2 glycogen forms have the same complement of protein but differing amounts of attached carbohydrate. Proglycogen represents the smaller size range of glycogen granules (<400 kDa), whereas MG represents the larger range up to 10,000 kDa (Lomako *et. al* 1993; Alonso *et al.* 1995). Studies on human and equine skeletal muscle have demonstrated that both PG and MG contribute to glycogenolysis but the magnitude of the degradation appears to be dependent on factors such as exercise intensity, duration and initial muscle glycogen concentration. In man, situations with intense exercise have demonstrated proglycogen to have accelerated rates of degradation compared with the more carbohydrate dense macroglycogen granule (Graham *et al.* 2001; Shearer *et al.* 2001). In a recent study on horses, PG and MG contributed equally to the glycogenolysis during maximal treadmill exercise (Bröjer *et al.* 2002b). In contrast, MG is degraded to a greater extent compared to PG during long-term exercise such as marathon races in man and endurance rides in horses (Asp *et al.* 1999; Essén-Gustavsson and Jensen-Waern 2002). In man, PG is the predominate form resynthesised early in the recovery after exhaustive exercise whereas the synthesis of MG is slower and more constant (Adamo *et al.* 1998; Battram *et al.* 2004). Compared to man, post exercise repletion of muscle glycogen appears to be slow in the horse.

Separation of glycogen into PG and MG allows for a more detailed look at the glycogen resynthesis process after exercise. An often-used training method of Standardbred trotters in Sweden is different forms of interval training such as uphill training. The aim of this study was to determine the resynthesis of PG and MG in skeletal muscle in racing Standardbred trotters after they had performed repeated bouts of exercise on a slope.

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Materials and methods

All experiments were conducted after approval by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

Horses

Ten Standardbred horses, 4 mares (age 6–8 years, weight 466–542 kg bwt) and 6 geldings (age 6–12 years, weight 476–514 kg bwt) were used. The horses belonged to the National Equine Centre, Wången. All horses had been in regular training for several months and at the time of the study all were in racing condition. They were fed their regular diet consisting of haylage (10–13 kg, dry matter 64%), cracked barley (0.5–2 kg), molassed sugar beet pulp (0.2– 0.8 kg) and a commercial pelleted feed[1] (1–4 kg) daily to meet the requirements of a working horse (Planck and Rundgren 2003). The average amount fed per horse a day was 11.3 ± 1.3 kg haylage and 3.2 ± 0.7 kg concentrated feed. The daily intake of metabolisable energy was 0.217 ± 0.008 MJ/kg bwt. The haylage was fed every day at 0600, 1200, 1700 and 2100 h. Concentrated feed was fed as for haylage except for the 1200 h feeding. Water and a salt block were provided *ad libitum*.

Experimental design

The horses performed a training session consisting of a warm-up period of slow trot over 4000 m, 7 repeated 500 m bouts of exercise on an uphill slope at a speed of 9 m/sec followed by a period of slow trot over 2000 m on a track. Between bouts of exercise the horses were walked downhill along the slope. The slope ascended 24 m per 500 m. The horses performed the training session in pairs based on their training condition. The heart rate was monitored with a pulsimeter[2]. The horses were stall rested for 3 days being walked daily for 60 min in the morning and 40 min in the afternoon.

Muscle biopsies

Muscle biopsies were taken percutaneuosly at a depth of approximately 6 cm from the left and right *gluteus medius* muscle according to the method described by Lindholm and Piehl (1974). Samples were taken at rest prior to and immediately post exercise and 1, 4, 8, 24, 48 and 72 h after exercise. The muscle biopsies were frozen rapidly in liquid nitrogen and stored at -80°C until further analysis. The samples were freeze-dried and dissected free of visible blood, connective tissue and fat before analyses.

Blood samples

Venous blood samples were taken from the jugular veins in heparinised tubes and in tubes without additive using a Vacutainer system[3]. Samples were taken before, immediately after the last bout of exercise on the slope, and at the time of each muscle biopsy. The heparinised tubes were kept on ice until centrifugation. Plasma and serum were harvested and stored at -80°C until analysed.

Muscle metabolites

Proglycogen and MG fractions were separated based on solubility in perchloric acid (PCA) with the method described by Bröjer *et al.* (2002a). The PG and MG fractions were then boiled for 2 h in 1 mol/l HCl and the formed glucosyl units were subsequently measured fluorometrically with the hexokinase method (Lowry and Passonneau 1973). The obtained MG fractions were corrected for free glucose and glucose-6-phosphate (G-6-P; Bröjer *et al.* 2002a). Muscle glucose and G-6-P were analysed according to Lowry and Passonneau (1973).

Insulin, lactate, and glucose

Plasma glucose was analysed using an automated analyser (Konelab 30)[4]. Plasma lactate was measured enzymatically with a lactate analyser (Analox GM7)[5]. Serum insulin concentration was analysed using a solid-phase radioimmunoassay with a commercial kit (Coat-A-Count Insulin)[6].

Calculations and statistical analysis

The total glycogen (G_t) in each sample was calculated as the sum of measured MG and PG. The net resynthesis rate was calculated for every 24 h. However, since the first hour of recovery had negative values for resynthesis rate, the first time interval was calculated between 1 and 24 h. A one-way repeated-measures ANOVA test was used to test differences in PG, MG, G_t, muscle glucose, G-6-P, plasma glucose and serum insulin between time points. Where significance was indicated the Tukey *post hoc* test was performed to compare specific means. A paired *t* test was used to compare degradation in glycogen fractions (Δ PG and Δ MG). The null hypothesis was rejected at P<0.05. All calculations were performed with Sigma Stat software[7]. Data are presented as mean ± s.d.

Results

Heart rate and exercise conditions

The exercise was performed in the winter under good weather and track conditions between 0900 and 1200 h. Four horses performed the exercise on Day 1 and 6 horses on Day 2. The weather was overcast with a temperature of -6°C on Day 1 and the sky clear with a temperature of -7°C on Day 2. The speed for all horses was set to 9 m/sec but was adjusted slightly in accordance with their training condition to achieve a HR between 210 and 220 beats/min at the end of each exercise bout. One horse was excluded from the study since he lost a shoe and could only complete 6 exercise bouts. The mean heart rates for the horses at the end of each of the 7 exercise bouts were 216 ± 15, 220 ± 12, 221 ± 9, 216 ± 13, 215 ± 11, 217 ± 8 and 214 ± 7.

Muscle glycogen, glucose and glucose-6-phosphate

Concentrations of G_t, PG and MG for all time points are shown in Figure 1. Before exercise MG represented 50% of G_t. A decrease in muscle content of G_t (Δ 200 ± 75 mmol/kg dwt), PG (Δ 63 ± 26 mmol/kg dwt) and MG (Δ 136 ± 68 mmol/kg dwt) was observed after exercise. The MG fractions contributed significantly more to the glycogenolysis compared to PG (68% and 32% of G_t degradation for MG and PG, respectively). During the first hour post exercise there was a further decrease in MG in 7 out of 9 horses (Δ 46 ± 62 mmol/kg dwt); however, the decrease was not statistically significant (P = 0.141). At 1 h post exercise the MG fraction accounted for 35% of G_t and at 24 h the percentage of MG had increased to 49% of G_t, which is the same percentage as before exercise. Between 1 and 24 h, the significant elevation of G_t was due to a net resynthesis rate of 4.7 ± 1.7 mmol glucosyl units/kg dwt/h in the MG fraction (Fig 2). In comparison to MG the rate of PG resynthesis was slower and remained unchanged over the 72 h of recovery. After 48 h of recovery G_t, MG and PG had reached its pre-exercise levels.

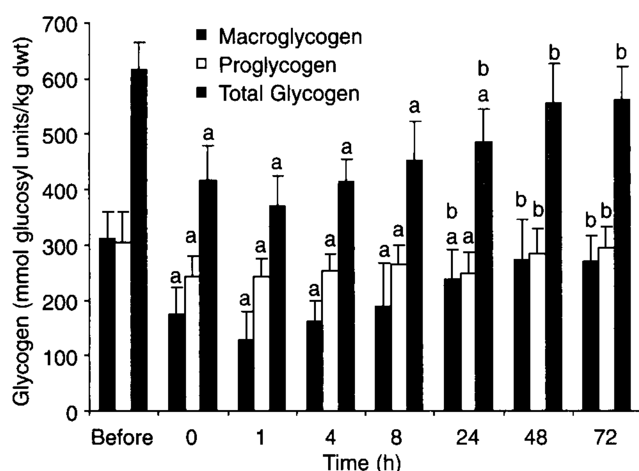

Fig 1: Macroglycogen (■), proglycogen (□) and total glycogen (■) concentrations in skeletal muscle before and after intermittent exercise and during 72 h of recovery. Values are mean ± s.d.; n = 9 muscle biopsies per time point. Within a type of glycogen: [a]significantly different from before exercise; [b]significantly different during the recovery period from value 0 hr (P<0.05).

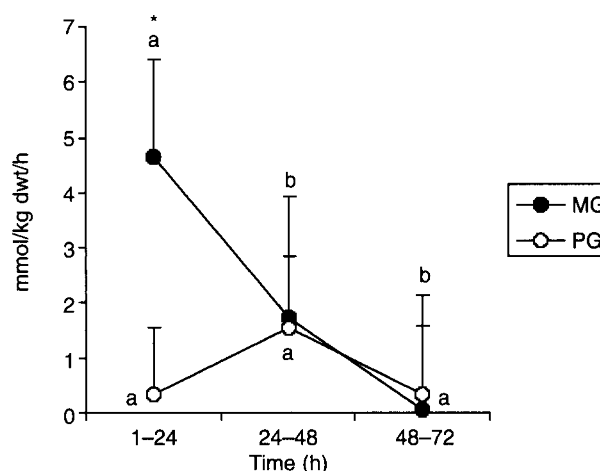

*Fig 2: Net synthesis rates of PG and MG during the time intervals 1–24, 24–48 and 48–72 h in muscle biopsy samples after intermittent exercise. Values represent mean ± s.d.; n = 9 muscle biopsies per time point. Values with same letter are not significantly (P<0.05) different within a glycogen fraction. *Significant difference (P<0.05) between PG and MG within a time point).*

There was an increase in free muscle glucose concentration post exercise, which decreased to pre-exercise levels 1 h after exercise. Glucose-6-phosphate concentration did not differ between the 4 different time points (Table 1).

Plasma lactate, glucose and serum insulin

An increase in plasma lactate (Δ 14.8 ± 2.5 mmol/l) and plasma glucose (Δ 2.5 ± 1.3 mmol/l) was observed immediately after the last bout of intermittent exercise on a slope (Table 2). The plasma lactate decreased to pre-exercise levels at the beginning of the recovery period whereas the plasma glucose decreased to pre-exercise levels at 1 h of recovery. The values for serum insulin increased significantly after completion of the exercise session (Δ 7.6 ± 6.8 miu/l) and decreased to values before exercise at 1 h of recovery.

Discussion

Glycogenolysis during repeated bouts of exercise on a slope occurred to a greater extent in the MG fraction compared to the PG fraction. The present study also confirms previous findings that glycogen resynthesis during recovery in horses is a slow process. The new finding was that the resynthesis of MG seems to be restricted during the first hour of recovery but then predominates during the remainder of the first 24 h. The resynthesis of PG was slower than MG. Therefore, the highest rate of resynthesis during the first day was found in the MG fraction, which was the glycogen fraction most depleted during exercise.

Previous studies in both man and horses have demonstrated that factors such as exercise intensity and duration are of importance for the degradation of PG and MG (Asp *et al.* 1999; Graham *et al.* 2001; Bröjer *et al.* 2002b; Essén-Gustavsson and Jensen-Waern 2002). The repeated bouts of exercise on a slope performed by the horses in the present study were intense as indicated by the increase in plasma lactate and decrease in muscle glycogen. The magnitude of glycogenolysis in the present study was twice as high in the MG compared to the PG fraction. In contrast, when horses perform short intense maximal incremental treadmill exercise to fatigue, there is a similar decrease in PG and MG (Bröjer *et al.* 2002b). Endurance type of exercise in horses, on the other hand, causes almost three times higher degradation in the MG compared to the PG pool (Essén-Gustavsson and Jensen-Waern 2002). It appears that there is a transition from a preferential utilisation of MG during long-term exercise towards an increase in the utilisation of PG during more intense exercise in both horses and man. However, in general horses rely to higher extent on MG degradation compared to man. This is probably related to the higher muscle glycogen concentration in horses and especially the higher MG fraction compared to human skeletal muscle (Bröjer *et al.* 2002a).

Glucose can be incorporated into glycogen molecules in 3 different ways. It can be incorporated into newly formed glycogen particles, where glucose is added to glycogenin, or glucose can be added to existing PG or MG molecules. The role of glycogenin in glycogen resynthesis is a topic of controversy. One study suggests that glycogenin is generally inactive in human skeletal muscle *in vivo* (Jiao *et al.* 1999). Another study on cultured rat myotubes shows a fast resynthesis of glycogenin (Elsner *et al.* 2002). If resynthesis of glycogen occurred predominantly in the PG fraction this would suggest that glucosyl units were added to existing PG molecules or that new glycogen granules were formed. In the present study, the largest increase in glycogen concentration after the first 24 h of recovery occurred in the MG fraction, indicating that existing PG grew into MG or existing MG particles grew larger by replenishment of the outer tiers.

TABLE 1: Pre- and post exercise muscle glucose and glucose-6-phospate (G-6-P) concentrations before exercise and during the first 4 h of recovery

	Rest	0 h	1 h	4 h
Glucose	1.0 ± 0.4	5.1 ± 2.5[a]	1.8 ± 1.6	1.6 ± 1.2
G-6-P	2.1 ± 0.6	2.0 ± 0.7	2.3 ± 0.8	2.1 ± 1.1

Values are presented as mean ± s.d. (mmol/kg dwt). [a]significant difference (P≤0.05) from resting value.

The overall patterns of PG and MG resynthesis as well as the rates of total glycogen resynthesis seem in the present study to differ from previous work performed in man during the recovery period. The general resynthesis pattern in human skeletal muscle after intense exercise is predominate resynthesis of PG early in recovery and a slower more constant rate of MG synthesis over the

TABLE 2: Plasma lactate, glucose and serum insulin before exercise, after last bout of intermittent exercise and during the recovery period

	Before exercise	After slope	0 h	1 h	4 h	8 h	24 h	48 h	72 h
Lactate	0.5 ± 0.2	15.3 ± 2.6[a]	2.0 ± 0.8	0.9 ± 0.3	ND	ND	ND	ND	ND
Glucose	5.6 ± 0.3	8.1 ± 1.1[a]	6.9 ± 1.2[b]	5.4 ± 0.4	5.4 ± 0.2	5.5 ± 0.4	5.6 ± 0.3	5.6 ± 0.6	5.8 ± 0.3
Insulin	7.0 ± 3.6	6.4 ± 2.9	14.6 ± 8.9[c]	8.4 ± 1.9	7.1 ± 2.7	9.3 ± 5.4	6.5 ± 4.2	10.3 ± 6.6	11.5 ± 5.1

Plasma lactate, glucose (mmol/l) and serum insulin (mIU/l) before exercise, after last bout of intermittent exercise (after slope) and during recovery (0–72 h), presented as mean ± s.d. [a]Significant difference from before exercise and recovery 0–72 h. [b]Significant difference from before exercise, after slope and recovery 1–72 h. [c]Significant difference from before exercise, after slope and 4 and 24 h of recovery. $P<0.05$; ND = not determined.

next 24 h (Adamo *et al.* 1998; Shearer *et al.* 2001; Battram *et al.* 2004). One explanation for why glycogen resynthesis in the horses in the present study did not appear to be a process involving new glycogen granule formation may be related to the fact that the fraction of MG in horse muscle is greater compared to man. Another explanation may be the level of glycogen depletion. The horses in the present study decreased their total glycogen concentration in muscle to approximately 400 mmol glucosyl units per/kg dwt whereas the humans that participated in the aforementioned recovery studies had post exercise concentrations between 50 and 100 mmol glucosyl units/kg dwt. Such extreme glycogen depletion probably requires the formation of new glycogen granules rather than the addition of glucose to existing glycogen molecules, which is more likely to occur when glycogen depletion is more modest.

The rates of total glycogen resynthesis over the first 48 h in the present study are in accordance with previous reports on horses fed a conventional diet (Snow and Harris 1991; Lacombe *et al.* 2004). Whereas resynthesis of muscle glycogen is complete within 24 h in most situations after exercise in man, the horses in the present study only had replenished their glycogen levels to 79% of the pre-exercise concentrations. The horses were not fed concentrated feed until 5–7 h after completion of the exercise bouts. In general, muscle glycogen resynthesis after exercise is dependent on factors such as carbohydrate substrate availability and interval from completion of the exercise to access to carbohydrate substrate. However, horses fed a high carbohydrate diet or supplemented orally with a glucose polymer did not have an enhanced muscle glycogen resynthesis over the first 24 h post exercise (Snow *et al.* 1987; Davie *et al.* 1994). The relative slower resynthesis rate during the post exercise recovery period in horses compared with other species has not been clarified. One potential explanation is that the horse has a limited ability to digest starch in the small intestine compared to other monogastric animals due to a slower rate of amylase secretion (Comline *et al.* 1969). However, oral glucose supplementation does not increase muscle glycogen resynthesis whereas i.v. administration does (Davie *et al.* 1994, 1995). Despite the administration of glucose i.v. during the recovery period muscle, glycogen did not return to the pre-exercise levels by 24 h indicating that there are other local factors within the muscle limiting glycogen resynthesis in the horse; local factors such as decreased glucose transport across the cell membrane, low activity of glycogen synthase and inability of the skeletal muscle to shift from carbohydrate to fat oxidation during the recovery period (Hyyppä *et al.*1997; Lacombe *et al.* 2004).

As demonstrated in previous studies, plasma glucose and serum insulin were increased at the beginning of the recovery period (Hyyppä *et al.* 1999; Pösö and Hyyppä 1999). The increased plasma glucose and serum insulin as well as low intramuscular G-6-P at the beginning of the recovery period gives the potential for intramuscular glucose uptake and glycogen resynthesis. Persistent glycogenolysis during the recovery period has been reported previously at 4 h post exercise in Standardbred trotters after repeated bouts of exercise (Hyyppä *et al.* 1997). The decrease in muscle glycogen stores during the early recovery period in the present study is in agreement with the above mentioned finding and indicates an inability of the skeletal muscle to shift from carbohydrate to fat oxidation during the early recovery period. Fat is an important energy supply for resting and contracting muscle. Relatively little attention has been given to lipid utilisation during the recovery period following glycogen-depleting exercise in man and horses. However, an earlier study showed low plasma concentrations of lipid metabolites, i.e. glycerol, nonesterified fatty acids (NEFA) and triglycerides, during the recovery period suggesting difficulties in the horse to switch from carbohydrate to lipid metabolism (Hyyppä *et al.* 1997). In contrast, studies in man have demonstrated plasma fatty acids, very low density lipoprotein and triacylglycerols to be important fuel sources for aerobic energy, particularly during the first hours of recovery (Kiens and Richter 1998; Kimber *et al.* 2003). Further, there are results indicating a greater contribution from fat oxidation during recovery in the presence of reduced muscle glycogen in man. Paradoxically, increased fat oxidation during recovery in man is even present during elevated plasma concentrations of insulin and glucose (Kimber *et al.* 2003). Interestingly, activation of glycogen synthase has been demonstrated in exercised human muscle, whereas the glycogen synthase ratio (active:total activity) muscle glycogen in horse muscle after intense exercise is increased to a lesser degree compared to man (Lacombe *et al.* 2004). Taken together, this information supports the theory that glycogen resynthesis is of high priority early in recovery in man but of low priority in the horse. However, there are situations in man where the rate of glycogen resynthesis is reduced e.g. after eccentric exercise (Komi and Viitasalo 1977). In one study in man a further glycogen decrease occurred during early recovery after eccentric exercise intended to cause delayed onset of muscle soreness (DOMS; Zehnder *et al.* 2004). The authors speculated that glycogen reduction was an effect of muscle damage. However, in the present study this is not a likely explanation for the further decrease in muscle glycogen during early recovery as the horses were in racing condition and accustomed to this type of exercise. In addition, clinical examination of the horses during the recovery period revealed no evidence of muscle soreness.

In conclusion, a greater fraction of MG than PG is utilised during repeated bouts of exercise on a slope and the resynthesis of glycogen during the recovery period is a slow process. The resynthesis of MG seems to be restricted early post exercise but predominates thereafter during the first 24 h of recovery.

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Manufacturers' addresses

[1]Krafft Hästfoder AB, Falkenberg, Sweden.
[2]Polar Electro OY, Kempele, Finland.
[3]Becton Dickinson, Meylan, France.
[4]ILS Laboratories AB, Sollentuna, Sweden.
[5]Analox Instruments Ltd, London, UK.
[6]Diagnostic Products Corporation, Los Angeles, California, USA.
[7]SPSS Inc. Chicago, Illinois, USA.

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