Two daily glucagon injections induce nonshivering thermogenesis in Muscovy ducklings

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BARRÉ, HERVÉ, FRÉDÉRIQUE COHEN-ADAD, AND JEAN-LOUIS ROUANET. *Two daily glucagon injections induce nonshivering thermogenesis in Muscovy ducklings.* Am. J. Physiol. 252 (Endocrinol. Metab. 15): E616–E620, 1987.—In 6-wk-old chronically glucagon-treated (GT) ducklings, the calorigenic effect of intraperitoneal test injection of glucagon was measured at 25 and 4°C ambient temperature (T_a). At 25°C T_a, the increase in metabolic rate (MR) due to test injection of glucagon (360 μg/kg) reached 5.3 W/kg (i.e., 98% above the saline control value) in GT ducklings and only 1.7 W/kg (i.e., 29% above the control value) in control (TN) ducklings. After the injection, GT ducklings developed a hyperthermia, reaching 2.4°C, accompanied by intense panting, whereas thermal body temperature did not change in TN ducklings. At 4°C T_a for the same dose of glucagon, no significant change in MR was observed in GT ducklings during 180 min of exposure, whereas a 25% decrease in MR occurred in the same conditions in TN ducklings. In the cold, glucagon injection inhibited shivering in both groups of ducklings but thermogenesis was not suppressed in GT ducklings, showing a true nonshivering thermogenesis in these birds. This nonshivering thermogenesis was estimated to be 3 W/kg (i.e., 55% above resting MR). Such changes produced by chronic glucagon treatment resemble the artificial cold acclimation of rats chronically treated by norepinephrine.

birds; acclimation to cold; calorigenic effect of glucagon; shivering; free fatty acids

NONSHIVERING THERMOGENESIS (NST) has long been considered as an exclusive attribute of mammals; birds resort only to shivering thermogenesis (14, 28, 30). In addition, birds are devoid of brown adipose tissue (2, 21), the main site of NST in mammals. However, in cold acclimated young birds (ducklings) an important muscle thermogenesis coupled to a reduced contractile activity has been observed (3). This potentiated shivering thermogenesis (PST) was accompanied by an increased sensitivity of muscle mitochondria to the uncoupling effect of free fatty acids (5) and in a later stage of cold acclimation by the development of a true NST in response to cold (4).

The role of glucagon in the control of heat production, particularly as a possible mediator of NST in birds, was first suggested by Freeman (12) on the grounds of the potent glycogenolytic and lipolytic action of this hormone, both in vivo and in vitro. A considerable increase in O_2 consumption was later reported after glucagon injection in 3-mo-old Japanese quails (22), in winter-acclimatized king penguin chicks (6), and in 2- and 4-wk-old chicks (1) exposed to thermoneutral ambient temperature (T_a). In addition, an increase in plasma glucagon level was found in ducklings acclimated to 4°C T_a for 3 wk (4).

If glucagon is a potential mediator of NST in birds, chronic treatment with glucagon should mimic the state of cold adaptation, as does treatment with norepinephrine, in rats (23). To solve this problem, we have tried to induce artificially cold acclimation by repeated daily injections of glucagon to ducklings maintained at thermal neutrality for 6 wk. We are reporting on the calorigenic effect of glucagon in these birds and on a true NST due to glucagon. The experiments were conducted at two T_a, one in (at 25°C T_a) and one below the thermoneutral zone (at 4°C T_a).

MATERIALS AND METHODS

Animals. Male muscovy ducklings (*Anas barbariae*, pedigree R 31, INRA, France) were obtained from a commercial stock breeder (Ets Grimaud). They had free access to water and a commercial mash (Sanders 5061). For chronic treatment, the following schedule was used: from the age of 1 wk the ducklings were caged for a period of 6 wk at 25°C T_a in a constant photoperiod (8:16 light:dark) and treated either with glucagon (GT; 360 μg/kg ip twice daily at 8 A.M. and 6 P.M.) or with saline solution (controls, TN). Cold acclimated ducklings (CA) were reared at 4°C T_a from the age of 1 wk (3). A total of 48 ducklings weighing between 1.450 and 1.750 kg was used.

Metabolic rate. Metabolic rate (MR) was measured by indirect calorimetry. We used an open-circuit system derived from that of Depocas et al. (11) and already used for measurement in ducklings (3). The duckling deprived of food in the night was positioned in a thermostatic chamber ventilated by a constant atmospheric airflow (air speed 0.05 m/s). Variable heat loss by conduction on the ground was reduced by a polystyrene bed. Airflow rates (10 l/min) were measured using a Tylan mass flowmeter model 261 (accuracy ± 0.5% of full scale), and converted to standard values (STPD). The fractional concentrations of O_2 were monitored using a Servomex OA 184 paramagnetic gas analyzer with full-scale response to a change from 21 to 20%. CO_2 concentrations were measured using a Hartmann-Braun model Uras 2 infrared gas analyzer with a linearizing circuit. The full-

scale response of this analyzer was to a change from 0 to 2%. The O_2 and CO_2 analyzers were calibrated before and after each experiment with known mixtures of O_2 (± 0.01%) and CO_2 (± 0.02%). The rates of O_2 consumption and CO_2 production were calculated according to the equation of Depocas and Hart (10). The caloric equivalent for O_2 was determined from the respiratory quotient using Lusk tables (25).

Shivering. Shivering was measured as integrated electromyogram (EMG) activity by the procedure described by Barré et al. (3). Briefly, the EMG signal that was received from two monopolar electrodes inserted into the muscle (the gastrocnemius in this study) 5 mm apart was monitored on an oscilloscope and recorded directly on one channel of a Racia pen polygraph (DUO 75). The EMG signal, rectified and integrated, was displayed on the other channel of the polygraph. This expression of the EMG activity as the mean rectified value was shown to give the best correlation with the MR in the pigeon (18). Air (T_a) and cloacal (T_b) temperatures were measured using copper-constantan thermocouples connected to a Chessell model 320 recording potentiometer (accuracy ± 0.2°C).

Experimental procedure. After 4 wk of chronic treatment, the effects of a separate glucagon test injection (similar to the injections for chronic treatment) on MR, T_b, and EMG activity were studied at 25°C T_a in the thermoneutral zone and at 4°C T_a in the cold zone, zones previously determined in ducklings reared at 25°C T_a (3). All measurements of MR, T_b, and EMG activity were taken in darkness during daytime (between 8 A.M. and 7 P.M.). To obtain metabolic steady state and thermal equilibrium, the duckling was left sitting in the thermostatic chamber for an initial 120-min adjustment period before the experiment was begun. This initial period was considered to be long enough, because there was no noticeable change of T_b and MRs over 3–4 h under constant T_a conditions. At the end of the initial period the duckling was usually very quiet. MR, T_b, and EMG activity were recorded during a 20-min control period and then for 240 min after the intraperitoneal glucagon injection. Control experiments were conducted with saline in place of glucagon. Test injections were made between midday and 2 P.M. via a polyethylene catheter, intraperitoneally implanted before the beginning of the experiment and fixed with adhesive tape.

Drugs. Saline dilutions of glucagon (Nova Industrie Pharmaceutique, Paris) were prepared for each experiment less than 2 min before injection. Glucagon was dissolved to a concentration of 1 mg/ml for injections. The injection volume was 500 μl, and the dose used was 360 μg/kg. Control saline injection volume was 500 μl.

Plasma determinations. Blood samples were collected on ducklings after decapitation and immediately centrifuged at 4°C after sampling. Plasma free fatty acids (FFA) and triglycerides were measured using the enzymatic Nefa-C test, Wako, and Triglyceride G test, Wako, from Wako Chemicals (Osaka, Japan), respectively. Aliquots of plasma were deproteinized in 7% perchloric acid for assays of glucose (GOD-perid enzymatic method, Boehringer) and glycerol concentrations (glycerol UV-method, Boehringer) from Boehringer-Mannheim (Mannheim, FRG).

Units and terms. International units and the glossary of Bligh and Johnson (7) have been used throughout this paper. Values have been presented as means ± SE. Student's t test and F test were used for statistical calculations.

RESULTS

Effect of chronic treatment. To determine the effect of chronic treatment on their growth rate, ducklings were weighed every day. Growth curves of both GT and TN ducklings (Fig. 1) were similar from *day 8* to *16*. But from *day 17*, body mass of GT ducklings was less ($P < 0.05$) than that of TN ducklings, and the difference between both GT and TN ducklings increased with increasing age. Growth rate of GT ducklings appeared to be slower with the establishment of a plateau from the age of 5 wk ($F = 1.99$, $P < 0.05$). If the treatment was pursued beyond 6 wk, some GT ducklings ceased to eat and finally died through hyperthermia after glucagon injection.

As shown in Table 1, chronic treatment with glucagon does not change the level of MR at either 25 or 4°C T_a as compared with controls. This result is at variance with the increased basal metabolic rate and metabolic response to cold in CA ducklings at 25 and 4°C T_a, respectively.

Effects of saline solution injection (Figs. 2 and 3). In GT ducklings receiving saline solution injection at 25 and 4°C T_a, no significant change in MR (at 25°C T_a, $F = 0.12$; at 4°C T_a, $F = 0.88$; NS) integrated EMG activity (at 4°C T_a, $F = 0.76$; NS) or T_b (at 25°C T_a, $F = 0.18$; at 4°C T_a $F = 0.08$; NS) was observed after the injection.

Effect of glucagon test injection. The effects of this hormonal test injection were studied comparatively in GT and TN ducklings at two values of T_a purposely chosen above and under the lower critical temperature (LCT) previously determined (3), i.e., at 25°C T_a (thermoneutral zone) (Fig. 2) and 4°C T_a (cold zone) (Fig. 3). Results of glucagon test injections were also given in CA

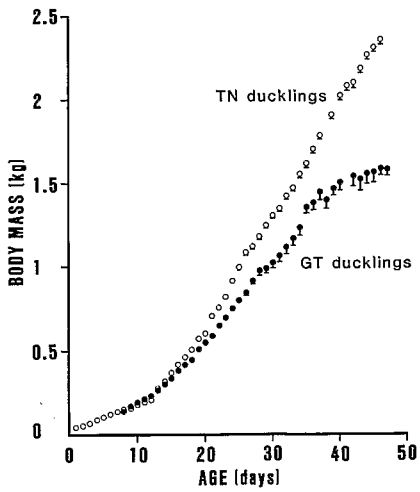

FIG. 1. Growth rate from glucagon-treated (GT) and control (TN) ducklings, both reared at 25°C ambient temperature (T_a). Values are means and *bars* are SE; $n = 6$ in each group.

TABLE 1. *Metabolic rates of ducklings at thermoneutrality or in the cold*

	MR Measured, W/kg			
	Without test injection		After glucagon test injection	
	25°C T_a	4°C T_a	25°C T_a	4°C T_a
GT ducklings	5.5±0.3	8.0±0.2	10.8±0.2*	8.4±0.2
TN ducklings	5.5±0.2	7.9±0.6	7.2±0.2*	5.4±0.2†
CA ducklings	6.1±0.3	8.7±0.4	8.7±0.4*	7.4±0.4

Values are means ± SE; $n = 6$ in each group of ducklings. Metabolic rates were measured at 25 and 4°C ambient temperature (T_a) in glucagon-treated (GT) and control (TN) ducklings (reared at 25°C T_a) and in cold-acclimatized (CA) ducklings (reared at 4°C T_a). In GT ducklings, the measurements were made at least 14 h after the last glucagon injection. MR, metabolic rate. Comparisons are made between MR values measured without or with glucagon test injection, at the same T_a. * $P < 0.001$; † $P < 0.01$.

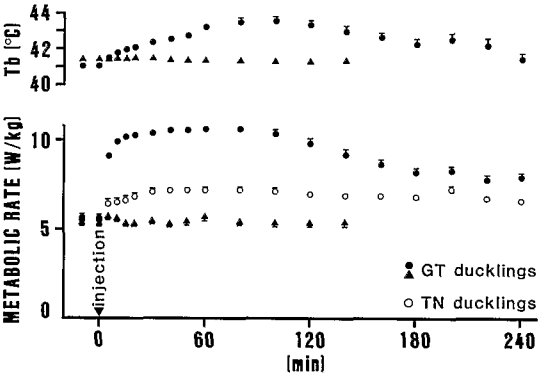

FIG. 2. Effects of glucagon test injection (360 μg/kg ip) on body temperature (T_b) and metabolic rate in glucagon-treated (GT; *closed circles*) and control (TN; *open circles*) ducklings exposed to neutral ambient temperature (25°C T_a). GT ducklings also received intraperitoneal saline solution injection (*closed triangles*). In GT ducklings, all points of T_b from *min 15* and of metabolic rate from *min 5* are significantly greater ($P_{Tb} < 0.01$ and $P_{MR} < 0.001$, respectively) than control values. In TN ducklings, all *points* of metabolic rate between *min 30* and *120* are significantly greater ($P < 0.05$) than control values. Values are means and *bars* are SE; $n = 6$ in each group.

ducklings for comparison (Table 1).

At 25°C T_a after the glucagon test injection in GT ducklings, there was a sudden increase in MR. Its maximum value (10.8 ± 0.2 W/kg, i.e., 98.5% above the saline control value) was reached 40 min after this injection. A plateau was maintained for the next 40 min, and then MR decreased progressively. In TN ducklings, the glucagon-induced change in MR followed the same pattern as in GT ducklings, but the maximum value (7.2 ± 0.3 W/kg) that was only 29% above the saline control value was significantly lower ($t = 6.52$; $P < 0.001$) than the maximum value in GT ducklings. In CA ducklings, the maximum value in MR (8.7 ± 0.4 W/kg) due to glucagon injection represented an intermediate increase in MR of 44% above the saline control value.

At 25°C T_a in GT ducklings, when MR increased from *min 5* ($F = 44.1$; $P < 0.001$), T_b increased significantly only from *min 15* ($F = 4.7$; $P < 0.01$) due to an active polypnea (breathing frequency: 230 ± 10 breaths/min in GT ducklings vs. 30 ± 2 breaths/min in TN ducklings) starting some minutes after glucagon injection. The glucagon-induced increase in T_b reached 2.4°C from *min 80* to *100* (43.6 ± 0.4°C in GT ducklings vs. 41.2 ± 0.2°C in Tn ducklings).

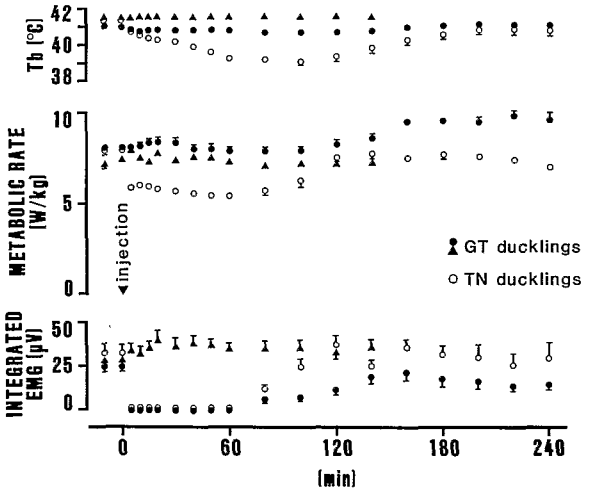

FIG. 3. Effects of glucagon test injection (360 μg/kg ip) on body temperature (T_b), metabolic rate, and integrated electromyogram (EMG) activity in glucagon-treated (GT; *closed circles*) and control (TN; *open circles*) ducklings exposed to 4°C T_a. GT ducklings also received intraperitoneal saline solution injection (*closed triangles*). Shivering activity was inhibited by glucagon in both GT and TN ducklings for 75 min after the intraperitoneal injection: in these ducklings, all *points* of integrated EMG activity between *min 5* and *140* and between *min 5* and *80*, respectively, are significantly lower ($P < 0.05$) than control values. But metabolic rate and T_b decrease only in TN ducklings after glucagon injection: all *points* of metabolic rate between *min 5* and *80* and of T_b between *min 30* and *160* are significantly lower than control values.

At 4°C T_a no significant change in MR was observed in GT ($F = 1.6$; NS) and CA ducklings ($F = 0.3$; NS) after glucagon injection during 180 min of exposure at 4°C T_a, although within 1 min after injection shivering activity was completely suppressed. Shivering activity was absent for 75 ± 15 min, and full recovery occurred 10–20 min after the first signs of reappearance, while MR increased. In TN ducklings, inhibition of shivering activity followed the same pattern as in GT ducklings but was concomitant with a 25.5% decrease in MR from *min 5* after glucagon injection ($F = 8.8$; $P < 0.05$). When shivering reappeared, MR increased to its initial rate.

At 4°C T_a, T_b was not affected by glucagon injection in GT ducklings ($F = 0.6$; NS). In TN ducklings, the T_b decrease was 2.5°C in the 100 min after glucagon injection (39.0 ± 0.4°C in TN ducklings at *min 100* vs. 41.5 ± 0.2°C before glucagon injection). With the reappearance of shivering and the increase of MR, T_b increased to its initial level.

Plasma metabolites. Table 2 shows the plasma metabolite concentrations in GT and TN ducklings exposed to 25°C T_a, 14 h after the last glucagon injection. In both groups, no significant changes occurred in blood glucose and plasma triglyceride levels. But in GT ducklings, plasma FFA and glycerol levels were, respectively, 112 and 250% above the levels of controls.

Cold resistance was assessed by measuring the change in cloacal temperature during a 180-min exposure to −15°C (Fig. 4). Whereas CA ducklings were better able to maintain T_b (40.3°C) than TN ducklings, GT duck-

TABLE 2. *Plasma metabolites in control and chronically glucagon-treated ducklings*

	TN Ducklings	GT Ducklings
Glucose, mM	13.02±0.42	14.17±0.69
FFA, mM	0.08±0.01	0.17±0.01*
Glycerol, mM	0.10±0.02	0.35±0.10†
Triglycerides, mM	2.49±0.25	1.72±0.26

Values are means ± SE; $n = 6$ in each group of ducklings. Plasma concentrations were measured 14 h after the last glucagon injection. See Table 1 for abbreviations. * $P < 0.001$; † $P < 0.05$.

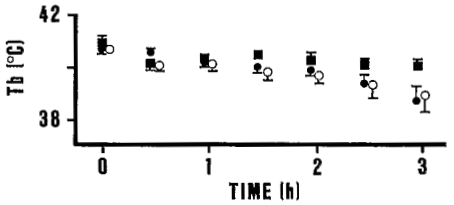

FIG. 4. Change of cloacal temperature (T_b) during 180-min exposure to cold (−15°C T_a) in cold-acclimated (CA; *closed squares*), glucagon-treated (GT; *closed circles*), and control (TN; *open circles*) ducklings. There was no significant decrease of T_b in CA ducklings ($F = 1.11$). But decrease of T_b is significant from *min 150* in both GT ($F = 17.48$, $P < 0.001$) and TN ducklings ($F = 4.20$, $P < 0.05$). Values are means and *bars* are SE; $n = 6$ in each group.

lings were not. Indeed, T_b of TN and GT ducklings decreased significantly from *min 150* to the end of the experiment, reaching 38.9 and 39.4°C, respectively.

DISCUSSION

Chronic treatment of ducklings. The dose of glucagon (360 μg/kg twice a day) was within the range of the doses used in rats (500 μg/kg twice a day) (31). Inasmuch as glucagon pool is about 10 times larger in birds (duck, goose) than in mammals (27) and plasma glucagon level is 90% higher in cold-acclimated ducklings than in controls (4), this dose, although it might not be regarded as physiological, was actually lower than that previously used in mammals (31).

Calorigenic action of glucagon. The calorigenic action of glucagon is well documented in mammals, newborn rabbits (15), rats (9), and dogs (29), but is not found in mice and guinea pigs (22). In birds, a considerable calorigenic effect comparable with that elicited by norepinephrine in mammals was induced by glucagon in winter-acclimatized penguin chicks (6), cold-acclimated ducklings (this study), 3-mo-old Japanese quail (22), and 2- and 4-wk-old chickens (1) exposed to neutral ambient temperature, but this action was not observed in 2-day-old chicks (26) nor in pigeons (19). Note that in any case, these calorigenic actions were much less than the calorigenic action observed in GT ducklings.

NST in GT ducklings. The prominent result of the present study was the potent thermogenic action of glucagon resulting in hyperthermia and leading sometimes to the death of the GT ducklings. This effect was observed despite the absence of shivering at thermoneutrality and even despite the suppression of shivering in the cold. As this inhibition of shivering produced a 2.5 W/kg decrease in MR in TN ducklings only and no significant MR variation in GT and CA ducklings, the metabolic effect of exogenous hormone during a test injection substituted for part of the regulatory thermogenesis at 4°C T_a, relieving the shivering thermogenesis in GT and CA ducklings, as previously described in winter-acclimatized king penguin chicks (6). Under these conditions, in GT ducklings glucagon was responsible for a true NST that may be estimated to be 3 W/kg (i.e., 55% above resting MR). As this calorigenic response was seen both in GT ducklings and, to a lesser extent, in CA ducklings, one is justified in considering it as an NST comparable with that described in cold-acclimated mammals receiving norepinephrine (review in Ref. 17). However, the previous observation of an ability of ducklings to develop an NST of muscular origin after cold acclimation (3) raised the question of the action mechanism of glucagon.

Sensitization to glucagon by chronic treatment. Ducklings injected for 6 wk with glucagon acquired from *day 23* (i.e., 2 wk after the beginning of the chronic treatment) an increased sensitivity to the calorigenic action of this hormone. The response to test injection was even larger than that of cold-acclimated ducklings at neutral ambient temperature (5.3 vs. 2.6 W/kg). The change in sensitivity to glucagon by chronic treatment is comparable with that described by Leblanc and Pouliot (23) for norepinephrine in rats treated for 45 days with this hormone (300 $\mu g \cdot kg^{-1} \cdot day^{-1}$), the mediator of NST in mammals. The same order of MR increase above basal values was obtained with both hormones, glucagon, and norepinephrine, respectively, 29 and 26% in control ducklings and rats, 98 and 75% in GT ducklings and norepinephrine-treated rats, and only 44 and 49% in cold-acclimated ducklings and rats. Although the effector tissue responsible for thermogenesis is different in both animals, because brown adipose tissue is lacking in birds (2, 21), a comparable mechanism of sensitization by repeated injections may be inferred for the hormone action.

Mechanism of the glucagon thermogenic effect. The powerful thermogenic response to glucagon exhibited by GT ducklings at 25°C T_a appears as the chief result. The intense panting and the stretched posture were signs of a struggle against the hyperthermia caused by this excess thermogenesis. In the cold, glucagon thermogenesis simply replaced shivering. Indeed, suppression of shivering was also observed in control ducklings at 4°C T_a. A similar inhibition of shivering after single glucagon injection has already been noted in the pigeon (19) and in the king penguin chick (6). A central neuromodulator action of glucagon, independent of the peripheral thermogenic effect similar to that observed in the rat (20), may explain the inhibition. On the other hand, if glucagon treatment of ducklings results in an enhanced thermogenesis from muscular activity, like PST observed in cold-acclimated ducklings (3), all muscular activity becomes a threat of hyperthermia and shivering should be strictly controlled or even suppressed.

Whereas norepinephrine does not produce any increase in MR and is not lipolytic in cold-acclimated (8) or in winter-acclimatized birds (6), glucagon is a potent lipolytic hormone in birds (13). At rest, 14 h after the

last glucagon injection, the higher plasma FFA level in GT ducklings could be due either to decreased utilization or increased mobilization from adipose tissue. Because an increase in plasma glycerol level usually roughly reflects the increase in the rate of lipolysis in adipose tissue (16), the higher blood glycerol concentration in GT ducklings favors the interpretation that the rate of FFA production is increased in these GT birds, even in absence of stimulation by exogenous glucagon. However, even after glucagon test injection, the increase in FFA blood level can provide the cells with more fuel available, but cannot trigger the utilization of this fuel. The rapid metabolic response to injected glucagon is to be ascribed to a direct effect at the cellular level in the thermogenic tissue. It must be pointed out that the sensitivity to the uncoupling effect of FFA on muscle mitochondria is increased in CA ducklings (5). A similar modification is presently under investigation in GT ducklings (H. Barré, G. Berne, P. Brebion, F. Cohen-Adad, and J. L. Rouanet, unpublished observations).

It is possible to parallel the artificial cold acclimation obtained in rats by chronic norepinephrine treatment (23) and the sensitization to glucagon observed in GT ducklings. Indeed the same differences between CA and GT ducklings are observed as in CA and chronically norepinephrine-treated rats: increased tolerance to low ambient temperatures only in CA ducklings, but not in GT and TN ducklings, and increase in resting MR in CA animals, but no changes in chronically hormone-treated animals. On the other hand, it is now acknowledged that cold acclimation in rats is triggered by continuous norepinephrine secretion during prolonged exposure to cold (24). Similarly, an increased secretion of glucagon was shown in CA ducklings (4). Thus glucagon has to be considered among the mediators of cold acclimation in birds during the period of growth.

The authors thank Prof. J. Chatonnet for helpful discussions and suggestions.

This work was supported by grants from Département de Biologie Humaine, Université Claude Bernard, Lyon I and Centre National de la Recherche Scientifique.

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Received 24 July 1986; accepted in final form 9 December 1986.

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