different reaction times. Since the color yield depends on a catalyzed reaction between protein and the assay reagents, an increase in temperature will increase the color yield at a given incubation time. We have tested the color yield at room temperature, 37 °C and 60 °C, and found that temperatures above 37 °C gave unsatisfactory results for the following two reasons; firstly, heating of the micro-titer plate caused uneven evaporation leading to erroneous results, and secondly, Triton X-100 clouded which resulted in a two-phase system.

Prolongation of the reaction time will also give an increase in color yield. In our hands optimal results were obtained when the plates were first incubated at room temperature for 20 min. If the unknown samples had reacted, i.e. if a color formation could easily be seen, the plate was read in the photometer. This was typically the case for samples with protein concentrations above 200 µg/ml. If the wells contained less protein (down to 10 µg/ml) the plate was either kept at room temperature for 20 h or at 37 °C for 2 h, and finally measured spectrophotometrically again. This two-step reading allows to the very rapid determination of protein concentrations in the range of 10 µg/ml to 2 mg/ml.

The influence of the non-ionic detergents Triton X-100 and Tween 20 was investigated since these additives are common in studies involving membrane proteins. The standard curves for protein in the concentration range of 10–1500 µg/ml was not influenced by either detergent in concentrations up to 5% (the highest measured). In all cases the standard curves could be superimposed on those obtained without detergents, i.e. within the standard error of the assay (± 5%). Nonetheless, we recommend that the standard curve be recorded in the presence of the same detergent at the same concentration as in the unknown sample, since some detergents may have (oxidative) impurities which could affect the determination. Since there was no indication of a concentration dependency it is likely that even higher detergent concentrations will not interfere. We have further compared the values obtained with our modified BCA assay to those obtained by the method of Lowry, as modified by Wang and Smith[3], a method which also excludes interference from non-ionic detergents. Detergent extracts from animal brains were analyzed for protein using both methods, and in all cases the results differed by less than ±10%.

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Acute cold exposure increases the glucagon sensitivity of thermogenic metabolism in the rat

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Summary. Administration of glucagon to rats at 25 °C had no effect upon their $\dot{V}O_2$, while administration of noradrenaline or noradrenaline plus glucagon raised the $\dot{V}O_2$. At 5 °C, noradrenaline had no effect upon the cold-enhanced $\dot{V}O_2$, while glucagon caused a rise of 13.7%, implying increased glucagon sensitivity at 5 °C. The glucagon-induced enhancement of $\dot{V}O_2$ was abolished by concurrent administration of noradrenaline.
Key words. Glucagon; thermogenesis; cold-exposure; $\dot{V}O_2$.

On exposure of animals to low environmental temperatures, thermogenic metabolism is increased in order to maintain body temperature[1]. Upon initial exposure, the primary method of additional heat production is that of shivering; with increased duration of cold exposure, non-shivering mechanisms, primarily attributable to brown adipose tissue (BAT) become predominant[2] and eventually replace shivering as the source of enhanced thermogenesis[3]. Activation of BAT is widely considered to result from increased adrenergic activity during cold exposure[4]. There is substantial evidence, however, that non-adrenergic factors may also be implicated in the activation of BAT[5]. Such factors may include the corticosteroids[6] and glucagon, the latter of which has been suggested to be an important component of a multifactorial activation of BAT in cold environments[7]. BAT appears to be active within 1 h of cold exposure[8], as evidenced by increased mitochondrial GDP-binding, indicating raised levels of 32 kdalton uncoupling protein[9]. Therefore, as corticosteroids are generally regarded as relatively slow-acting agonists, while plasma glucagon has been shown to be significantly elevated after cold exposure for 1 h[10], the experiments described below were undertaken to obtain an indication of the relative potencies of noradrenaline and glucagon as promoters of thermogenic metabolism. The potencies were assessed by measurement of oxygen consumption ($\dot{V}O_2$) of intact animals at thermoneutrality and in acute cold exposure.

Materials and methods. Male Wistar albino rats of body weight 210 ± 6.8 (SEM) g were used. They were held at an ambient temperature of 22.5 ± 1.0 °C, with a 12 h photoperiod (08.00–20.00 h), and received food and water ad libitum. The animals were divided into 2 groups: one for experiments at thermoneutrality (25 °C) and one for experiments in acute cold exposure (5 °C). Neither group had previous experience of cold exposure. All experiments were performed between 10.00 h and 16.00 h, at the nadir of their circadian cycle as indicated by plasma corticosterone concentration[11]. Four treatment regimes were employed at each experimental temperature: 1) Control, consisting of the vehicle used in drug administrations (NaCl 250 µg/ml and lactose 1.07 mg/ml in sterile distilled water, pH adjusted to 3.5 with 0.1 M HCl) administered at a dose of 0.1 ml/100 g b.wt, i.p.; 2) Noradrenaline as the acid tartrate (Levophed: Winthrop Laboratories, U.K.), was diluted with the vehicle to 250 µg/ml and given at a dose of 25 µg/100 g b.wt, i.p.; 3) Glucagon (Novo Industri A/S, Denmark) as the hydrochloride at 1 mg/ml in the vehicle, given at 100 µg/100 g b.wt, i.p. (12); and 4) Noradrenaline plus glucagon at the doses indicated above, given in a total volume of 0.1 ml/100 g b.wt, i.p. Immediately following administration of the drug or vehicle, the animals were placed individually into the temperature-controlled chambers of a high-precision respirometric system as previously described[13]. Measurements of $\dot{V}O_2$ were noted at 10-min intervals during the first hour after the administration of vehicle or drugs. Data from the second hour were expressed as ml O_2/min/kg$^{0.75}$ at standard temperature and pressure of dry gas. Data from the second hour were used because elevations of $\dot{V}O_2$ following handling and

Second-hour oxygen consumptions of rats at 25 °C and 5 °C. Data are means ± SEM; in all cases n = 6. Values for $\dot{V}O_2$ are at STPD

Environmental temperature	$\dot{V}O_2$ (ml min^{-1} kg$^{-0.75}$) 25 °C	5 °C
Treatment:		
Control	9.67 ± 0.57	18.30 ± 0.80
Noradrenaline	12.68 ± 0.63* #	18.34 ± 0.35 #
Glucagon	9.72 ± 0.34	20.82 ± 0.49*
Noradrenaline + glucagon	12.98 ± 0.64** # #	17.99 ± 0.40 # #
Statistical analysis:		
vs control	*p < 0.006	*p < 0.04
	**p < 0.004	
vs glucagon-treated	# p < 0.004	# p < 0.003
	# # p < 0.003	# # p < 0.002

drug administration were not consistent among animals, and reached steady values only after 40–50 min. Significances of differences were assessed using Student's t-test for paired samples; statistical significance was assumed at $p \leq 0.05$.

Results and discussion. The results are presented in the table. At 25 °C, a clear enhancement of $\dot{V}O_2$ is evident following treatment with noradrenaline, consistent with earlier reports[12]. However, administration of glucagon at 25 °C did not effect any change in $\dot{V}O_2$, in contrast to the report of Doi and Kuroshima[12], who found enhanced $\dot{V}O_2$ in response to glucagon at the same dose as that used here. Following the concurrent administration of noradrenaline and glucagon, the $\dot{V}O_2$ rose to the same level as that seen with noradrenaline alone. In the cold-exposed group, in all cases the $\dot{V}O_2$s were higher than those recorded at thermoneutrality. However, the pattern of response to glucagon and noradrenaline was dissimilar to that seen at thermoneutrality. Noradrenaline failed to enhance $\dot{V}O_2$, while the glucagon-treated group exhibited a further elevation of $\dot{V}O_2$ amounting to a factor of 13.7%. Thus cold exposure appeared to increase the sensitivity of thermogenic processes to glucagon, as indicated by its effects upon oxygen consumption, while concurrent administration of noradrenaline with glucagon abolished the enhanced glucagon sensitivity.

Two principal issues arise with respect to these data. First, the reasons for the discordance between these data and those of Doi and Kuroshima[12] in relation to the effect of glucagon upon $\dot{V}O_2$ in the warm-acclimated animal at thermoneutrality, and second, the functional significance of the cold-induced elevation of glucagon sensitivity.

With reference to the first issue, two factors may be relevant. First, Doi and Kuroshima[12] measured $\dot{V}O_2$ over a period of 60 min immediately after giving glucagon, whereas in the present report the data were obtained over a period of 60 min, starting 60 min after drug administrtion. Their data (in loc. cit.) showed a decline of $\dot{V}O_2$ towards basal values 60 min after giving glucagon; possibly by measuring the second-hour $\dot{V}O_2$, as here, an initially raised $\dot{V}O_2$ might have subsided. However, the 10-min observations of $\dot{V}O_2$ made during the first hour after giving glucagon revealed no enhancement; to the contrary, at 30 and 40 min after glucagon administration, the $\dot{V}O_2$ of that group was significantly less ($p < 0.05$) than that of the control group. The reason for the difference may relate to the methods used. Doi and Kuroshima[12] used glucagon dissolved in 0.01 M HCl, which was then diluted with saline. In the absence of pH buffering in the diluent, it may be that the increased $\dot{V}O_2$ reported by them was a result of systemic stress caused by the i.p. administration of a solution of pH approximately 2.0. Immobilisation stress, as well as cold stress, is a potent activator of BAT[14]; other stressors may act in a similar manner. Thus, the elevated $\dot{V}O_2$ seen in animals at thermoneutrality may have been a consequence of the i.p. administration of a solution of low pH, rather than the contained glucagon per se. However, intravenous infusion of glucagon into warm acclimated rats at thermoneutrality caused elevation of plasma free fatty acid (FFA) levels in the venous effluent of interscapular BAT in the presence of β-adrenergic blockade[15]. Nonetheless, a non-specific stress component of raised $\dot{V}O_2$ cannot be excluded. The reason(s) for the evident lack of glucagon sensitivity with respect to $\dot{V}O_2$ at thermoneutrality remain unclear, although unpublished data from the author's laboratory has shown that isolated brown adipocytes from warm-acclimated animals from the same breeding stock as those used in this report show no enhancement of $\dot{V}O_2$, measured polarographically, in response to glucagon, consistent with the data here reported.

With reference to the second issue, that of the functional significance of the cold-induced increase of glucagon sensitivity, factors other than BAT require consideration. Upon initial exposure to cold, while BAT is activated in the rat with no prior cold experience[16], the thermogenic capacity of that tissue, in such an animal, is limited[2], and it is considered that shivering thermogenesis is the primary source of the initially enhanced heat production[17]. In either case, an important prerequisite is substrate mobilization. In acute cold exposure, plasma FFA has been shown to provide some 75% of the energy supply for thermogenic processes[18]. Glucagon possesses potent lipolytic effects in both white[19] and brown[20] adipose tissues, in both cases liberating FFA from triglyceride stores. While the cold-induced increase of glucagon sensitivity cannot readily be explained on the basis of the present data, it is tempting to speculate that in view of the fact that the lipolytic actions of glucagon are maximal at low insulin concentrations[21], as found in acute cold exposure[22], the enhanced glucagon sensitivity seen under such conditions relates to reduced insulin secretion. However, as reduced insulin secretion is associated with increased adrenergic activity[23], the reason why concurrent noradrenaline administration should abolish the cold-induced enhancement of glucagon sensitivity remains unclear.

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