ing the samples and allowing them to stand for another 10–15 min, they were centrifuged at 17.000 × g, 30 min, 0 °C, to prepare final pellet and supernatant fractions. Radioactivity due to ^{3}H and ^{14}C was determined by a previously-described method[12] and protein was determined by the method of Lowry et al.[14]. ^{14}C-sucrose distribution ratios provided measures of the supernatant fluid in the pellets. The data are expressed as mole ^{3}H-glycine bound/mg protein and as mole ^{3}H-glycine bound/g P_2, corrected for sucrose space[12]. Values were corrected further for 'nonspecific' binding of ^{3}H-glycine that occurred in the presence of 10^{-3} M unlabelled glycine[11].

Results and discussion. 'High-affinity' mechanisms for ^{3}H-glycine binding to 'synaptosomal-mitochondrial' fractions of 6 regions of rat CNS were evident in data presented on a protein basis (figure 1a) and on a weight basis (figure 1b). Corresponding K_D values were similar for all regions, and ranged from about $0.8–1.6 \times 10^{-7}$ M. It is noteworthy that the order of potency of these binding mechanisms varied with respect to both the region analyzed and the method used for expressing the data. Correction of the data for the amounts of ^{3}H-glycine bound in the presence of 10^{-3} M unlabelled glycine revealed that these binding mechanisms involved positive cooperativity. This is evident from the parabolic, concave-upward Lineweaver-Burk plots shown in figure 2. Hill plots of these data possessed slopes that ranged from about 1.25–1.50. These results support the observation of Werman[15,16] that more than one glycine molecule is necessary for activation of glycine-receptors. Such cooperativity is also in accord with the finding of Giambalvo and Rosenberg[17] that GABA binding to post-junctional complexes of rat cerebellum occurred with a Hill coefficient of about 2.2 and with physiological results which have indicated that 2 GABA molecules are required to activate the GABA-receptor of the crayfish neuromuscular junction[18]. Results shown in the table revealed further that competition of several amino acids for these ^{3}H-glycine-binding sites paralleled their relative potencies at mimicking the post-synaptic depressant action of glycine[2,19], but not their relative potencies at inhibiting glycine-binding to CNS transport-receptors; e.g., β-alanine exerts an effect similar to that of glycine on spinal neurones[19], whereas it does not compete significantly with glycine for binding to CNS membrane fragments[10]. As observed previously with higher concentrations of glycine[20], this binding was sensitive only to $10^{-5}–10^{-3}$ M strychnine-SO_4 (data not shown), indicating that glycine and strychnine are bound to distinct CNS sites.

14 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
15 R. Werman, Comp. Biochem. Physiol. *30*, 997 (1969).
16 R. Werman, Ass. Res. nerv. ment. Dis. *50*, 147 (1972).
17 C. T. Giambalvo and P. Rosenberg, Biochim. biophys. Acta *436*, 741 (1976).
18 A. Takeuchi and N. Takeuchi, J. Physiol., Lond. *205*, 377 (1969).
19 D. R. Curtis, L. Hösli and G. A. R. Johnston, Exp. Brain Res. *5*, 235 (1968).
20 F. V. DeFeudis, P. Madtes and J. Gervas-Camacho, Experientia *33*, 340 (1977).

Tolerance to cold and glucose homeostasis in adrenal demedullated dogs[1]

J. Forichon, M. J. Jomain, M. C. Patricot and Y. Minaire[2]

Laboratoire de Thermorégulation (C. N. R. S.), Domaine Universitaire Rockefeller, 8, avenue Rockefeller, F–69373 Lyon Cédex 2 (France), 2 February 1977

Summary. The rise in O_2 consumption and in glucose turnover, induced by acute cold exposure is not suppressed by adrenal demedullation in dogs. However, both at neutral and cold ambient temperature, the mean plasma glucose concentrations are higher in normal (N) than in adrenal-demedullated dogs (ADMX). In the cold, the fall in rectal temperature is larger in ADMX than in N dogs.

When acutely exposed to low ambient temperatures, dogs are able to increase their metabolic rates up to 10 times the BMR[3]. Such an energy expenditure is covered by a parallel increase in substrates supply, mainly FFA and glucose. Previous experiments conducted in cold-exposed normal dogs have shown that there is an almost linear relationship between glucose turnover, as measured by U-^{14}C-glucose, and energy expenditure for O_2 consumption, ranging from 5 to 40 $ml \cdot kg^{-1} \cdot min^{-1}$ [4]. In addition, no significant drop in plasma glucose concentration has been observed in these dogs, even at times when they were exposed for more than 3 h to ambient temperatures far below − 20 °C. This seems to suggest a rather strict balance between glucose production and utilization. These results prompted us to look for the mechanisms permitting glucose metabolism to be so perfectly adapted to the requirements of energy needs. Adrenomedullary secretion is increased during cold exposure[5], and the hyperglycemic effect of catecholamines is well-documented (for review, see Himms-Hagen[6]). The aim of this investigation was to compare energy expenditure, tolerance to cold and glucose turnover in normal and adrenal-demedullated dogs exposed to neutral and cold ambient temperatures.

Methods. 16 unanesthetized adult female mongrel dogs, with body weights ranging from 7.8 to 16.1 kg (mean: 11.3 kg) were used in 49 experiments. They were housed in a + 22 °C temperature-controlled room and fed about 300 g of a dry commercial pet food (U.A.R. 121, containing 42.5% of its calories in carbohydrate form). The animals were fed daily between 17.00 h and 18.00 h, before the experiment on the following day, so that the period of fasting was about 15 h at the beginning and 19 h at the end of the experiment. They were given tap water ad libitum. 10 dogs were used in 18 experiments

1 This work was supported by a grant from Université Claude Bernard (U.E.R. Lyon Nord and U.E.R. Biologie Humaine).
2 The authors thank D. Rougier and G. Dallevet for their excellent technical assistance and A. Brillant for secreterial assistance.
3 J. Chatonnet and Y. Minaire, Fed. Proc. *25*, 1348 (1966).
4 Y. Minaire, J. C. Vincent-Falquet, A. Pernod and J. Chatonnet, J. appl. Physiol. *35*, 51 (1973).
5 J. Klepping, M. Tanche and J. F. Cier, C. r. Soc. Biol. *151*, 1539 (1957).
6 J. Himms-Hagen, in: Handbook of Physiology, section 7, vol. VI. Ed. H. Blashko, G. Sayers and A. D. Smith. Williams and Wilkins Company, Baltimore 1975.

Comparison of data collected at neutral and cold ambient temperature in normal (N) and adrenaldemedullated (ADMX) dogs

	Ta = +25 °C N (n = 10)		ADMX (n = 17)		Ta = −21 °C N (n = 8)		ADMX (n = 14)
$\dot{V}O_2$ (ml · kg$^{-0.75}$ · min^{-1})	13.4 ± 0.61		14.1 ± 0.41		63.0 ± 2.89		58.9 ± 2.10
ΔTr (°C)	0.39 ± 0.132		0.57 ± 0.175		0.31 ± 0.268	*	1.15 ± 0.259
Plasma cortisol (μg · 100 ml^{-1})	0.68 ± 0.163		0.43 ± 0.081		2.45 ± 0.363		1.83 ± 0.345
Plasma glucose (mg · 100 ml^{-1})	94.8 ± 1.57	**	90.2 ± 0.83		93.7 ± 2.44	*	87.8 ± 1.72
Turnover rate (mg glucose · kg$^{-0.75}$ · min^{-1})	7.7 ± 0.63		7.9 ± 0.43		14.1 ± 1.56		12.3 ± 0.62

Values are means ± SEM; n = number of experiments. Symbols: * $p < 0.05$ (t-test for unpaired data); ** $p < 0.01$. ΔTr: Tr at the beginning minus Tr at the end of the experiment.

and are designated in the text as normal (N). In 11 animals, bilateral adrenomedullary inactivation was accomplished by a one-step surgical procedure involving demedullation and denervation of the right gland, and total adrenalectomy of the other[7]. Such animals are designated in the text as adrenal-demedullated (ADMX) and were used for 31 experiments at least 2 weeks after demedullation. A post-mortem histological control of the right gland was performed on each dog. It never disclosed any remaining medullary tissue.

For 3–5 weeks, the animals were trained to lie calmly for 4 h on a table set within a large thermostatic chamber, with their heads enclosed in a ventilated plastic mask[8,9]. During the experiments, the respiratory mask was connected to an open-circuit system[10] for measurement of O_2 consumption (Paramagnetic analyzer Magnos 2, Hartmann-Braun). 2 ambient temperatures, Ta = +25 °C (air flow in the mask: 25 l/min) and Ta = −21 °C (air flow: 70 l/min), were selected. In the cold environment, the animals, which were sheared 1 day before each experiment, developed a sustained shivering that increased the energy expenditure to nearly 4.4 times the BMR. About 1 week before the beginning of the experiments, after the animals had been adequately trained, two vascular catheters (one in a carotid artery and one in a jugular vein) were chronically implanted[11] for serial sampling of arterial blood and for venous infusion of U-^{14}C-glucose.

Each experiment was started between 09.00 h and 10.00 h. Rectal temperature was measured at the beginning and at the end of the experiment. After body weight was noted, the animal was positioned in the experimental set-up, and an initial adjustment period of 45 min was allowed, in order to obtain metabolic steady-state and thermal equilibrium. The experimental procedures were carried out according to the priming dose-infusion technique[12]. Uniformly labelled ^{14}C-glucose (238 mCi · mmole^{-1}) diluted in saline was employed. The priming dose was immediately followed for 3 h by a continuous infusion at a constant rate. The ratio priming dose/infusion rate was respectively 80:1 and 58:1 at neutral and cold ambient temperatures. Arterial blood samples were drawn in chilled tubes containing a NaF-heparine mixture 75, 105, 135 and 165 min after the start of the infusion. Plasma glucose was measured by a glucose oxidase method. Plasma glucose ^{14}C specific activity was determined by liquid scintillation counting, using hexadecane-^{14}C as internal standard. During these experiments, even at neutral ambient temperature, an isotopic steady-state was reached 70 min after the beginning of the infusion, and the data obtained from samples collected at 105, 135 and 165 min were used to calculate specific activities. Mean plasma cortisol concentrations in samples collected at 105, 135 and 165 min were determined by a radiocompetition method[13].

Results (table). At neutral ambient temperature (Ta = +25 °C), oxygen consumption was not significantly different in N and in ADMX dogs. Moreover the rise in oxygen consumption induced by the lowering of the ambient temperature was the same in these 2 groups of animals, the O_2 consumption being not significantly different in N and in ADMX dogs at Ta = −21 °C. Whatever the ambient temperature, hypothermia never occurred in N or in ADMX dogs; the lowest rectal temperature (Tr) observed was 36.2 °C in an ADMX dog, after 225 min of cold exposure. However, the Tr slightly decreased throughout the experiments in both N and ADMX dogs, the decrease being greater and significant in ADMX than in N animals exposed to Ta = −21 °C.

Plasma cortisol concentrations were not significantly different in N and in ADMX dogs at both Ta = +25 °C and Ta = −21 °C, the values obtained during cold exposure being 4 times higher than those observed at a neutral ambient temperature. Mean plasma glucose concentrations were significantly lower in ADMX than in N dogs at both Ta = +25 °C and Ta = −21 °C. However, comparisons of data collected at 75 min and 165 min after the beginning of the experiment showed that no significant decrease occurred at Ta = +25 °C both in N and in ADMX dogs and at Ta = −21 °C in N dogs. Actually, in ADMX dogs exposed to Ta = −21 °C, a significant ($p < 0.05$) but slight increase (0.046 mg · 100 ml^{-1} · min^{-1}) was observed during the experiment.

Finally measurements of glucose turnover showed that glucose production and utilization which equal glucose

7 W. B. Cannon, A. Querido, S. W. Britton and E. M. Bright, Am. J. Physiol. *83*, 84 (1927).
8 Y. Minaire, A. Pernod, M. J. Jomain and M. Mottaz, Can. J. Physiol. Pharmac. *49*, 1063 (1971).
9 J. Chatonnet, Y. Minaire, A. Pernod and J. C. Vincent-Falquet, J. appl. Physiol. *32*, 170 (1972).
10 F. Depocas, Y. Minaire and J. Chatonnet, Can. J. Physiol. Pharmac. *47*, 603 (1969).
11 F. Depocas and Y. Minaire, Can. J. Physiol. Pharmac. *47*, 492 (1969).
12 G. L. Searle, E. H. Strisower and I. L. Chaikoff, Am. J. Physiol. *185*, 589 (1956).
13 R. Leclercq, G. Copinschi and J. R. M. Franckson, Revue fr. Etud. clin. biol. *14*, 815 (1969).

turnover in such a metabolic steady state did not show any difference between N and ADMX dogs both at neutral and at cold ambient temperature.

Discussion. Demedullation, as performed by the surgical procedure of Cannon et al.[7], did not induce any symptom of cortical deficiency. The animal remained in good health for the duration of the experiments, i.e., for a period of 3 to 12 months following surgery. Moreover, measurements of plasma cortisol showed that basal concentration, as well as the adrenocortical response to cold stress, were not impaired by adrenal demedullation. On the other hand, this type of surgery does not leave any adrenomedullary tissue, and the catecholamine secretory response to cold exposure is greatly reduced in adrenal-demedullated dogs[14,15]. The role of the adrenal medulla in thermogenesis is often difficult to evaluate, because of the lack of a satisfactory method for determination of the intensity of the cold stress. In the present experiments conducted on clipped dogs exposed to ambient temperatures of +25 °C and −21 °C, O_2 consumption increased to 4.4 times the BMR, and hypothermia was never observed. Similar results have been previously observed in adrenal-demedullated dogs[8,16] and cats[17]. In rats, the consequences of adrenal demedullation depend on the age (ref. in Himms-Hagen[6]): old rats are capable of shivering and piloerection, and they increase their O_2 uptake (just as controls do), while young ones do not maintain their body temperature when they are cold-exposed. During the present experiments, the body temperature of ADMX dogs decreased slightly, but significantly, at Ta = −21 °C. Therefore, it can be concluded that dogs deprived of their adrenal medulla are capable of making compensatory adjustments, in order to cope with their caloric requirements. However, their thermogenetic capacities are nevertheless reduced, and it is likely that intense cold stresses would disclose thermogenetic deficits due to adrenal demedullation which would induce hypothermia for a cold exposure of long duration.

A discussion of plasma glucose concentration should take into account several factors, such as the timing of the experiments and the length of previous fasting. Present data suggest that although the glucose pool, as determined from plasma glucose level, is somewhat smaller after adrenal demedullation, ADMX dogs can reach an equilibrium between production and utilization, which is not different from that of N dogs. Jarratt and Nowell[18] observed a decrease in glucose concentration in 24-h-fasted rats exposed to a +4 °C ambient temperature. This decrease was the same in controls and ADMX animals 2 h after the start of cold exposure, but was greater in ADMX animals than in controls 10 h after. On the other hand, adrenal demedullation can eliminate the hyperglycemia sometimes induced by intense cold exposure in short-fasted rats[19,20] or in dogs[14].

The tracer methodology employed in the present experiment allows an overall picture of glucose metabolism, but does not provide any insight into the sites of glucose release and uptake. However, with reference and analogy to data collected in man (*ref. in* Felig[21]), it is likely that, in 15 h-fasted dogs, the almost totality of glucose production has a hepatic origin, the renal contribution being negligible. At neutral or cold ambient temperature, plasma glucose concentrations remained fairly constant both in normal and ADMX dogs. Therefore, it is possible to consider that, under these experimental conditions, hepatic glucose output was almost equal to glucose utilization.

The present study shows that dogs deprived of adrenal medulla are able to increase glucose production and utilization in the same manner as normal dogs. However, it cannot be inferred from these results that, in normal dogs, circulating epinephrine has no effect on glucose irreversible production by the liver. Actually, as recently stated by Goodner[22], the regulation of hepatic glucose production probably reaches a high degree of redundancy and includes many systems, such as secretion of pancreatic glucagon, reduction of insulin secretion, and stimulation of the direct autonomic innervation of the hepatic parenchymal cells. One may postulate that, when present, as in normal dogs exposed to cold, adrenal medullary secretion affects glucose output and plasma concentration. However, when it is not present, these animals are capable of making other adjustments, in order to maintain their glucose homeostasis.

In conclusion, it appears from these experiments that the thermogenetic capacities and the glucose homeostasis are not clearly affected by adrenal demedullation in dogs submitted to a cold severe enough to rise the energy expenditure 4.4 times above the resting metabolic rate.

14 G. Morin, Biol. med. *37*, 196 (1948).
15 Y. Satake, Nazando Co Hongo, Tokyo (1955).
16 T. Onti, Tohoku J. exp. Med. *40*, 506 (1941).
17 S. Kojima, Tohoku J. exp. Med. *40*, 353 (1941).
18 A. M. Jarratt and N. W. Nowell, Can. J. Physiol. Pharmac. *47*, 1 (1969).
19 A. Gilgen, R. P. Maickel, O. Nikodijevic and B. B. Brodie, Life Sci. *1*, 709 (1962).
20 K. Stock and E. Westermann, Arch. exp. Path. Pharmak. *251*, 465 (1965).
21 P. Felig, Metabolism *22*, 179 (1973).
22 C. J. Goodner, New Engl. J. Med. *292*, 1022 (1975).

An electrophoretic investigation of the binding of 3-^{14}C coumarin to rat serum proteins

N. B. Piller and L. H. Schmitt

Electron Microscope Unit, University of Adelaide, and Department of Genetics, University of Adelaide, G. P. O. Box 498, Adelaide (S. Australia 5001), 3 January 1977

Summary. The binding of coumarin to serum proteins of the rat has been demonstrated. Of the total bound coumarin (37% of injected dose), 36% was bound to the slow and fast α_1 globulins, 11% to the post albumins, 10% to globulin and 9% to albumin.

The binding of coumarin (5-6-benzo-α-pyrone) to purified serum and plasma albumins has been reported by Garten and Wosilait[1] and by O'Reilly[2]. The report of Garten and Wosilait[1] suggests ~40% of coumarin binds to bovine serum albumin in vitro. Bauer-Staeb and Niebes[3] reported a range of binding for the related O-β-hydroxyethyl derivatives ranging from 5% for the tetra-hydroxyethyl rutoside to 71% for rutin. They used human serum in its unpurified form.

Piller[4] reported the binding of coumarin to rat serum proteins. In vitro, over a dose range of 3.3–50 μg/ml binding remained constant at ~40% while in vivo over the