0000 silk. Each ligated gall bladder was fixed for 4 h in 10% neutral formalin. Thereafter, the gall bladder was opened, and after an additional 24-h fixation at 4 °C the specimens were embedded in paraffin. Transverse sections, 3–4 µm thick, were taken from the midportion of each gall bladder. Sections were covered with photographic emulsion (Ilford K5), developed after 16 days exposure at 4 °C (Kodak Dektol Developer) and stained with haematoxylin-eosin. In each gall bladder, the percentages of labeled cells (labeling index) and of mitoses (mitotic index) were counted on a total of 2000 consecutive epithelial cells.

Analysis of variance was used to test the statistical significance of the data.

Results. There was no difference in body weight between both groups of mice before the experiment was started. A significant loss of weight after 48 h of fasting was noted, whereas the body weight increased after free access to food. The weight of food intake of the fasting animals during refeeding was 67% higher than this value in the controls.

At the 0-h mark, the difference in labeling indices between fasting and control animals did not reach statistical significance. After refeeding, a considerable increase in DNA synthesis activity was found at 12 h ($p < 0.01$), and this effect was still significant at 20 h ($p < 0.05$) and at 28 h ($p < 0.05$) (fig.). Mitotic indices (table) were also higher in the refed group, but the difference was significant ($p < 0.05$) only at the 12-h mark.

Discussion. Our data indicate that proliferative activity in the gall bladder epithelium is stimulated by refeeding mice after fasting; not only DNA synthesis activity but also mitotic indices were increased during refeeding. The fact that no significant difference in the kinetic parameters was found between normally fed mice and fasting animals suggests that the observed postprandial burst in proliferative activity may not occur in normal circumstances, when physiological eating habits are maintained. Food intake by the animals after 48-h fasting period was higher than in the controls; the stimulus was more intense than in the controls, and it was more intense than it was after a normal meal. The rapid increase in DNA synthesis may also indicate that a number of cells in a resting phase of the cell cycle were probably synchronized to enter the DNA synthesis phase after refeeding.

A wide series of gastrointestinal hormones is known to be released after the meal, and cholecystokinin is only one of them. Caerulein, its synthetic analogue, has been recently shown to stimulate DNA synthesis in the epithelium of the gall bladder[7]. This observation may lead to the hypothesis that cholecystokinin is the hormone determining the postprandial increase in proliferative activity. However, the influence of other hormones cannot be excluded by the present data. Gastrin[8], secretin[8], and pancreatic polypeptide[9] are known to exert a trophic influence on the pancreas. The effect of these polypeptides on the gall bladder epithelium is unknown at the present time. On the other hand, feeding provokes a vagal stimulation, and the effect of vagal stimuli on cell proliferation has not been investigated. It is probable that a complex system of humoral and neurogenic influences is involved in the postprandial stimulation of cell renewal in the gall bladder.

Mitotic index values (‰) in gall bladder epithelium of mice refed after fasting (group 1) and of non fasted, normally fed controls (group 2)

Time after refeeding	Mitotic index (‰) Group 1	p - value	Group 2
0 h	0.05 ± 0.05	NS	0
12 h	0.55 ± 0.17	< 0.05	0.05 ± 0.05
20 h	0.35 ± 0.20	NS	0
28 h	0.20 ± 0.08	NS	0.10 ± 0.07

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3 T. Whalin, G.D. Bloom and A. Danielsson, Cell Tissue Res. *171*, 425 (1976).
4 V. Erspamer, Gut *11*, 79 (1970).
5 T.E. Solomon, M. Petersen and M.I. Grossman, Scand. J. Gastroent. *13*, 172 (1978).
6 D.L. Mainz, O Black and P.O. Webster, J. clin. Invest. *52*, 2300 (1973).
7 Ph. Putz, L. Bazira and G. Willems, Experientia *36*, 429 (1980).
8 A.B. Dembinski and L.R. Johnson, Endocrinology *106*, 323 (1980).
9 G.R. Greenberg, Mitznegg, S.R. Bloom, Experientia *33*, 1332 (1977).

Independence of circulating insulin levels of the increased glucose turnover in shivering dogs

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Summary. In dogs, selective insulin deficiency induced by simultaneous somatostatin and glucagon infusion does not alter the high rate of glucose utilization provoked by acute cold exposure. However, both in resting and in shivering dogs, lowering of plasma insulin decreases plasma glucose metabolic clearance significantly.

Shivering is usually not mentioned in papers devoted to the influence of insulin upon glucose utilization by muscular activity[1]. However, shivering, which plays a major part in cold-induced thermogenesis in large animals, shows many similarities to running, with respect to glucose production and utilization[2]. Recently, Therminarias et al.[3] suggested that a non-increase in plasma insulin concentration was responsible for depressed shivering and hypothermia observed in dogs immersed in cold water; they suggested that in spite of hyperglycemia, glucose uptake by shivering muscle might be limited because of an epinephrine-induced inhibition of insulin secretion. Actually, little information on glucose uptake can be obtained by just measuring plasma concentrations. Using labelled glucose, we pre-

viously observed in cold-exposed dogs that epinephrine infusion resulted in hyperglycemia without a parallel hyperinsulinemia while the rate of glucose utilization was not affected and glucose metabolic clearance was significantly decreased[4]. However it cannot be excluded that these changes could be at least partly due to epinephrine-induced metabolic changes such as lipolysis and muscular glycogenolysis.

In order to analyze more specifically the effects of insulinopenia on the rate of utilization of glucose and metabolic clearance during shivering, simultaneous somatostatin and glucagon infusions were performed in acute cold-exposed dogs.

Methods. A total of 8 unanesthetized female mongrel dogs (8.3-12.5 kg), housed in a temperature-controlled room, were fed daily with a standard diet and fasted for 20 h at the beginning of the experiment[5]. They were trained to lie calmly in a thermostatic chamber with their heads enclosed in a ventilated mask[6]. 2 ambient temperatures TaN = +25 °C and TaC = −21 °C were selected. A 4-fold increase in the metabolic rate and a sustained shivering were observed at TaC[7].

About 1 week before the beginning of the experiments, once the animals had been adequately trained, 3 vascular catheters (1 in a carotid artery, 1 in a jugular vein and 1 in the portal vein) were chronically implanted under thiopental sodium anesthesia[8]. These catheters were used for serial sampling of arterial blood, for portal infusion of glucagon and for jugular infusion of somatostatin and tracer. Only dogs with WBC less than 16,000/mm^3, hematocrit above 35%, b.wt constant for 3 weeks, and a good appetite, were used. Each experiment began between 09.00 and 10.00 h. Rectal temperature was measured at the beginning and at the end of each experiment. After b.wt had been noted, the animal was positioned in the experimental set-up. An initial adjustment period of 45 min was allowed before beginning tracer infusion, so as to obtain a metabolic steady state and thermal equilibrium. During the experiments, the respiratory mask was connected to an open-circuit system[9] for measurement of O_2 consumption. According to the priming dose-infusion technique[10], 2.5-3.4 μCi · kg^{-1} of D-3-^{3}H glucose in saline was injected rapidly into the jugular vein and followed by continuous infusion of the same solution at a rate of 35 nCi · kg^{-1} · min^{-1}. The infusion began at t = 0 and continued until the end of the experiment, i.e. for 230 min. The priming dose/infusion rate ratios were fixed to 120 and 90 at TaN and TaC spectively. 3 successive periods were observed: preexperimental (t_0-t_{135} min) experimental (t_{135}-t_{205} min) and recovery (t_{205}-t_{230} min). Saline was infused (i.v.) during the preexperimental and recovery periods. An isolated insulin deficiency was obtained during the experimental period by changing the jugular saline infusion for a somatostatin infusion (1 μg · kg^{-1} · min^{-1} linear somatostatin, Serono) and by simultaneously infusing glucagon (Novo) through the portal vein (1 ng · kg^{-1} · min^{-1} in saline added with 1 ml of the own dog's plasma for 15 ml of solute). Glucagon replacement was considered adequate when the resulting mean values for arterial hormone concentration did not differ by more than 18% from the mean value collected during the control period. It appeared that only 14 out of 23 experiments performed met this criterion, and the data collected during the 9 other experiments were deliberately rejected. Arterial blood samples were with drawn at 90 min after the beginning of the tracer infusion and every 10-min thereafter until the end of the experiment. Packed cell volumes were measured by the microhematocrit method. Blood for measurement of glucose concentration and glucose specific activity was collected in chilled tubes containing a lyophilized NaF-heparin mixture. Plasma glucose concentration was measured by a glucose oxidase method (Boehringer Co.) and plasma (^{3}H) glucose specific activity was assayed as previously described[4]. Immunoreactive insulin (IRI) was measured in duplicate in heparinized plasma samples, using the sephadex bound-antibody procedure[11]. Immunoreactive glucagon (IRG) was assayed in triplicate in heparinized plasma samples to which 13.000 U/ml i.p. of aprotinin (Iniprol Choay, Paris) had been added. The assay procedure included an alcoholic extraction with lyophilisation and the use of the K 964 antiserum regarded as specific to pancreatic glucagon and as having a low affinity for gut extract containing glucagon-like material[12].

The rates of endogenous glucose production (rate of appearance, Ra) and utilization (rate of disappearance, Rd) were calculated according to Steele's equations[13,14]

Insulin, glucagon and glucose concentrations and glucose kinetics during selective insulin deficiency (experimental period) at 2 ambient temperatures. Number of experiments: 7 both at TaN and TaC. Values given during the experimental period are means of data collected at 15, 25, 35, 45, 55 and 65 min after the beginning of the somatostatin-glucagon infusion

	Neutral ambient temperature (TaN = +25 °C) $\dot{V}O_2 = 8.4 \pm 0.94$ ml · kg^{-1} · min^{-1}			Cold ambient temperature (TaC = −21 °C) $\dot{V}O_2 = 32.9 \pm 0.75$ ml · kg^{-1} · min^{-1}		
	Pre-experimental period	Experimental period	Recovery period	Pre-experimental period	Experimental period	Recovery period
Glucose production rate (mg · kg^{-1} · min^{-1})	4.22 ± 0.322	5.89 ± 0.822	4.75 ± 0.611	9.27 ± 0.936*	10.33 ± 0.193*	8.63 ± 1.014
Glucose utilization rate (mg · kg^{-1} · min^{-1})	4.26 ± 0.316	4.51 ± 0.471*	5.85 ± 0.879	9.37 ± 0.932	9.57 ± 0.844	10.01 ± 0.949
Glucose metabolic clearance (ml · kg^{-1} · min^{-1})	4.64 ± 0.374*	3.71 ± 0.305	4.13 ± 0.477	10.02 ± 1.099*	8.53 ± 0.971	9.16 ± 0.954
Plasma glucose (mg · dl^{-1})	92 ± 3.4*	135 ± 16.0	137 ± 19.6	94 ± 1.8**	120 ± 5.9**	104 ± 3.1
IRI (μU · ml^{-1})	12.1 ± 1.94*	8.0 ± 2.16**	24.4 ± 5.70	15.2 ± 3.13**	12.3 ± 2.60**	18.5 ± 2.00
IRG (pg · ml^{-1})	144 ± 21.5	151 ± 23.3	182 ± 15.0	130 ± 19.7	129 ± 19.4*	181 ± 24.5
IRI/plasma glucose (μU · mg^{-1})	13.2 ± 2.25**	5.9 ± 1.94**	18.0 ± 4.23	18.5 ± 3.55**	11.5 ± 2.06**	17.6 ± 1.56

* $p < 0.05$; ** $p < 0.01$ (non parametric Wilcoxon test for pair values).

using a glucose pool fraction of 0.5 and expressed as $mg \cdot kg^{-1} \cdot min^{-1}$ [15]. We thus obtained an average value for each time interval between 2 consecutive samples. The volume distribution of glucose was assumed to be 300 $ml \cdot kg^{-1}$ b.wt[16], and the pool size was calculated from the plasma glucose concentration. The glucose utilization rate is governed by both the plasma glucose concentration and the intrinsic ability of the tissues to remove glucose. The latter factor can be assessed by calculating the glucose metabolic clearance as MCR ($ml \cdot kg^{-1} \cdot min^{-1}$) = Rd/plasma glucose concentration ($mg \cdot ml^{-1}$). Data collected between t = 90 and t = 135 min, t = 145 and t = 205 min and t = 215 and t = 230 min were considered as representative respectively of the preexperimental, experimental and recovery periods and used for calculations. Statistical analyses were carried out using the nonparametric Wilcoxon tests for paired values.

Results. During the preexperimental period, glucose production, utilization and metabolic clearance were about twice higher in shivering (TaC) than in resting dogs (TaN) (table).

During the experimental period, both in dogs shivering in the cold and in those resting at TaN, simultaneous somatostatin and glucagon infusion resulted in a significant reduction in plasma insulin and insulin/glucose ratio, while plasma glucagon remained fairly constant. This selective insulin deficiency induced a significant hyperglycemia which persisted during the recovery period at TaN but faded away in the cold, and an increase in glucose production which was significant in cold conditions. Glucose utilization was not impeded by insulin deficiency (experimental period) neither at TaN (resting dogs) nor at TaC (shivering dogs). It can be stressed that in this latter condition in spite of low plasma insulin concentration, glucose utilization reaches levels twice as high as at TaN. However, both at TaN and TaC a slight but significant reduction in metabolic clearance was observed during the insulin deficiency.

Discussion. In dogs, the continuous infusion[13] of 3^3H glucose[17] is a reliable method (see Vranic[18]) for the measurement of glucose kinetics in non-steady states. It is rather difficult to obtain total insulin deprivation without the aid of pancreatectomy; however, pancreatectomy induces permanent metabolic disorders such as lipolysis or ketosis, which can mask the specific effect of insulin deprivation upon glucose uptake. Alternatively, a transient and reversible insulin deficit, without other apparent metabolic changes, can be obtained by the present method involving simultaneous somatostatin and glucagon infusions, and results in a significant decrease in insulin and in the insulin/glucose ratio and a postsomatostatin rebound. In addition, the plasma insulin concentrations obtained were clearly below those (18 μU/ml) collected by Therminarias et al.[3]. Thus, present data suggest that a diminished glucose uptake, possibly due to a non-increase in insulinemia, is not the factor responsible for impeded shivering and hypothermia in cold-water immersed dogs. During muscular exercise, glucose uptake and metabolic clearance increases while insulinemia remained within the physiological ranges (Schultz[19] and references therein). However when insulin-concentration reaches abnormally low levels (5–10 μU/ml) such as reported in insulin-withdrawn diabetic patients[20,21] and in pancreatectomized dogs[22] a large increase in glucose uptake remains possible, although the usual rise in metabolic clearance does not occur. In rats, shivering increases glucose uptake by skeletal muscles of normal or well controlled diabetic rats, but this accelerated disposal of carbohydrate is limited by conditions favoring excess lipolysis and ketogenesis, such as occur in severely diabetic animals[23]. It is possible that a very low insulin concentration is sufficient to allow an increase in glucose utilization by skeletal muscle[24], provided that muscular activity or shivering maintains glucose-6-phosphate utilization at a rate high enough to avoid intracellular accumulation and therefore hexokinase inhibition[25].

Finally it is worth mentioning that until now, the inhibitory effect of basal insulin level upon glucose production has been demonstrated only in resting animals with basal glucose output[26,27]. The present findings suggest that even in view of the increased glucose turnover rates of shivering dogs, basal insulin exerts a restraining effect on glucose production since it increased upon induction of insulin deficiency.

1 M. Vranic and M. Berger, Diabetes *28*, 147 (1979).
2 Y. Minaire, A. Pernod, J.C. Vincent-Falquet and M. Mottaz, Horm. Metab. Res. *5*, 80 (1973).
3 A. Therminarias, M.F. Chirpaz, A. Lucas and M. Tanche, J. appl. Physiol. *47*, 342 (1979).
4 J. Forichon, M.J. Jomain, G. Dallevet and Y. Minaire, J. appl. Physiol. *43*, 230 (1977).
5 J. Forichon, M.J. Jomain, J. Schellhorn and Y. Minaire, Experientia *33*, 1171 (1977).
6 Y. Minaire, A. Pernod, M.J. Jomain and M. Mottaz, Can. J. Physiol. Pharmac. *49*, 1063 (1971).
7 J. Forichon, M.J. Jomain, M.C. Patricot and Y. Minaire, Experientia *33*, 1070 (1977).
8 F. Depocas and Y. Minaire, Can. J. Physiol. Pharmac. *47*, 492 (1969).
9 F. Depocas, Y. Minaire and J. Chatonnet, Can. J. Physiol. Pharmac. *47*, 603 (1969).
10 G.L. Searle, E.H. Strisower and I.L. Chaikoff, Am. J. Physiol. *185*, 589 (1956).
11 L. Wide and J. Porath, Biochim. biophys. Acta *130*, 257 (1966).
12 L.G. Heding, Diabetologia *7*, 10 (1971).
13 R.C. De Bodo, R. Steele, N. Altszuler, A. Dunn and J.S. Bishop, Recent Prog. Horm. Res. *19*, 445 (1963).
14 R. Steele, Ann. N.Y. Acad. Sci. *82*, 420 (1959).
15 K. Schmidt-Nielsen, Fedn Proc. *29*, 1524 (1970).
16 B. Issekutz, Jr, and M. Allen, J. appl. Physiol. *31*, 813 (1971).
17 N. Altszuler, A. Barkai, C. Bjerknes, B. Gottlieb and R. Steele, Am. J. Physiol. *229*, 1662 (1975).
18 M. Vranic, Fedn Proc. *33*, 1837 (1974).
19 T.A. Schultz, S.B. Lewis, D.K. Westbie, J.D. Wallin and J.E. Gerich, Am. J. Physiol. *233*, E514 (1977).
20 J. Lyngsøe, J.P. Clausen, J. Trap-Jensen, L. Sestoft, O. Schaffalitzky de Muckadell, J.J. Holst, S.L. Nielsen and J.F. Rehfeld, Clin. Sci. molec. Med. *55*, 73 (1978).
21 J. Wahren, L. Hagenfeldt and P. Felig, J. clin. Invest. *55*, 1303 (1975).
22 M. Vranic and R. Kawamori, Diabetes *28* (suppl. 1), 45 (1979).
23 O.L.K. Smith, Proc. Meeting Int. Un. phys. Sci. Budapest 1980, p. 710.
24 M. Berger, S. Hagg and N.B. Ruderman, Biochem. J. *146*, 231 (1975).
25 B. Issekutz, Jr, Diabetes *29*, 348 (1980).
26 H.L.A. Lickley, G.G. Ross and M. Vranic, Am. J. Physiol. *236*, E255 (1979).
27 A.D. Cherrington, J.L. Chiasson, J.E. Liljenquist, A.S. Jennings, U. Keller and W.W. Lacy, J. clin. Invest. *58*, 1407 (1976).