Anaerobic Dormancy Quantified in *Artemia* Embryos: A Calorimetric Test of the Control Mechanism

STEVEN C. HAND AND ERICH GNAIGER

Continuous measurement of heat dissipation from brine shrimp embryos during reversible transitions from aerobic development to anaerobic dormancy demonstrates a primary role for intracellular pH (pH_i) in this metabolic switching. Artificially elevating the depressed pH_i during anoxia by adding ammonia markedly reactivates metabolism, as judged by increases in heat dissipation, trehalose catabolism, and the ratio of adenosine triphosphate to adenosine diphosphate. Energy flow during anaerobic dormancy is suppressed to 2.4 percent of aerobic values, which is the lowest percentage thus far reported for euryoxic animals. Use of diguanosine tetraphosphate stores cannot account for this observed heat dissipation. Thus, mobilizing trace amounts of trehalose may explain the energy metabolism during quiescence.

QUIESCENT, RESTING FORMS ARE common during the early life stages of numerous organisms (*1*) and, in some taxa, even mature adults can enter such hypometabolic states (*2*). The broad phylogenetic distribution of organisms with this ability suggests the existence of fundamental mechanisms for reversible suppression of metabolism and development. But with few exceptions, our understanding of the regulation of these periods of dormancy, which occur in response to harsh environmental conditions, is lacking. By using microcalorimetry (*3*), we have made a direct test of the proposed role of intracellular pH (pH_i) in metabolic switching in brine shrimp embryos and have obtained new information quantifying the degree of metabolic arrest during anaerobic dormancy.

During the life cycle of the brine shrimp *Artemia*, the development of postdiapause embryos (cysts) is sometimes interrupted by anaerobic dormancy, an extreme state of quiescence under environmental anoxia. As reviewed elsewhere (*4, 5*), reversible shifts from aerobic development to anaerobic dormancy in hydrated gastrula-stage embryos are characterized by cessation of carbohydrate metabolism (*6, 7*) and by intracellular acidification from $pH \geq 7.9$ to as low as 6.3 (*8*). In the laboratory, artificial acidification of the pH_i of aerobically developing cysts to 6.8 by exposure to elevated levels of CO_2 induces a quiescent condition (aerobic acidosis) comparable to anaerobic dormancy, as judged by several indicators (*7, 9, 10*). Recent evidence for pH-induced blockage of the catabolic pathway for trehalose (*7*) and for the kinetic and structural sensitivity of brine shrimp trehalase to pH (*11*) further implicates pH_i as a regulator of these metabolic transitions.

However, key questions remain regarding the regulation of this metabolic switching. What influence does modulation of embryo pH_i have on energy metabolism during anaerobic dormancy? If the shutdown of energy metabolism under anoxia is indeed due to intracellular acidification, then artificial alkalinization in the absence of oxygen would be expected to cause an elevation of metabolism. An affirmative result is important in demonstrating a regulatory role for pH_i during anoxia, but, until now, a direct approach to this point has not been experimentally feasible. To what degree is total metabolic rate of *Artemia* embryos suppressed during quiescence? In the hydrated state, one would expect some minimal level of metabolism to occur in cysts to provide for cellular maintenance (for example, DNA repair and protein and membrane turnover). Such information would provide a better estimate of the energy requirements for maintenance-level function in eukaryotic cells. Finally, what is the identity of the substrate (or substrates) supporting the "pilot light" metabolism? The application of microcalorimetry, coupled with respirometric and biochemical measurements, affords an opportunity to address these points.

The instrumentation we used for open-flow microcalorimery and respirometry (*3, 12, 13*) and the methods for embryo preparation (*7*) have been described. Figure 1 shows that a continuous increase in heat dissipation occurred in the *Artemia* embryos during the first 7 hours of aerobic development, after which a steady state was reached (hour 7 to 9). This pattern was reflected in the oxygen consumption values obtained simultaneously. At steady state, the heat dissipated per unit of oxygen consumed was −495 kJ/mol O_2, in close agreement with the theoretical oxycaloric equivalent of −478 kJ/mol O_2 for catabolism of carbohydrate under these conditions (*14*). Trehalose is the exclusive catabolic fuel used by these embryos during preemergence development, and net changes in protein and lipid levels do not occur (*15*). Heat dissipation decreased precipitously during aerobic acidosis (Fig. 1), reaching a minimum value after 8 hours that was only 9% of control. Oxygen consumption decreased simultaneously [consistent with data in (*9*)] resulting in a calorimetric-respirometric ratio of −481 kJ/mol O_2. Since this ratio did not increase during aerobic acidosis, nonoxidative metabolism was not stimulated to compensate for this suppression of aerobic metabolism.

Uninterrupted measurement of metabolic heat dissipation during transitions from aerobic to anaerobic states allowed us to quantify the actual depression of energy flow under anaerobic dormancy. An immediate drop in heat dissipation occurred upon the removal of

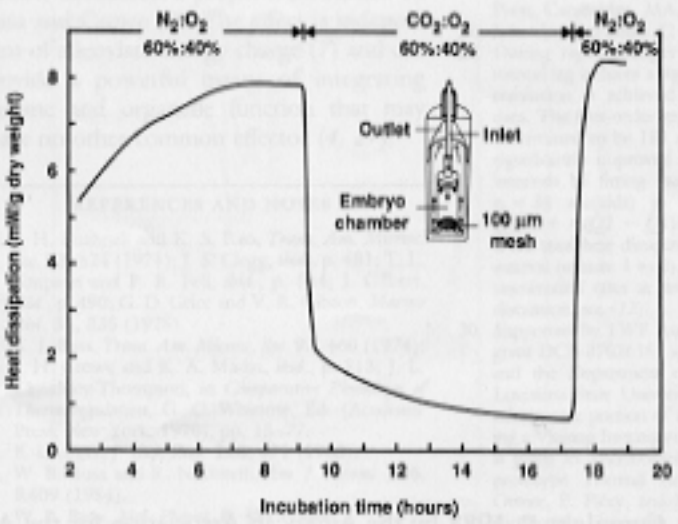

Fig. 1. Continuous record of heat dissipation by *Artemia* embryos during aerobic development (hour 0 to 9), aerobic acidosis (hour 9 to 17.5), and recovery (hour 17.5 to 19). Embryos from the Great Salt Lake, Utah, were perfused (23.5 ml/hour) in 0.25*M* NaCl at 25°C throughout the experiment. Inset depicts the 3.5-ml perfusion chamber adapted to ensure uniform flow through the sample of embryos (75 to 100 mg dry weight) and normal development under aerobic conditions (*8*). Hatchability of cysts was 60% based on a separate study (*28*). The stainless steel chamber was coupled to the respirometer with stainless steel capillaries.

S. C. Hand, Department of Environmental, Population and Organismic Biology, Campus Box B-334, University of Colorado, Boulder, CO 80309.
E. Gnaiger, Institut für Zoologie, Abteilung Zoophysiologie, Universität Innsbruck, A-6020 Innsbruck, Austria.

oxygen (Fig. 2). After 4 hours of anaerobic dormancy, the rate declined to 2.4 ± 0.4% (SD) of controls in three experiments (two of which are shown in Fig. 2). Compared to other euryoxic invertebrates, this is the lowest percentage so far reported (*3*). The quantitative relation between calorimetric and biochemical measures of metabolic rate depends on the operative anaerobic pathways (*3*). If one considers that anoxia can be tolerated by *Artemia* embryos for weeks (*6*), the heat dissipation we measured during anaerobic dormancy [0.13 μW per million cells; (*16*)] probably reflects maintenance-level metabolism for these eukaryotic cells. For comparison, the aerobic rate of *Artemia* embryos is 5.4 μW per million cells. Human T lymphoma cells in suspension exhibit an average value of 7.5 ± 0.6 (SD) μW per million cells at 37°C (*17*).

If decline of pH_i is the primary regulatory mechanism for these transitions, then artificial alkalinization of pH_i during anaerobic dormancy should elevate the anoxic energy flow, resulting in a state of "activated anoxibiosis." After a transition to perfusion conditions with ammonia (hour 9.5 to 12, Fig. 2) known to alkalinize anaerobic pH_i (*18*), a fivefold increase in heat dissipation occurred (hour 10.5 to 12). Since oxygen was not present for mitochondrial-based metabolism during this treatment, an increase in heat dissipation to control (aerobic) levels was not expected. Blank experiments (no organisms in the measuring chamber) gave stable baselines across these transitions, demonstrating that the changes in heat dissipation were not a result of exothermic mixing of the two perfusion media. After switching to anoxic perfusion conditions with ammonia, a small pulse of oxygen was recorded in the inflow. This oxygen uptake can quantitatively explain the small overshoot of heat dissipation at the beginning of activated anoxibiosis. During anoxic steady states, oxygen diffusion into the system was extremely low (typically below 0.5% air saturation). Thus, elevating the pH_i of embryos during strict anoxia (*19*) promoted a marked deinhibition of metabolic rate.

The microcalorimetric data were supported by a parallel series of experiments in which selected metabolites were measured. Trehalose utilization, which is blocked during anaerobic dormancy (*6*, *7*), was reinitiated upon addition of ammonia under anoxia (Table 1). The observation is probably explained by deinhibition of trehalase (*11*). Relative to levels during anaerobic dormancy, concentrations of adenosine triphosphate (ATP) increased approximately 50% during activated anoxibiosis, as did the ratio of ATP to adenosine diphosphate (ADP). Also, there is an increase in the ratio of glucose-6-phosphate:glucose, suggesting partial deinhibition of the hexokinase reaction (*7*).

Previous workers have suggested that energy for cellular maintenance during anaerobic dormancy comes from the catabolism of an unusual nucleotide found in *Artemia* yolk platelets—diguanosine tetraphosphate (Gp_4G) (*20*). Until now, it had not been established whether Gp_4G utilization is sufficient to account for the level of energy metabolism under these conditions or whether there are other bioenergetic contributions. Metabolism of Gp_4G proceeds at a rate of approximately 0.58 μmol per hour per gram dry weight during anaerobic dormancy at 25°C (*20*). If we assume complete catabolism of Gp_4G via the pathway described by Denbos and Finamore (*21*) [Gp_4G + adenosine → Ap_4G + guanosine; Ap_4G → ATP + guanosine monophosphate (GMP); ATP → adenosine monophosphate (AMP); AMP → adenosine], the caloric equivalent of the process at pH 6.5 and pMg 2.5 would be approximately −140 kJ/mol Gp_4G (*22*). Thus, the expected heat dissipation derived from Gp_4G metabolism under anaerobic dormancy would be 0.002 mW per gram of dry cyst, as compared to our lowest value of 0.12 mW/g. Interbatch variability of cysts might explain part of the discrepancy. But based on these values, Gp_4G metabolism can account for <2% of the observed heat dissipation from *Artemia* embryos. We suggest that a more likely explanation for this metabolic rate is the catabolism of trace amounts of trehalose. Although a decline in trehalose cannot be detected after several hours of anaerobic dormancy (Table 1) (*6*, *7*), the amount required to explain the heat dissipation is within the error of the assay.

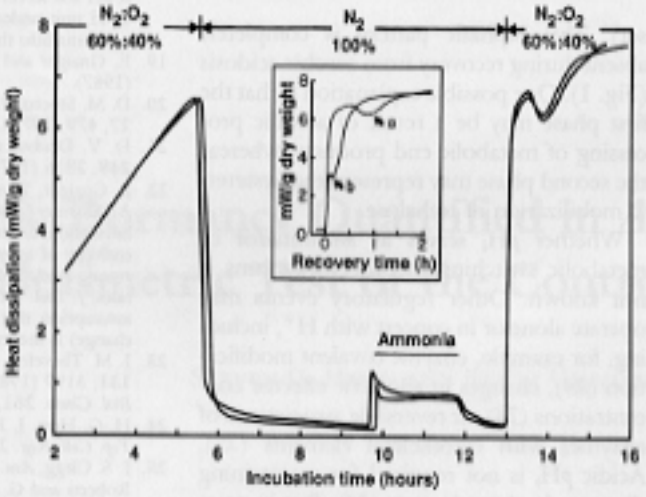

Fig. 2. Superimposed traces of two independent experiments showing heat dissipation of *Artemia* embryos during aerobic development (hour 0 to 5.5) and anoxia (hour 5.5 to 13). During the continuous period of anoxia, the acidotic state of anaerobic dormancy (hour 5.5 to 9.5) was interrupted with the addition of ammonia, which serves to alkalinize pH_i. This period of activated anoxibiosis (hour 9.5 to 12) was reversed by the removal of ammonia (hour 12 to 13). The recovery response (hour 13 to 16) showed a reproducible biphasic pattern. The inset contrasts this recovery pattern (a) to that observed after exposure to a shortened (5-hour) period of anaerobic dormancy alone (b). The perfusion medium consisted of 0.20*M* NaCl and 0.05*M* tricine (pH 8.5) except during activated anoxibiosis, where the composition of the medium was 0.16*M* NaCl, 0.05*M* tricine, and 0.04*M* NH_4Cl. For further experimental details see legend to Fig. 1.

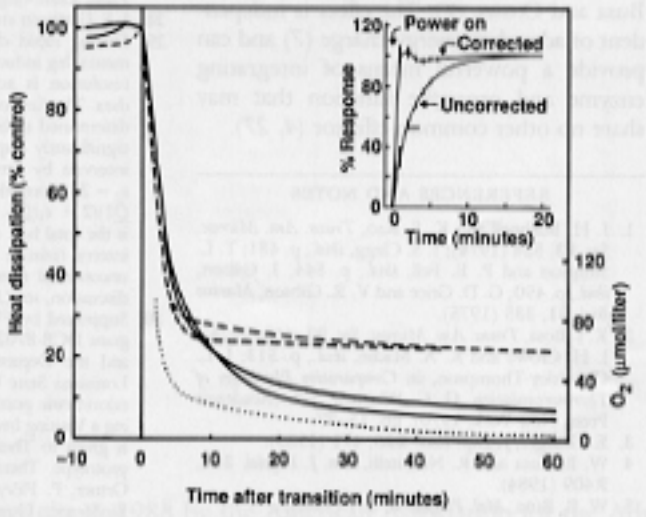

Fig. 3. High resolution of the time course of heat dissipation during pH-induced metabolic transitions in *Artemia* embryos. Superimposed traces from two experiments are presented for each of the following conditions: aerobic acidosis (···) and anaerobic dormancy (——). Heat dissipation is calculated as percent of the aerobic control prior to each transition. Time course for washout of oxygen from the perfusion system after switching to anoxic medium is depicted by the dotted line. The inset shows the accuracy of time resolution for the calorimetry signal achieved by correcting for instrumental lag (*29*). The internal calibration heater was activated at time 0 ("Power on"). Vertical bars represent ±1 SD (n = 10).

Table 1. Changes in selected metabolite levels and ratios in *Artemia* embryos during 12.5-hour incubations. After an initial 6-hour period of aerobic development (60% N_2:40% O_2), conditions were switched to anaerobic dormancy (100% N_2). After hour 10, NH_4Cl (40 m*M* final concentration) was added to the anoxic flasks, and incubation continued under anaerobic conditions through hour 12.5. Values are means ± SEM for three independent samples. Analyses were performed as previously described (*7*).

Period and time (hours)	Trehalose (μmol glucosyl U/mg protein)	ATP (μmol/ mg protein)	ATP:ADP ratio	Glycogen (μmol glucosyl U/mg protein)	Glucose-6 phosphate: glucose ratio
Aerobic development					
0	2.42 ± 0.02	0.94 ± 0.02	0.149 ± 0.010	0.166 ± 0.002	0.030 ± 0.003
6	1.94 ± 0.03	5.09 ± 0.03	1.50 ± 0.075	0.323 ± 0.003	0.236 ± 0.003
Anaerobic dormancy					
10	2.01 ± 0.04	0.84 ± 0.01	0.196 ± 0.018	0.326 ± 0.002	0.022 ± 0.002
Activated anoxibiosis (with 40 m*M* NH_4Cl)					
10.5	1.74 ± 0.01	1.30 ± 0.02	0.283 ± 0.018	0.331 ± 0.008	0.030 ± 0.002
12.5	1.76 ± 0.02	1.13 ± 0.02	0.306 ± 0.012	0.326 ± 0.005	0.010 ± 0.001

Based on thermochemistry, a second point to note is that the trehalose utilization measured during activated anoxibiosis (Table 1, hour 10 to 12.5) is sufficient to account for the total heat dissipation by the embryos during this period. If trehalose is metabolized completely to succinate or propionate (the actual end products are unknown), the expected heat dissipated would conservatively be 1.4 mW per gram of dry cyst (*3*), compared to our observed rate of 0.9 to 1.0 mW/g (Fig. 2). This observation is taken as additional evidence that artificial alkalinization under anoxia serves to reinitiate trehalose metabolism.

The rates and profiles of these metabolic transitions suggest several points about the mechanisms involved. Kinetic data indicate that 90% of the metabolic depression that occurs after onset of aerobic acidosis is complete within 5 minutes (Fig. 3). Transitions into anaerobic dormancy are somewhat slower for at least three reasons. Whereas ATP levels remain constant under aerobic acidosis (*7*), decreases in ATP contribute 6% of the total heat dissipation during the initial 30-minute transition into anoxia. In contrast to aerobic acidosis where the pH_i decline is rapid (exogenously controlled with CO_2), the pH_i shift during anaerobic dormancy is metabolically produced and consequently slower (*8*). Also, in vitro data showed that inhibition of brine shrimp trehalase by pH is hysteretic (*11*), involving a time-dependent change in assembly state. For the artificial state of aerobic acidosis, where the level of the inhibitory effector ATP is maintained high, the hysteresis may be less pronounced. Interestingly, the kinetics of the recovery from anaerobic dormancy are at least biphasic (Fig. 2, inset). This biphasic pattern is completely absent during recovery from aerobic acidosis (Fig. 1). One possible explanation is that the first phase may be a result of aerobic processing of metabolic end products, whereas the second phase may represent the hysteretic mobilization of trehalose.

Whether pH_i serves as an initiator of metabolic switching in other organisms is not known. Other regulatory events may operate alone or in concert with H^+, including, for example, enzyme covalent modification (*23*), changes in allosteric effector concentrations (*24*), or reversible associations of enzymes with cytoskeletal elements (*25*). Acidic pH_i is not required for maintaining diapause in *Artemia* cysts (*26*). But in postdiapause embryos, all biochemical, metabolic, and pH_i data indicate that H^+ reversibly initiates the metabolic shutdown during anaerobic dormancy, a proposal forwarded by Busa and Crowe (*9*). The effect is independent of adenylate energy charge (*7*) and can provide a powerful means of integrating enzyme and organelle function that may share no other common effector (*4*, *27*).

REFERENCES AND NOTES

1. J. H. Bushnell and K. S. Rao, *Trans. Am. Microsc. Soc.* **93**, 524 (1974); J. S. Clegg, *ibid.*, p. 481; T. L. Simpson and P. E. Fell, *ibid.*, p. 544; J. Gilbert, *ibid.*, p. 490; G. D. Grice and V. R. Gibson, *Marine Biol.* **31**, 335 (1975).
2. K. J. Boss, *Trans. Am. Microsc. Soc.* **93**, 460 (1974); J. H. Crowe and K. A. Madin, *ibid.*, p. 513; J. L. Cloudsley-Thompson, in *Comparative Physiology of Thermoregulation*, G. C. Whittow, Ed. (Academic Press, New York, 1970), pp. 15–77.
3. E. Gnaiger, *J. Exp. Zool.* **228**, 471 (1983).
4. W. B. Busa and R. Nuccitelli, *Am. J. Physiol.* **246**, R409 (1984).
5. W. B. Busa, *Mol. Physiol.* **8**, 351 (1985).
6. J. Dutrieu and D. Crestia-Blanchine, *C. R. Acad. Sci. Ser. D* **263**, 998 (1966); R. D. Ewing and J. S. Clegg, *Comp. Biochem. Physiol.* **31**, 297 (1969).
7. J. F. Carpenter and S. C. Hand, *J. Comp. Physiol. B* **156**, 451 (1986).
8. W. B. Busa, J. H. Crowe, G. B. Matson, *Arch. Biochem. Biophys.* **216**, 771 (1982).
9. W. B. Busa and J. H. Crowe, *Science* **221**, 366 (1983).
10. P. J. Utterback and S. C. Hand, *Am. J. Physiol.* **252**, R774 (1987).
11. S. C. Hand and J. F. Carpenter, *Science* **232**, 1535 (1986).
12. J. Szarkezak and I. Wadsö, *Chem. Scr.* **20**, 155 (1982).
13. E. Gnaiger, in *Polarographic Oxygen Sensors: Aquatic and Physiological Applications*, E. Gnaiger and H. Forstner, Eds. (Springer-Verlag, New York, 1983), pp. 134–166.
14. ———, *ibid.*, pp. 337–345.
15. J. Dutrieu, *Arch. Zool. Exp. Gen.* **99**, 1 (1960); S. Muramatsu, *Embryologia* **5**, 95 (1960); D. N. Emerson, *Proc. S.D. Acad. Sci.* **42**, 131 (1963); J. S. Clegg, *J. Exp. Biol.* **41**, 879 (1964).
16. This calculation is based on the observations that there are 100,000 embryos per gram wet weight [(*20*); S. C. Hand, unpublished observations], each embryo contains 4,000 cells [C. S. Olson and J. S. Clegg, *Experientia* **32**, 864 (1976)], and the dry weight percentage is 31.8.
17. A. Schön and I. Wadsö, *Cytobios* **48**, 195 (1986); *J. Biochem. Biophys. Methods* **13**, 135 (1986).
18. Addition of NH_4Cl to perfusion medium buffered at pH 8.5 serves to raise embryo pH_i approximately 1 pH unit under anoxia, as a result of diffusion of ammonia into the cyst (*9*).
19. E. Gnaiger and I. Staudigl, *Physiol. Zool.* **60**, 659 (1987).
20. D. M. Stocco, P. C. Beers, A. H. Warner, *Dev. Biol.* **27**, 479 (1972).
21. D. V. Denbos and F. J. Finamore, *J. Biol. Chem.* **249**, 2816 (1974).
22. E. Gnaiger, *Thermochim. Acta* **40**, 195 (1980); R. A. Alberty, *J. Biol. Chem.* **244**, 3290 (1969). Our calculation is based on the observation that the enthalpy of anhydride bond splitting is comparable among nucleotides [R. J. Podolsky and M. F. Morales, *J. Biol. Chem.* **218**, 945 (1956)] and on the assumption that the first reaction (nucleotide exchange) is thermally neutral.
23. J. M. Thevelein and M. Beullens, *J. Gen. Microbiol.* **131**, 3199 (1985); M. A. Luther and J. C. Lee, *J. Biol. Chem.* **261**, 1753 (1986).
24. H.-G. Hers, J. Francois, E. van Schaftingen, *Curr. Top. Cell. Reg.* **27**, 399 (1985).
25. J. S. Clegg, *Am. J. Physiol.* **246**, R133 (1984); S. J. Roberts and G. N. Somero, *Biochemistry* **26**, 3237 (1987); W. C. Plaxton and K. B. Storey, *J. Comp. Physiol. B* **156**, 635 (1986).
26. L. E. Drinkwater and J. H. Crowe, *J. Exp. Zool.* **241**, 297 (1987).
27. P. W. Hochachka and M. Guppy, *Metabolic Arrest and the Control of Biological Time* (Harvard Univ. Press, Cambridge, MA, 1987), pp. 146–165.
28. J. S. Glasheen and S. C. Hand, *J. Exp. Biol.*, in press.
29. During rapid changes in heat dissipation, instrumental lag induces a significant error. Accurate time resolution is achieved by deconvoluting the raw data. The first-order exponential time constant was determined to be 151 seconds. Deconvolution was significantly improved to <5% error for 1-minute intervals by fitting two terms (t_1 = 128 seconds, t_2 = 33 seconds) in the equation: $\dot{Q} = (\dot{Q}2 + \dot{Q}1)/2 + t_1(\dot{Q}2 - \dot{Q}1)/60 + t_2(\dot{Q}2 - \dot{Q}0)/120$. $\dot{Q}$ is the total heat dissipation during the 1-minute time interval (minute 1 to 2), and $\dot{Q}0$, $\dot{Q}1$, and $\dot{Q}2$ are the uncorrected rates at times 0, 1, and 2. For further discussion, see (*12*).

30. Supported by FWF Austria grant J0187B and NSF grant DCB-8702615. Special thanks to W. B. Stickle and the Department of Zoology and Physiology, Louisiana State University, Baton Rouge, where the calorimetric portion of the work was performed during a Visiting Investigator Program. Acknowledgment is given to ThermoMetric, Stockholm, for use of a prototype Thermal Activity Monitor and to M. Ortner, P. Flöry, and M. Kronhammer (Cyclobios-BioMetric, University of Innsbruck) for efforts in development of the Twin-Flow respirometer.

9 November 1987; accepted 26 January 1988