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HYPOTHERMIA AS A MECHANISM FOR DRUG-INDUCED RESISTANCE TO HYPOXIA*

FREDERICK N. MINARD† and DENNIS S. GRANT
Department of General Pharmacology, Abbott Laboratories, North Chicago, IL 60064, U.S.A.

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Abstract—A linear regression line relating the logarithm of survival time in a standard hypoxia survival test (4.6% oxygen) with hypothermia was obtained upon cooling mice by exposure to either a graded hypoxia or a cold environment. When S/BT values (log minutes survival versus body temperature) of drug-treated animals were compared with this regression line, values for many drugs lay on the line, indicating that their effects upon hypoxic survival were no greater than those expected from the concomitant hypothermia. Among the drugs whose anti-hypoxia effects were explained by their production of hypothermia were adenosine, diazepam, diphenylhydantoin, pentobarbital and physostigmine. The anti-hypoxia activity of each drug had been reported previously by other investigators and ascribed to numerous mechanisms. The present observations relating hypothermia with increased survival in hypoxia provide a rationale for the protective effect of the above drugs in animals suddenly exposed to lethal hypoxia.

Cerebral hypoxia is a component of the ischemia syndrome which follows stroke from vascular spasm and from thrombotic occlusion. An apparent correlation exists between anti-ischemia activity in the brain and anti-hypoxia activity in whole animals because several drugs under investigation for their anti-ischemia actions after experimental stroke are also protective against alveolar hypoxia in non-stressed animals. Pentobarbital [1, 2] and diphenylhydantoin [3, 4] are examples of such drugs.

During evaluation of a variety of drug classes in a survival test involving the exposure of mice to an hypoxic atmosphere, evidence was obtained which demonstrates that the anti-hypoxia action of many drugs, including pentobarbital, is explained by their elicitation of whole body hypothermia. This was unexpected because in several studies [5] of experimentally induced stroke the ameliorative action of pentobarbital upon the cerebral ischemia was demonstrated while a normal body temperature was purposely maintained. It is evident, despite the apparent pharmacological correlation, that any causal relation between such anti-hypoxia and anti-ischemia activities is unlikely.

MATERIALS AND METHODS

Metered flows from cylinders of compressed nitrogen gas and either air or oxygen gas were combined at a T joint and bubbled through a bottle of water via a diffuser into a 500-ml empty glass jar from which twelve outlets were connected with 3-ft lengths of tubing to twelve test cylinders. These were plastic tubes (5 cm diameter × 30 cm length) to which was cemented a saddle support at the inlet end to produce an angular elevation of approximately 5°. The gas inlet tube passed through a rubber stopper and ended in a T so that two streams of the entering gas were directed against the cylinder walls. Animals were inserted and removed from the other end which was closed by a rubber stopper with a 0.5 cm gas escape hole. All plastic tubing was 0.5 cm ID Tygon. Because of the dimensions of the tubing and stopper hole, the removal or replacement of stoppers did not affect the flow of gas in the other cylinders, and thus identical flows were maintained in each of the cylinders at all times.

Non-fasted male ICR mice (Harlan Sprague–Dawley) were used in all the experiments. Their body weights ranged from 23 to 28 g but were grouped over a 2-g range for individual experiments. Drugs and vehicles (0.9% saline or 0.5% methylcellulose in 0.9% saline) were administered by intraperitoneal (i.p.) injection in a volume of 0.15 ml/25 g body weight. Following the injections, the animals were usually kept as groups of three in metal cans (30 cm diameter) with wood shavings over the bottom. Animal–animal interaction was lessened by this use of small groups. Rectal temperature was measured with a Tele-thermometer (Yellow Springs Instruments Co., Inc., Yellow Springs, OH) whose thermistor was inserted 28 mm. Room temperature was 21–23°.

Experiments were usually performed in the mornings because there was less variability than when performed in the afternoons. The survival time of saline control animals would occasionally (4 to 8-month intervals) be randomly increased over several days, and experiments were then postponed until the problem disappeared. A spot check of body temperature would reveal that many of the animals

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† Author to whom correspondence should be addressed.

were mildly hypothermic for some unknown reason.

Hypothermia of graded severity was produced by cooling animals in a refrigerator at 6° for times ranging from 15 to 60 min. Body temperatures remained near normal unless the animals were restrained during this period—mice were kept in individual plastic cylinders (4.4 cm diameter × 15 cm length) that were closed on each end with rubber stoppers drilled with a 1.8 cm hole.

In a typical survival experiment, twelve mice were injected with either a test drug or vehicle at 30-sec intervals, and 30 min later, unless otherwise noted, they were sequentially inserted every 30 sec into individual test cylinders through which a gaseous mixture was flowing at a rate of 3.3 ft^3/hr. At this flow rate, with a mixture of 4.6% oxygen/95.4% nitrogen, the oxygen concentration of 4.6% was regained within 25 sec following the insertion of a mouse into a cylinder. The survival time of each mouse was noted; a last gasp served as the measure of death. A concentration of 4.6% oxygen was adopted for comparing the effects of various treatments upon hypoxic survival because at this concentration the survival of untreated animals was relatively brief (usually 4–6 min), and the precision was correspondingly large. The composition of each gas mixture was monitored continuously with a mass spectrometer, the Perkin Elmer 1100 Medical Gas Analyzer.

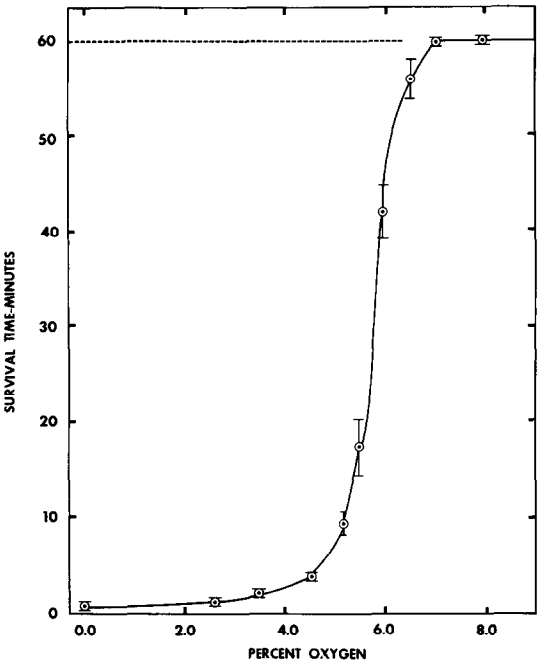

Fig. 1. Survival time of mice subjected to graded concentrations of oxygen. Values are the means ± S.E.M. for groups of six animals (0.0 to 3.5% oxygen) and for thirty-six animals (4.5 to 7.0% oxygen). Note that the maximum survival time was arbitrarily limited to 60 min.

RESULTS

Survival time during continuous exposure to graded oxygen concentrations. Groups of six or twelve mice were tested for survival time in graded percentages of oxygen that ranged from 0.0 to 8.0. When a plot of the resulting times versus percentages was prepared (Fig. 1), a smooth curve could be drawn that clearly illustrated the critical oxygen concentration between 5.0 and 6.0%. This curve closely resembles that reported by Secher and Wilhjelm [6]. It should be noted that the upper limit for survival time was arbitrarily set at 60 min for convenience in experimentation. This caused an increasingly negative bias with survival times above 45 min, as revealed by an inflection point in a plot of S.E.M. values versus minutes of survival from numerous experiments.

The pronounced variation in the individual survival times with 4.5 to 6.5% P_2 is illustrated by a frequency plot relating the number of animals with the minutes of survival (Fig. 2). This plot reveals a gradual change in survival time with increasing percentage of oxygen in the ambient atmosphere and, in addition, an apparent separation of the animals into two groups, one having considerably longer survival times than the other. This division is most clearly seen with the 5.5 and 6.0% oxygen atmospheres, and it shows the difficulty of expressing valid survival values with a small group (e.g. six) of animals, especially for times longer than 20 min. An identical division of animals was revealed in a replicate study.

Physiological relationship between body temperature and survival time in 4.6% *oxygen.* Hypothermia of graded severity was produced in groups of six animals maintained in concentrations of oxygen below 15% (Fig. 3); a fixed period of 90 min was chosen because at this time a minimum temperature had been reached upon continuous exposure to a 7% oxygen atmosphere. However, in order to obtain the minimum temperature with 4.6% oxygen, it was necessary to lower the temperature of the animals by exposure to 7% oxygen for 90 min after which they were treated with 4.6% oxygen for only 60 min because of 50% mortality. The survivors exhibited normal behavior at 24 hr after the double exposure to hypoxia, and all the animals tested at higher oxygen concentrations also survived and appeared normal after testing. No change in temperature was produced upon exposure of animals to 100% oxygen for 90 min.

Hypothermia of graded severity was also produced by the exposure of groups of twelve animals to a cold environment while under restraint (described in Materials and Methods). To study the effects of pyrexia upon survival time, groups of twelve were warmed for 30 min under an infrared heat lamp adjusted to varied intensities.

To obtain valid data relating body temperature and survival time in 4.6% oxygen, it was necessary that animals tested for survival be handled as little as possible because the stress of temperature measurement in hypothermic animals was sufficient to reduce their survival times (and to elevate body temperatures [7]). Thus, these measurements could not be obtained in individual animals and, therefore, the following protocol was used on groups of twelve animals subjected to the same hypoxic pretreatment: the body temperatures of six animals were measured while the other animals were quickly inserted into

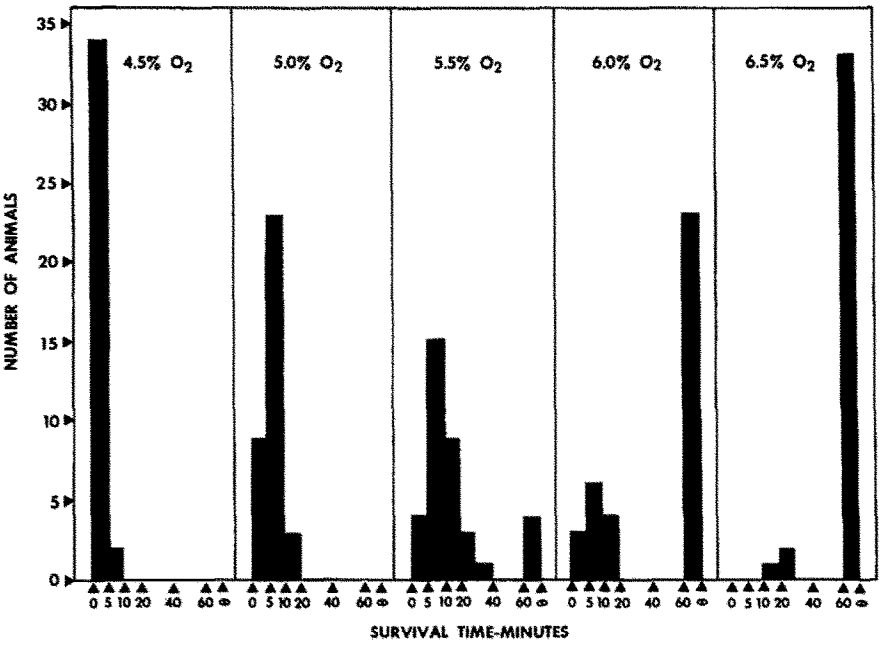

Fig. 2. Distribution of survival times of mice subjected to graded concentrations of oxygen. Thirty-six animals were tested at each concentration (their mean survival times were reported in Fig. 1). Note the reduced scale of the abscissa from 0 to 5 and 5 to 10 min as compared to later scales.

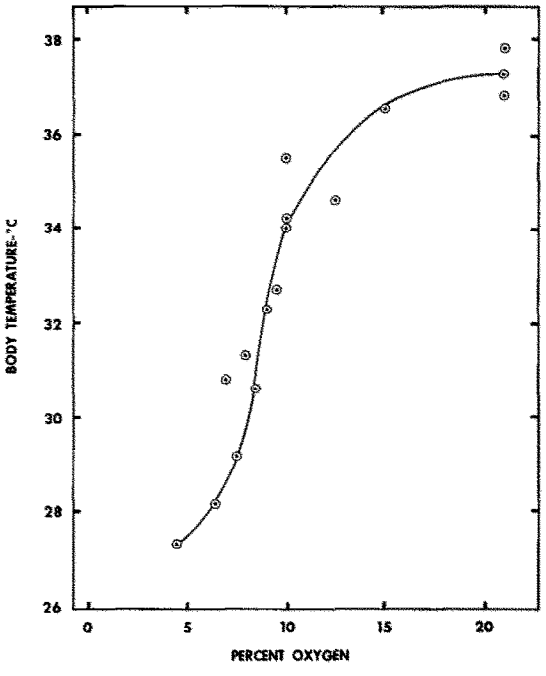

Fig. 3. Body temperatures of mice subjected to graded concentrations of oxygen. The value at 4.6% oxygen resulted from a 60-min exposure at 4.6% preceded by a 90-min exposure at 7.0%. All other values resulted from 90-min exposure to the designated concentration of oxygen. Values are means for groups of six animals; average S.E.M. for the sixteen body temperature values was 0.35. The correlation line was drawn by visual estimation.

or remained in the plastic test cylinders and their survival times in 4.6% oxygen were measured.

A plot of the average values of body temperature and survival time for each of the above sets of twelve animals (hypo-, normo-, and hyperthermic) revealed an asymptotic relationship that was converted into a linear relationship by a semi-logarithmic plot (Fig. 4) which can be expressed by the equation:

$$\log_{10} \text{min} = 6.743 - 0.1618 \times \text{°C body temperature}$$

having $R = 0.962$ ($P < 0.001$). An inverse relation between body temperature and survival time was reported previously by Kottke *et al.* [8], when mice were placed in simulated high altitude chambers.

Correlation of survival time in 4.6% *oxygen with body temperature following treatment with nucleic acid derivatives.* To examine the correlation of survival time with body temperature after treatment with various nucleic acid derivatives, each substance was administered i.p. to a group of twelve animals at a fixed dose of 0.4 mmole/kg (e.g. 107 mg/kg of adenosine). Body temperature was measured in six of these animals 30 min later, and the other six animals were then subjected to the 4.6% oxygen survival test. Again, handling was reduced to a minimum.

Of the many nucleic acid derivatives tested, those containing adenosine were the most effective in increasing the resistance to hypoxia and in producing hypothermia (Fig. 5), especially the 2′(3′)-cyclic-AMP and its acyclic derivative, 2′(3′)-AMP. With the exception of 5′-AMP and 5′-ADP, whose elevated values were considered to result from experi-

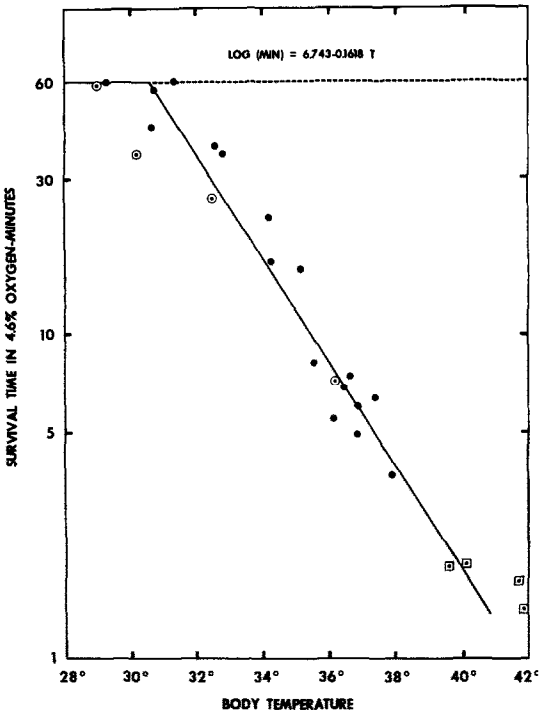

Fig. 4. Relation between survival time in 4.6% oxygen and body temperature after physiological treatments. Key: (●): exposure to graded concentrations of oxygen; (⊙) restraint in refrigerator; and (⊡) heat lamp. Values for each variable are means for groups of six animals. The average S.E.M. for the twenty-six survival time values was 2.52; that for body temperature values was 0.34. The regression line was calculated by the method of least squares. Note that maximum survival time was arbitrarily limited to 60 min.

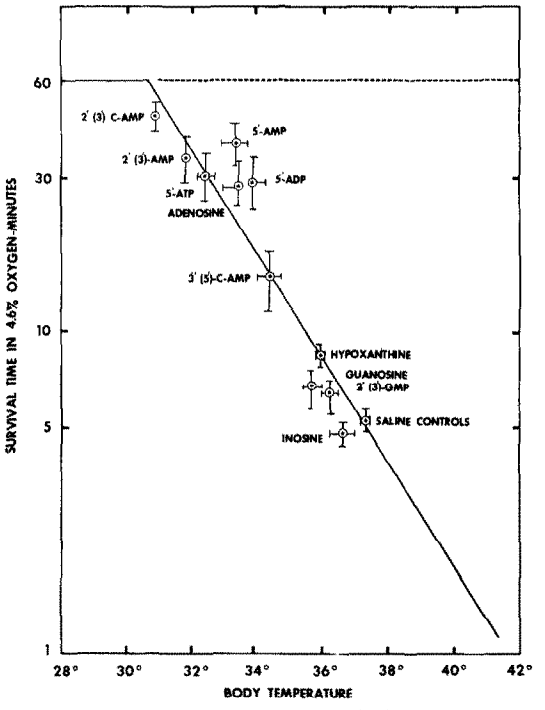

Fig. 5. Relation between survival time in 4.6% oxygen and body temperature following treatment with nucleosides and nucleotides. Survival testing was performed 30 min after administration of substances at 0.4 mmole/kg. Values for each variable are means ± S.E.M. from twelve animals. The physiological regression line is that shown in Fig. 4. Note that maximum survival time was arbitrarily limited to 60 min.

mental variation, the S/BT value (log minutes survival versus body temperature) for each nucleic acid derivative fell either on or near the physiological regression line but not above it. The anti-hypoxia activities of adenosine [9] and its 5′-phosphates were reported previously. Adenine, cytidine, uridine, 5′-CMP, 5′-UMP and adenosine-5′-sulfate were inactive when tested under the same conditions.

An unexpected finding was that, although adenosine and its phosphates produced both hypothermia and increased hypoxic survival, there was no overt indication of the anesthesia or tranquilization seen after the administration of barbiturates, reserpine or chlorpromazine. The animals were a little sluggish, perhaps because of the hypothermia, but they readily moved when disturbed and showed a startle response to noise. They also exhibited a slight piloerection.

Correlation of survival time in 4.6% *oxygen with body temperature following treatment with hypothermia-inducing drugs.* The duration of the increased survival produced by many known therapeutic drugs was found to correlate with the extent of the pharmacologically induced hypothermia, and the resulting S/BT values lay close to the physiological regression line described previously. This was observed 30 min after the administration of benzodiazepine tranquilizers related to diazepam and chlordiazepoxide (Fig. 6), some of which have been reported to increase resistance to hypoxia [10]. It was also observed 30 min after the administration of many other drugs that have been reported previously to increase resistance to hypoxia or ischemia (Fig. 7), among which were pentobarbital [2], diphenylhydantoin [11], and physostigmine [12]. The value for hydralazine also lay near the regression line. Trifluoperazine, a neuroleptic agent reported [13] to ameliorate experimental stroke in gerbils, significantly increased survival time, but its S/BT value was clearly below the regression line.

In contrast, tranquilizers such as reserpine and chlorpromazine shortened survival time to less than the control values even though they also lowered body temperature. Similarly, the S/BT value from a large dose of phenoxybenzamine was also considerably below the physiological regression line. A lowered blood pressure *per se* was not involved in these disparities because the S/BT value for a very large dose of hydralazine (8 mg/kg, i.p.) fell near to the line. In addition, the S/BT value of adenosine was also near the regression line (Fig. 5) and yet it exerts a significant hypotensive action at the same doses used in the present experiments [14].

DISCUSSION

The reason for the pronounced variation in susceptibility to hypoxia among a group of untreated

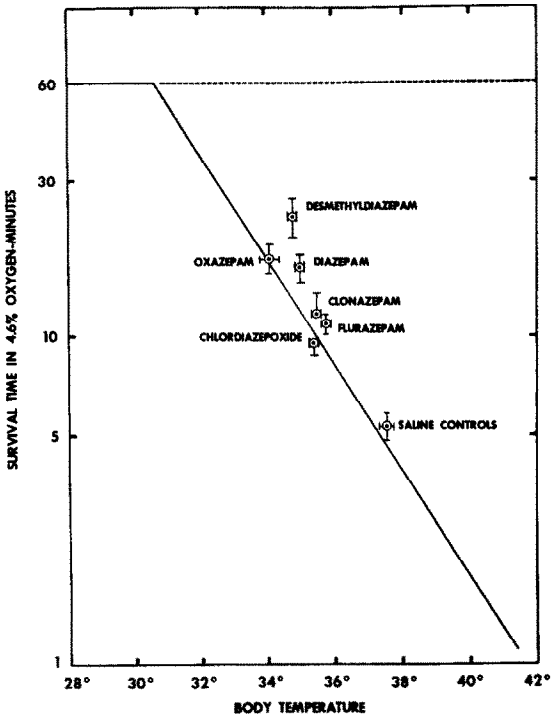

Fig. 6. Relation between survival time in 4.6% oxygen and body temperature following treatment with benzodiazepine drugs. Survival testing was performed 30 min after i.p. administration of drugs at 0.08 mmole/kg. Values for each variable are means ± S.E.M. from twelve animals. The physiological regression line is that shown in Fig. 4.

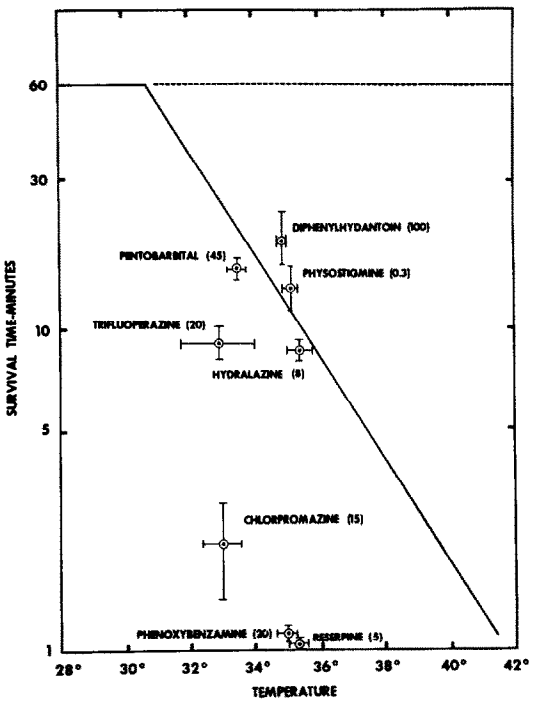

Fig. 7. Relation between survival time in 4.6% oxygen and body temperature following treatment with reported anti-hypoxia, anti-ischemia drugs and reference drugs. Survival testing was performed 30 min (60 min for phenoxybenzamine and 15 hr for reserpine) after i.p. administration at doses indicated in parentheses. Values for each variable are means ± S.E.M. from twelve animals for anti-hypoxia, anti-ischemia drugs (see text) and from six animals for the others. The physiological regression line is that shown in Fig. 4.

ICR mice remains unidentified. It could be due to individual differences in normal body temperature—the temperature of the individual animals when placed in the 4.6% oxygen atmosphere—or perhaps to variation in the rate of temperature fall upon exposure to the hypoxic environment. Unfortunately, these are not readily examined on an individual basis because of the (previously mentioned) interaction of the physical stress of rectal temperature measurement with survival time. An alternative explanation is that the complete division of animals into two groups, most clearly seen upon pretreatment with 6.0% oxygen, may have reflected two substrains of the ICR mouse which differ in their resistance to hypoxia. A precedent for this lies in the breeding of a Long–Evans strain of rat into substrains having low and high erythrocyte levels of diphosphoglyceric acid [15], a known endogenous determinant of the oxygen affinity curve for hemoglobin.

Treatment with a non-lethal hypoxic atmosphere provides a very convenient and reproducible physiological method to produce and maintain a low body temperature in the absence of manipulations such as drug-induced anesthesia and external cooling. The animals can apparently be maintained indefinitely over a wide range of subnormal temperatures by continuous exposure to graded oxygen concentrations and at even as low a temperature as 27° by exposure to 4.6% oxygen after a preparatory exposure to 7% oxygen. It is evident that the body temperature becomes stabilized at a level depending upon the quantity of available oxygen.

The data show that survival time in a 4.6% oxygen atmosphere decreased asymptotically as body temperature was lowered from its control value; the linear relationship presented here was obtained only when survival time was expressed in logarithms. This finding does not agree with the linear decrease in cerebral oxygen utilization ($CMRO_2$) with decreasing body temperature reported by Hagerdal *et al.* [16], and it may indicate an unexpected separation of hypoxic survival from reduced oxidative metabolism under conditions of mild hypothermia.

The linear semi-logarithmic relation between survival in the standardized 4.6% oxygen test and the lowered body temperature resulting from pretreatment with hypoxia reflected a physiological action as opposed to a pharmacological action. The premise can thus be made that a drug whose S/BT value lies on or near to this correlation line can exert anti-hypoxic action only by whole-body hypothermic activity. Similarly, the farther below the line an S/BT value lies, the stronger the indication that the drug will exert some noxious action that will prevent the induced hypothermia from expressing its full effect upon hypoxic survival. Thus, an S/BT line derived from a simple hypoxic pretreatment clearly

forms the upper survival limit for the drugs whose activity depends solely upon hypothermia.

To carry the interpretation further, a drug whose S/BT value lies above the physiological regression line would presumably have a biochemical or pharmacological action that increases hypoxic resistance in addition to lowering body temperature. For example, such a drug might divert the limited amount of oxygen or of an energy-yielding substrate from nonessential processes in the brain to those that are directly involved with survival, or provide disproportionate quantities of blood and/or oxygen to the brain. There is no indication of the efficacy of any drug having such actions at the present time although ketone bodies have been reported to increase hypoxic survival to a limited extent [17], perhaps by serving as alternative sources of metabolic energy.

To examine the usefulness of the above premise, the actions of numerous hypothermia-inducing drugs were tested. Except for phenoxybenzamine and reserpine, this was performed 30 min after their administration, a time when pilot studies usually showed the beginning of a period of minimum body temperature. Sufficient members of each group were discovered to have S/BT values falling on or below the physiological regression line to demonstrate that this line did, indeed, form an upper limit for the duration of hypothermia-induced hypoxic survival.

This regression line, relating survival time in hypoxia with whole-body temperature, can be used to explain certain pharmacological and physiological observations reported in the literature. For example, physostigmine was suggested recently [12] to protect mice against hypoxic stress by cholinergic-induced increases in cerebral blood flow and oxygen availability, but because its S/BT value fell on the physiological regression line, the anti-hypoxic action appears to result from nothing more than a previously reported hypothermia. A similar situation exists for pentobarbital, whose anti-hypoxic action has been ascribed to various mechanisms, including free radical scavenging, changes in cerebral blood flow and membrane stabilization. Steen and Michenfelder [18] concluded from a study of mice made tolerant to pentobarbital that barbiturate protection against hypoxia is due to a decreased cerebral metabolic rate and is a direct function of anesthetic depth. However, the present observation that the S/BT value for pentobarbital was near the physiological regression line together with drugs that did not cause anesthesia, such as adenosine, certain benzodiazepine tranquilizers and hydralazine, suggests that the protection by pentobarbital is a result of the induced hypothermia and is independent of the state of anesthesia. Indeed, animals, whose body temperatures were maintained within a normal range by external warming after treatment with pentobarbital and representative members of other drug classes, did not exhibit the increased resistance to hypoxia that appeared when drug-induced hypothermia was allowed to develop (unpublished observations). Finally, it appears that the protection of newborn animals against a lethal hypoxia by a preliminary exposure to a less severe hypoxia can be explained by the hypothermia produced during the initial hypoxic episode [19].

Although large pharmacological doses of many drugs were used to obtain the data reported here, response studies with lower doses revealed that, if a S/BT value for a large dose lay on the regression line, those from lower doses did also (unpublished observations). Similarly, at doses higher than those shown here, S/BT values for many drugs remained on the line, while those for other drugs, e.g. pentobarbital, fell significantly below the line due to a decreased resistance to hypoxia while yet reflecting a greater degree of hypothermia, indicating toxic effects.

It is likely that such diverse agents produce hypothermia by a wide variety of mechanisms. Vasodilators such as hydralazine probably cool by peripheral vasodilation, pentobarbital by a reduction in metabolism, and certain other drugs perhaps by actions upon temperature regulation centers in the anterior hypothalamus. It appears likely that the 2′(3′)- and 5′-adenosine phosphates act via adenosine itself, a known peripheral vasodilator, because all of them exhibited similar activities on a molar basis. Although there may be an inclination to relate the hypothermic response to changes in 3′,5′-cyclic-AMP metabolism, this appears untenable because its activity upon hypoxic survival was lower than that of the other adenosine phosphates.

The present study does not provide any decisive evidence that the protection exerted by hypothermia against hypoxia is due to a selective action in either the brain or the heart or results from a generalized reduction of oxygen utilization throughout the body. Changes in temperature and in oxidative metabolism of these two organs would be expected to parallel those of the body very closely. Hearts remained beating 30 sec after the last gasp in many of the animals subjected to the hypoxia survival test following a variety of pretreatments, as was also observed by Himwich, but the circulatory effectiveness of such terminal contractions is unknown. However, anecdotal evidence suggests that the brain is the critical organ in hypoxia (Himwich *et al.* [20]) and interpretation of the described drug actions has been loosely made on this basis.

Although both hypothermic and anti-hypoxia activities are exhibited by many drugs, including some with known anti-cerebral ischemia activity, the present study suggests that a causal relation of the former with anti-ischemia activity is unlikely. However, it does appear that any rationale for the treatment of experimental cerebral ischemia with existing drugs must accommodate their apparent potential for slowing unidentified metabolic processes, probably within the brain, as reflected by their ability to produce whole-body hypothermia.

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