A COMPARISON OF THERMOGENESIS BY SELECTED SUBSTRATES ON HYPOTHERMIC RAT LIVER

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THESIS

Presented to the Graduate Council of the

North Texas State University in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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August, 1979

Long, James T., A Comparison of Thermogenesis by Selected Substrates on Hypothermic Rat Liver. Master of Science (Biology), August, 1979, 32 pp., 1 table, 6 illustrations, bibliography, 61 titles.

The thermogenic effects in hypothermia of four substrates--alanine, glycine, ethanol, and pyruvate--were studied in seventeen experiments. Albino rates were decapitated, and their livers were removed. The livers were homogenized with phospate buffer at -5^{o} C. After equilibration in a refrigerated Warburg apparatus at 20^{o} C, the substrates were added and tissue respiration was recorded over three hours. Heat production was calculated from O_2 uptake and CO_2 production. Results showed that alanine, glycine, and pyruvate yielded 93.19, 89.86, and 89.89 X 10^{-6} kg-cal compared to a control value of 86.11 X 10^{-6} kg-cal. Ethanol provided 110.31 X 10^{-6} kg-cal, a value significantly greater than for the other substrates. The substrates studied, especially ethanol, did, therefore, increase heat production in an artifically hypothermic environment in homogenized rat livers.

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A COMPARISON OF THERMOGENESIS BY SELECTED SUBSTRATES ON HYPOTHERMIC RAT LIVER

The *in vitro* thermogenic effects of biochemical substrates on hypothermic animals is a subject that has not been extensively investigated. "Hypothermic," used in context, refers to an abnormally low body temperature in a homeothermic animal. Several investigators (1, 10, 11, 23, 25, 30) consider hypothermia in humans to be a body temperature of less than 35° C. Rapport and Beard (50), using crude hydrolystates of casein, found that ingestion of alanine, glycine, phenalalnine, and tyrosine increased the metabolic rate and heat production in normal dogs by 16 to 21 per cent, 30 per cent, 40 per cent, and 16 to 21 per cent respectively. Gubner, DiPalma, and Moore (27) observed that oral ingestion of 20 grams of glycine increased heat production and blood flow in patients with peripheral vascular disease. Laufman (40) reported a case of severe accidental hypothermia in a human being. The patient had been drinking heavily before exposure to an air temperature ranging from - 18° to - 24° C for eleven hours that resulted in a body temperature of 18° C. The author speculated that the high blood alcohol level (exact amount unknown) was in some way at least partially responsible

for the survival of his patient. In support of that speculation, Beavers and Covino (3), in an in vitro study using dogs at a body temperature of 26° C, discovered that intravenous injection of a 5 per cent glycine solution significantly increased heat production. As a result, they noted a prolonged cooling time (to 26°) and a shorter rewarming time compared to nonglycine treated dogs. Another investigation having to do with a biochemically increased thermogenesis was an in vivo study by White and Nowell (61). These workers injected rats intraperitoneally with 1.5 ml of 25 per cent ethyl alcohol per 100 grams body weight. Time to cool in crushed ice from 30° to 15° C, the temperature at which cardiac arrest took place, and the time to cardiac arrest were both significantly increased in the alcohol-treated group. Webb, et al. (59) administered 2 ml of 50 per cent ethanol intraperitoneally to each of 250 gram rats. Cooling in an iced saline bath and rewarming resulted in a lower temperature to cardiac arrest for the alcohol-treated rats (3.1° C, cf. 5.1° C for control rats) and a lower temperature at which spontaneous cardiac reactivation took place for the alcoholic rats (12-15° C, cf. controls of 25-30° C). Miller and Miller (44) determined the same basic facts in their study of guinea pigs. MacGregor, et al. (42) demonstrated an increased tolerance to ventricular fibrillation in dogs under hypothermic conditions (16° C) with administration of 2 grams/kilogram of ethanol.

Methods

The metabolically thermogenic effect of the substrates was studied in seventeen experiments, using one rat per substrate, in addition to a control, in each experiment. Four-month-old 300-gram male albino rats from Lewisville Animal Supply, Lewisville, Texas, were used. The rats were fasted for 24 hours before killing.

A Precision Scientific, refrigerated Warburg apparatus, capable of maintaining a water bath temperature within $\pm 0.05^{\circ}$C, was used. Five Warburg manometers were employed for determining oxygen uptake, five manometers for CO_2 production by Umbreit's direct method (56), and one manometer was a thermobarometer for temperature and barometric pressure corrections.

Stock solutions of the four substrates and Krebs-Ringer phosphate buffer were prepared just prior to each experiment and adjusted to give a pH of 7.20 in the final solution of homogenate. As a final concentration of 0.01 M of each substrate in the Warburg flasks was desired, and since there would be a tenfold dilution upon addition of the substrates from the sidearms of the flasks, the solutions were made up as 0.10 M.

The rats were killed by a blow to the head and rapidly decapitated with a Harvard Apparatus Co. guillotine. The livers were removed whole as quickly as possible, put into sealed weighing vials,

and immediately weighed. After weighing, the vials were packed in crushed ice. For each sample, the amount of buffer for a 20 per cent dilution with the liver tissue was pipetted into a Chemical Rubber Company Micro Mill; the whole liver was added; and the contents were homogenized for three minutes at 20, 000 RPM. Ethylene glycol at -5°C was circulated continuously through the cooling chamber of the Micro Mill by a Wilkins-Anderson Lo-Temp Bath. A 1.8 ml aliquot of liver homogenate was pipetted into each of two single sidearm Warburg flasks. The flasks were stoppered and set into crushed ice. When all of the five liver samples had ben processed, 0.2 ml of each substrate were added to the sidearms of their two respective flasks and 0.2 ml of fresh 20 per cent KOH together with a filter paper wick were put into the center well of those flasks used for O_2 determination. The eleven flasks were then attached to their respective manometers which were in turn attached to the water bath-shaker apparatus at 20°C for equilibration at a shaking rate of 100 per minute (56). After fifteen minutes of equilibration, the manometer Kreb's fluid (56) levers were adjusted and the stopcocks were closed. Readings were taken every fifteen minutes for three hours. Three initial readings, before the buffer substrates were added, were taken as a check on a normal and constant metabolism. After these readings, the contents of the sidearms were introduced

into the liver homogenate-buffer solution and the mixtures were poured back into the sidearms and back again into the main bodies of the vessels in order to assure a quantitative addition of the substrates. Agitation of the flasks in the water bath continued. A Yellow Springs model 42 Tele-thermometer and a model 80 recorder constantly monitored the temperature of the water bath. At the completion of each experiment, the flasks and weighing vials were cleaned by the dichromate method of Umbreit, et al. (56). After rinsing, the vessels were oven dried for one hour at 120° C and stored in a silica gel desiccator for use in the next experiment.

Heat production was calculated from equation 12 of Weir (60) --Total kg-cal = 3.9 X liters O_2 used + 1.1 X liters CO_2 produced.

Results

The accumulated data were processed by hand and by computer program utilizing an Olivetti model Programma 101. As can be seen from Table I, the control sample yielded a mean heat production of 86.11 X 10^{-6} kg-cal. Alanine, ethanol, glycine, and pyruvate yielded 93.19, 110.31, 89.86, and 89.89 X 10^{-6} kg-cal respectively. Statistical analyses were performed on a Hewlett-Packard model 9810 A computer. The two statistical tests utilized were the randomized complete block F test of Steel and Torrie (upon which the experimental design was based) and Dunnett's test for judging the

TABLE I

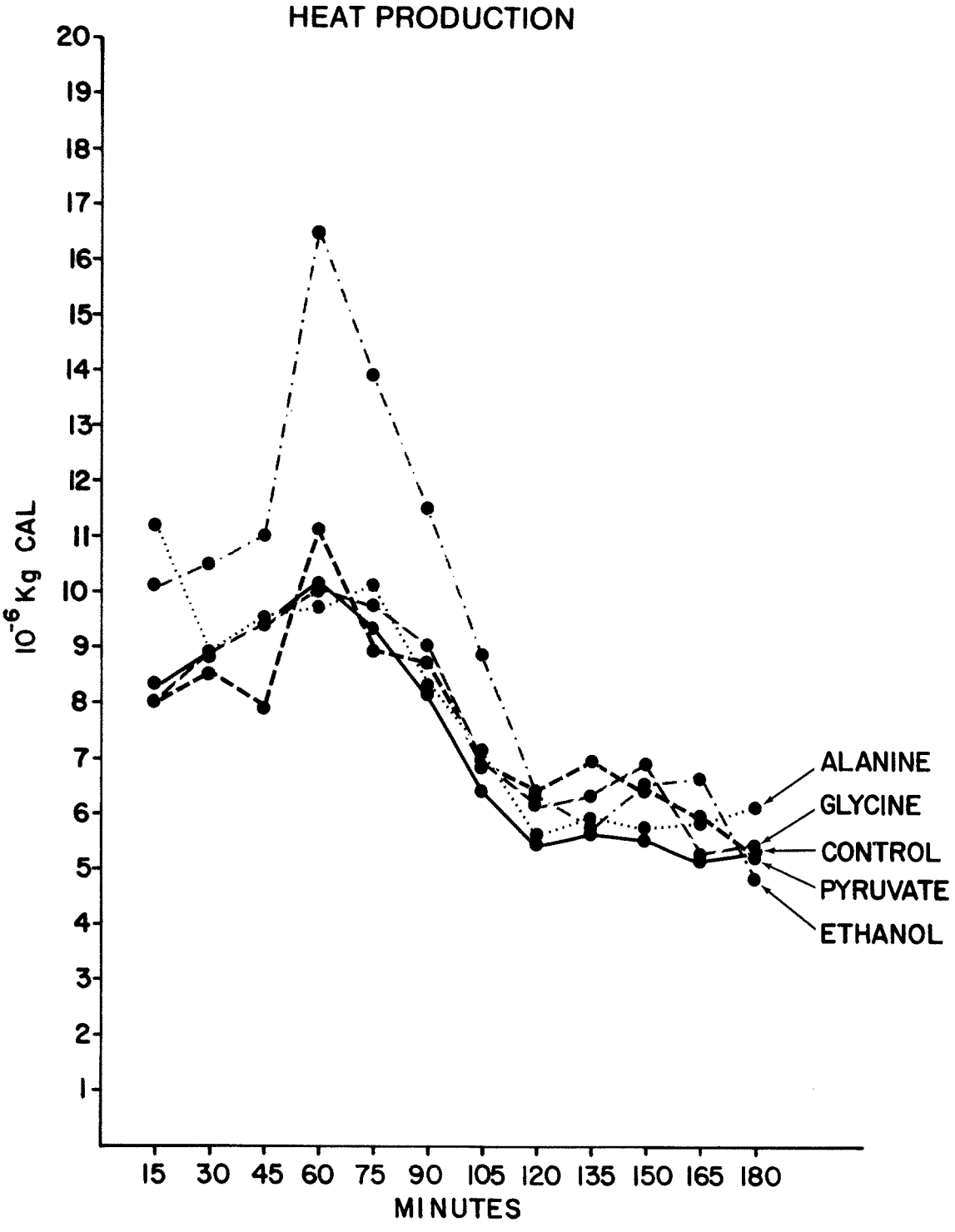
HEAT PRODUCTION
(KgCal X 10^{-6})

Substrate	Experiment No.							
	1	2	3	4	5	6	7	8
Control	89.51	74.70	72.07	104.52	106.93	90.41	83.38	97.43
Alanine	102.65	74.68	76.78	111.81	103.54	104.87	88.51	99.44
Ethanol	133.50	127.94	84.55	119.16	136.36	101.50	94.13	121.47
Glycine	95.61	75.60	68.13	113.02	114.76	100.63	82.26	100.81
Pyruvate	86.20	74.61	61.03	114.07	92.19	107.85	79.51	84.44

TABLE I continued

9	10	11	12	13	14	15	16	17	$\bar{X}$	$S_{\bar{X}}$
136.65	62.73	89.65	111.68	72.91	69.90	80.72	68.98	51.65	86.11	±5.07
150.29	78.89	91.31	110.37	75.94	73.29	85.00	90.91	66.36	93.19	±4.93
139.63	100.32	129.10	143.43	84.03	89.69	102.52	95.60	72.58	110.31	±5.41
138.51	79.23	84.22	126.99	70.63	61.80	79.88	74.99	60.59	89.86	±5.55
135.10	75.50	89.53	125.54	70.87	77.01	95.40	97.92	61.37	89.89	±5.10

significance of treatments compared with a control (53). The experimental F value of 20.54 compared with the accepted value at the 99 per cent level of 3.65 (53) indicated a high degree of significance of treatment effectiveness. The most effective treatment, that is, the most effective substrate in the sense of producing a thermogenic effect, was indicated by Dunnett's test. A comparison of the treatment means with the control mean gave a highly significant value at the 99 per cent level for only the ethanol treatment. The mean ethanol heat production value of 110.31×10^{-6} kg-cal exceeded the calculated significant Dunnett value of 95.44 (53). At the 95 per cent level, the alanine treatment (93.19) only closely approached significance. Glycine and pyruvate fell even shorter of significance. Figures 1 and 2 show graphically that substrate heat production did indeed exceed that of the control with ethanol showing the greatest gain. The ethanol solution, therefore, yielded the largest and the most significant increase in thermogenesis in the hypothermic, homogenized rat liver. Figures 3 and 4 show O_2 utilization during each 15-minute period of the experiments and the total utilization of O_2 during the experimental period, respectively. Figures 5 and 6 show CO_2 production during each 15-minute period and the total production of CO_2.


HEAT PRODUCTION
20
19
18
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16
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14
13
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10
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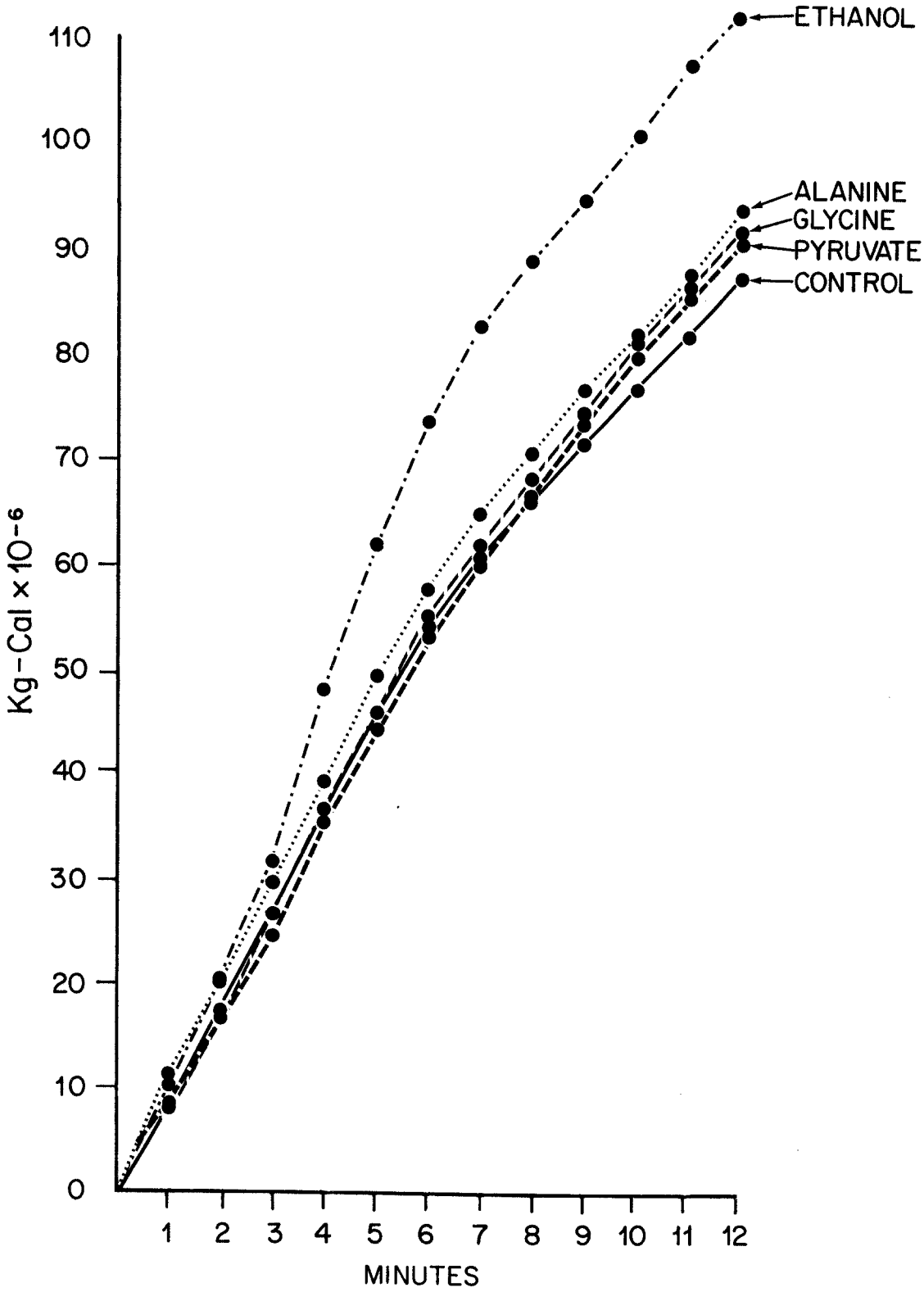
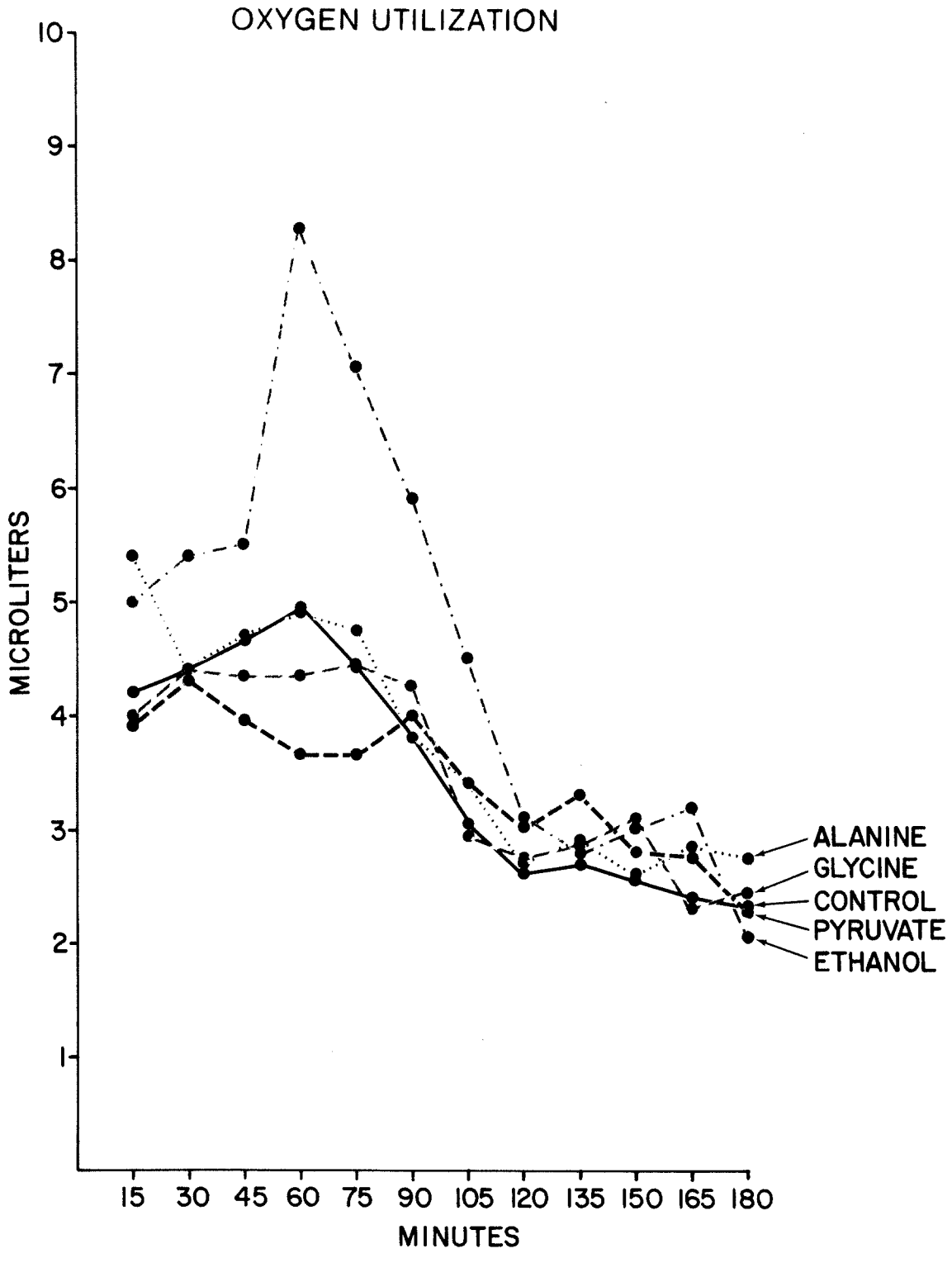
8
7
6
5
4
3
2
1
10^{-6} Kg CAL
15 30 45 60 75 90 105 120 135 150 165 180
MINUTES
ALANINE
GLYCINE
CONTROL
PYRUVATE
ETHANOL

CUMULATIVE HEAT PRODUCTION




OXYGEN UTILIZATION
MICROLITERS
10
9
8
7
6
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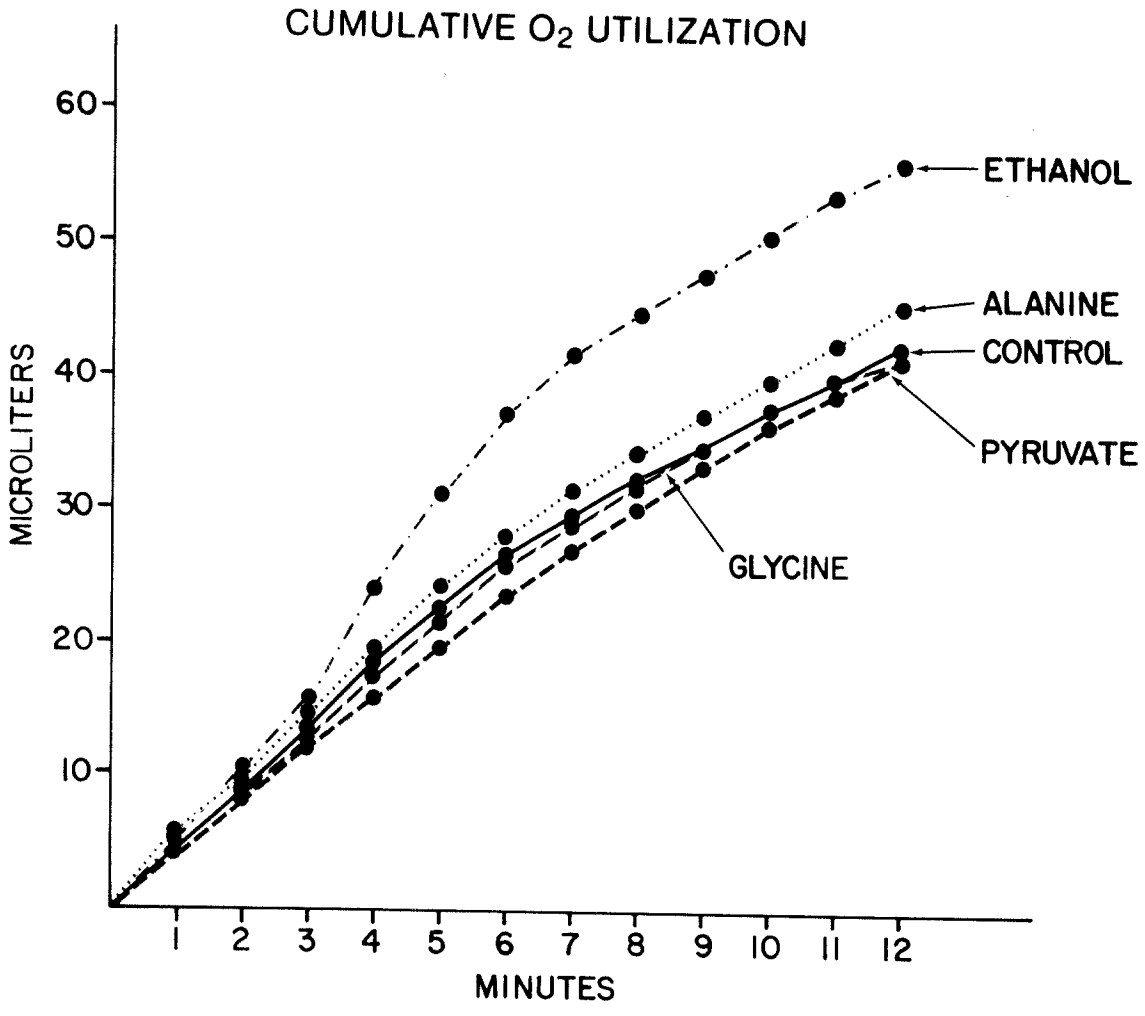
15 30 45 60 75 90 105 120 135 150 165 180
MINUTES
ALANINE
GLYCINE
CONTROL
PYRUVATE
ETHANOL


CUMULATIVE O_2 UTILIZATION
MICROLITERS
60
50
40
30
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10
1
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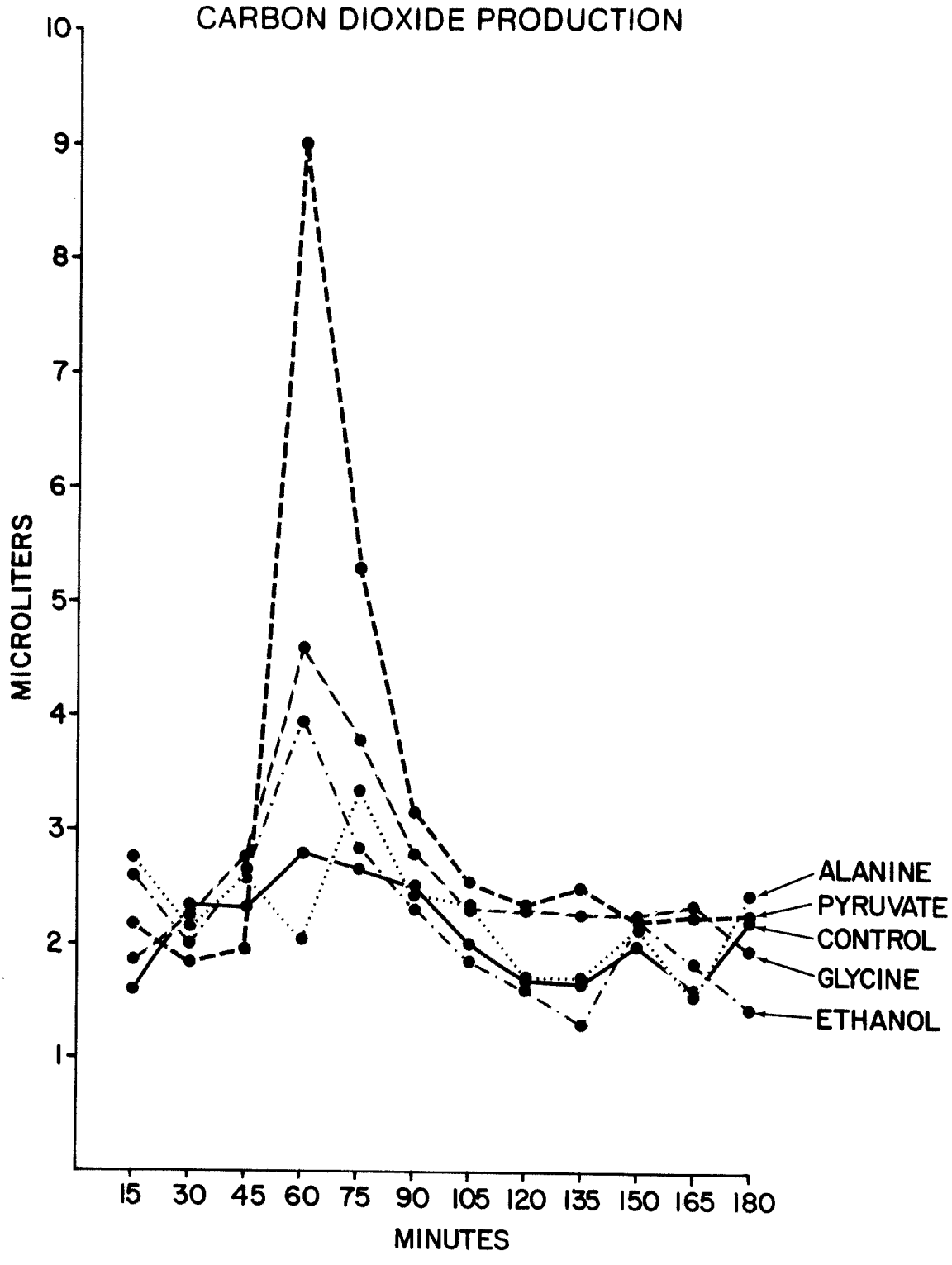
MINUTES
ETHANOL
ALANINE
CONTROL
PYRUVATE
GLYCINE


CARBON DIOXIDE PRODUCTION
MICROLITERS
10
9
8
7
6
5
4
3
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1
15
30
45
60
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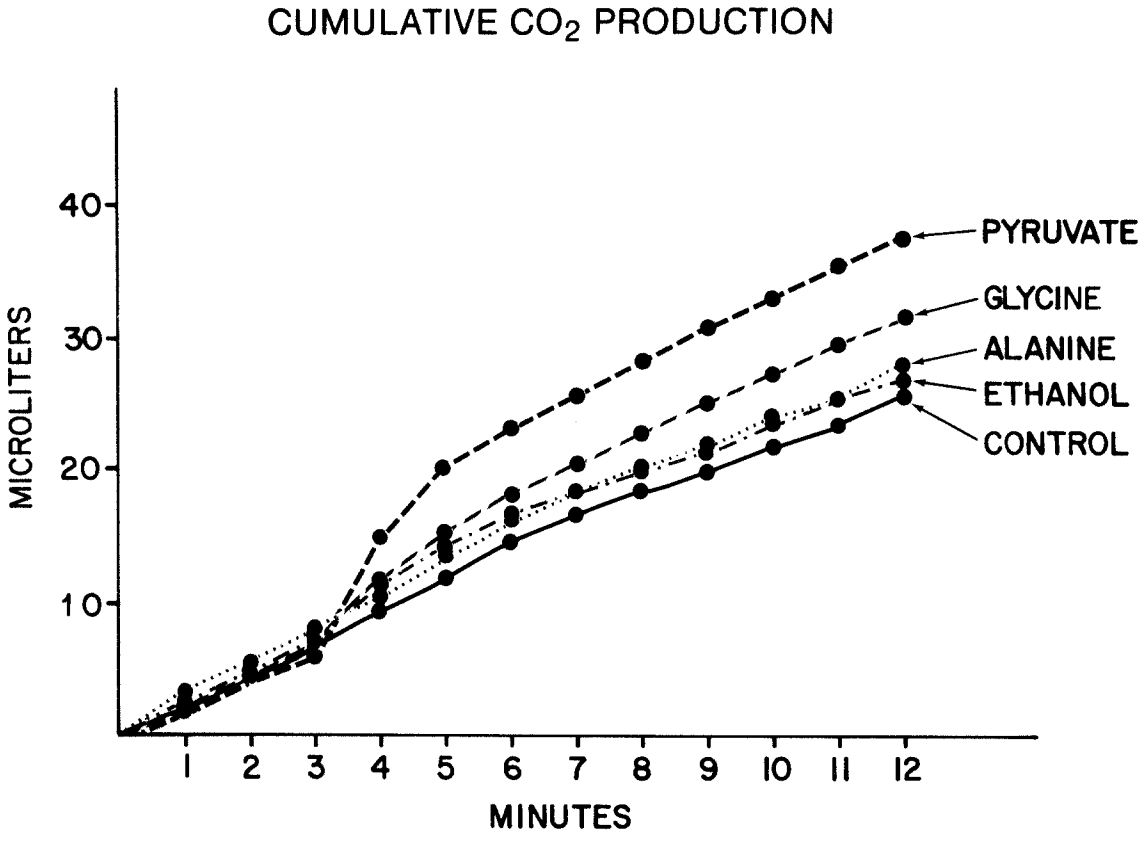
90
105
120
135
150
165
180
MINUTES
ALANINE
PYRUVATE
CONTROL
GLYCINE
ETHANOL


CUMULATIVE CO_2 PRODUCTION
MICROLITERS
40
30
20
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1
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11
12
MINUTES
PYRUVATE
GLYCINE
ALANINE
ETHANOL
CONTROL

Discussion

There are several technical details pertaining to the methods section of this study that should be elucidated. Kaden, et al. (34) used a pyruvate concentration of 0.011 M, since 0.103 - 0.105 M pyruvate induced a severe alkalosis in their isolated, perfused nonhypothermic (37° C) rat livers. It was decided to use .011 M as a working concentration, and since this study was a comparative one, the concentrations of the other metabolites were adjusted to the same value. A liver homogenate was preferred to tissue slices because of the increased homogeneity of the medium. The 20 per cent dilution of liver tissue with buffer gave a satisfactory metabolic rate with the time involved and provided an acceptable working range for calculations. Stunning the rats before decapitation usually reduced the violence of the convulsions resulting from spinal shock upon decapitation, therefore presumably creating less of a stress response on the animals' endocrine system and metabolism. The water bath temperature of 20° C used to simulate hypothermic conditions in vivo was chosen for several reasons. It was found that this temperature resulted in an average decrease of 70 per cent in metabolic rate compared to the rate of 37.3° C, normal rat body temperature, while still giving an adequate rate for the determinations. The metabolic rate at 15° C was very slight. Furthermore, White

and Nowell (61) found that cardiac arrest in their in vivo experiments occurred around 13.2° C. Since it was desired to approximate in vivo conditions in an in vitro process (an often difficult proposition), this lower temperature of 15° C was not used. Several investigators (21, 32, 44, 46, 61) using animals in hypothermic experiments have used a body temperature of 20 to 22° C. Additionally, many of the victims of accidental hypothermia that have been reported had body temperatures in the region of 20° C (1, 20, 24, 40). Another reason was the fact that in spite of the good temperature regulating qualities of the Warburg apparatus used, a water bath temperature of 20° C would not alter significantly over several hours in an ambient room temperature of 21° C. Weir's method for calculating heat production is based upon and applies to in vivo practices, i.e., an actively respiring person or animal. It was felt, however, that the same method should be applicable to an in vitro procedure such as the one followed in this study.

Another technical factor which deserves attention was the selection of a final working solution pH in the Warburg flasks of 7.20. Many investigators (1, 2, 4, 7, 8, 9, 13, 15, 16, 17, 18, 19, 21, 24, 30, 31, 32, 36, 39, 40, 43, 45, 47, 52, 54, 55, 59) have observed acidosis in their hypothermic subjects, both human and animal. This stemmed partly from a metabolic acidosis caused by

the fact that hypothermia decreases the efficiency of the capillary circulation creating widespread tissue ischemia and resultant tissue hypoxia. The latter, in turn, results in an accumulation of metabolic waste acids (lactic acid, notably). This effect seems to be especially noticeable upon rewarming of the subject when capillary circulation is restored and the metabolic acids wash out into the general circulation. Respiratory acidosis can occur from CO_2 retention due to an increased solubility of CO_2 in the blood, impaired cardiopulmonary function, and, according to Fleming (21), inhibition of the carbonic anhydrase system due to a lowered temperature. Another contributing factor in hypothermic acidosis is the liver's inability to metabolize lactate at a sufficient rate. Shivering, with its increased muscular metabolic activity and mobilization of myocapillary circulation, can be another source of acidosis. The pH values for hypothermic subjects cited in this bibliography ranged from 7.08 to 7.34 in accidentally exposed humans (8, 24, 42, 43, 45, 55) and from 6.9 to 7.29 in controlled animal experiments (21, 32, 36). It was therefore decided to use a pH value of 7.20 for this study.

The four substrates--ethanol, alanine, glycine, and pyruvate--produced different amounts of heat, as derived from Weir's formula (60), when added to liver homogenate. During the thirty-minute control period, the liver tissue maintained a reasonably

constant rate of respiration. The rate increased with the addition of the four test substrates and returned to near basal level at the end of the three-hour experiments. In the mostly cell-free (verified by microscopic examination) liver homogenates, it was assumed that intact mitochondria were present.

CO_2 production returned to basal levels approximately 15 minutes prior to the time the O_2 utilization returned to basal state. This time difference was interpreted as the continued uptake of oxygen by the mitochondria for some time after the completion of breakdown of the substrates through the tricarboxylic acid cycle. Observing Figures 1, 3, and 5, one can see a fall-off or decline of all parameters at approximately 105 minutes. This decline could have been the result of mitochondrial aging.

In the in vitro test utilizing liver homogenates, ethanol produced the greatest increase in calculated thermogenesis (110.31 $\times 10^{-6}$ kg-cal/mole) followed by alanine (93.19), glycine (89.86), and pyruvate (89.89). The complete combustion of ethanol, alanine, glycine, and pyruvate in the laboratory yields 328, 387, 233, and 279 kg-cal/mole, respectively (7, 11, 12, 48, 58).

In considering the results of this study, it is interesting to note that ethanol produced more heat than pyruvate, a natural mitochondrial metabolite. The inner membrane of liver mitochondria

permits pyruvate to pass through readily, through its specific tricarboxylate transport system, down and against concentration gradients, the energy being derived from electron transport (35). Ethanol, lipid-soluble and mainly diffusion-oriented, does not need such an active transport system and can equilibrate with the intramitochondrial space in less than a millisecond (37). There seem to be several possible explanations for the higher heat production of ethanol. Kalant and Israel have shown that ethanol does not uncouple oxidative phosphorylation per se, but rather inhibits (Na^+-K^+)-ATPase by competitive antagonism with K^+ ions (35). The sodium pump present in the cell membrane is also inhibited. Perhaps the pump overcompensates, and utilizing more O_2, produces heat in its attempt to maintain electrolyte balance within the cell (6, 35). Bernstein, et al., have demonstrated that in chronically ethanol-treated rats, there is a marked increase in the rate of liver O_2 consumption, together with an increase in sodium pump activity. In addition, the phosphorylation potential,

$$\frac{[ATP]}{[ADP]\ [Pi]}$$

is decreased by chronic ethanol treatment. This effect is due solely to changes in cellular [ATP] and [Pi], no change in [ADP] being found. This decrease in the phosphorylation potential increases the

rate of respiration and the utilization of O_2 (5). Other investigators (33, 38, 57) and we, in our laboratory, suggest that the lipid-soluble property of ethanol alters the permeability of the inner mitochondrial membrane, perhaps by increasing pore size, and permits more sodium to enter the cell. The mitochondria are forced to use the energy of respiration to pump cations, and release heat as a byproduct, instead of making ATP (41). Related studies have shown that in cold-acclimatized rats, there is a 100 per cent increase in (Na^+-K^+)-ATPase with an increase in sodium pump activity and a 30-80 per cent increase in O_2 consumption in rat liver and diaphragm. A cold-acclimatized liver metabolizes ethanol at a higher rate (57). Perhaps the true mechanism of the intramitochondrial action of ethanol involves both inhibition of (Na^+-K^+)-ATPase/sodium pump activity and altered membrane permeability. More studies need to be done to elucidate this phenomenon.

It has been suggested (3, 46, 59, 61) that the introduction of glycine and ethanol into persons subjected to hypothermia increases internal heat production. The results of this investigation indicate that, on a molar basis, ethanol might be a better material for the treatment of hypothermia than the other substrates. The data, however, do not indicate a direct extrapolation to intact organisms.

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