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THE UNIVERSITY OF ALBERTA

Effects of Fasting and Aminophylline on Norepinephrine-Stimulated Thermogenesis

by

(C) Michael L. Jourdan

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

Maximum thermogenesis which is depressed by mild fasting cannot be restored by sympathomimetics but can be restored and further enhanced by either substrate feeding or aminophylline (85% Theophylline, 15% Ethylenediamine). Since aminophylline potentiates cyclic AMP's action on substrate mobilization at a post-receptor level, it is possible that fasting depresses adrenergic efficacy, resulting in reduction of cAMP formation, substrate mobilization and maximum thermogenesis. To test this possibility, adrenergic efficacy under fed and fasted conditions was characterized in vivo, utilizing the established assay of norepinephrine (NE)-stimulated nonshivering thermogenesis (NST) in cold-acclimated rats (Depocas et al., 1980). Cold acclimated, male Spraque-Dawley rats (400 gm) cannulated in either the carotid or jugular vessel were used for characterization of the dose-response relationsnip of NE-stimulated NST. Following this the effects of aminophylline on NE-stimulated NST were assessed. Each rat was used at least twice serving as it's own control under well-fed, 1-day fasted and 2-day fasted conditions.

A typical dose-response relationship between log dose NE and heat production was observed under both feeding conditions. No significant difference was observed in resting heat production between the well-fed and fasted states (2.02±.05 and 1.81±.04 $Kcal(Kg^{.75} \cdot h)^{-1}$, respectively)

measured at 25°C under phenobarbital sedation. In the fasted state, NE-stimulated NST was significantly less than that found in the fed state at all NE doses tested (0.6-8.0 $ug(Kg^{.75}\cdot h)^{-1}$). The decrease in maximum response of 1-day fasted rats at a dose of 6.4 $ug(Kg^{.75}\cdot min)^{-1}$ was 18.2% (6.87±.47 vs 5.81±.39 $Kcal(Kg^{.75}\cdot h)^{-1}$). However, the Ke (dose of NE at 50% maximum response; well-fed = 0.85, fasted = 1.09 $ug(Kg^{.75}\cdot min)^{-1}$) was not significantly different between the two states.

Pretreatment with aminophylline ($15mg(rat)^{-1}$) increased the maximum NE-stimulated NST by 9, 10 and 18%, respectively, in the well-fed, 1-day fasted and 2-day fasted states. The 10% increase in 1-day fasted rats alleviated the depression of maximum thermogenesis consequent to fasting. The 18% increase in 2-day fasted rats resulted in an elevation of NE-stimulated thermogenesis to a level which was not significantly different from that of NE-stimulated thermogenesis in 1-day fasted rats.

These results indicate that the fasting-depressed adrenergic efficacy in stimulating thermogenesis is not due to a reduction in adrenergic-receptor affinity. It could be due to a reduction in adrenergic receptor numbers or adenylate cyclase activity but available studies from the literature suggest this is also unlikely. Since aminophylline can alleviate the fasting depression of adrenergic efficacy in stimulating thermogenesis, it is more likely that phosphodiesterase activity is increased after

fasting resulting in decreased intracellular cAMP concentration and substrate mobilization. The greater sensitivity to aminophylline in enhancing NE-stimulated NST in 2-day fasted rats also suggests that phosphodiesterase activity may increase with the duration of fasting.

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I. BACKGROUND

When a mammal is exposed to increasingly colder temperatures a point is eventually reached where heat loss exceeds maximum heat production and progressive hypothermia ensues (Popovic and Popovic, 1974). At this point one asks what physiological mechanisms might limit maximum heat production. There appear to be three major possibilities (Wang, 1978): 1) the maximum capabilities of the respiratory and cardiovascular functions for transport of oxygen and carbon dioxide; 2) the maximum cellular oxidative capabilities; and 3) the availability of substrates for oxidation.

Respiratory and cardiovascular limitations on maximum thermogenesis were investigated and effectively discounted by Wang and Peter (1975). Systemic hypoxia or acidemia was not observed in rats during HeO_2 (21% oxygen, balance helium) and cold (-10°C) induced hypothermia when maximum thermogenesis was maintained while body temperature (Tb) was continually decreasing. Thus, it appears that neither the maximum capacity for gas transport nor the maximum buffering capacity of the blood were exceeded under these conditions. Wang (1978) argued against maximization of cellular oxidative capabilities as a limiting factor by demonstrating that, while Tb could be 3.4 to 9.7°C lower at the end of a 135 min HeO_2-cold exposure, the total heat production during the final 15 min period was, in the majority of cases,

higher than the initial period when Tb was 37°C. This indicates that the cellular oxidative capabilities have not been exhausted prior to the onset of hypothermia; if they were, this should be manifest by a decrease in total heat production in the final period following the depression of Tb and the Q_{10} effect on metabolism. Finally, by utilizing four feeding regimes (overnight well-fed, rationed, fasted and overnight fasted plus a 15 Kcal substrate mixture 60 min. prior to cold exposure) Wang (1980) was able to modulate the cold tolerance in the same rats by varying the total as well as the maximum heat production. Thus, in the overnight fasting state, the thermogenic capacity and final body temperature were significantly lower than their respective values when fed ad libitum overnight or fasted overnight but given a substrate mixture prior to cold exposure (Wang, 1980). Since the thermogenic capabilities of rats can be modulated by short term variations of nutritional status, substrate availability is indicated as a limiting factor before the exhaustion of cellular oxidative capabilities or respiratory-cardiovascular functions.

Many factors influence the processes of substrate mobilization and utilization during cold exposure. Of these, sympathetic activities (catecholamines) are of primary importance for the mobilization and utilization of glucose (Depocas, 1960, 1962) and free fatty acids (FFA) (Himms-Hagen, 1967; Young and Landsberg, 1977a). Recent evidence indicates that "basal" sympathetic activity is

itself modulated by nutritional status. Young and Landsberg (1977b, 1979) found that fasting (48h) decreased, and one day of refeeding restored norepinephrine (NE) turnover (a measure of sympathetic activity) in the rat heart, pancreas, liver and more recently in brown adipose tissue (BAT) (Young et al., 1982). Overfeeding, on the other hand, increased NE turnover above that of controls. From this they suggested that the decrease, during fasting, and increase, during overfeeding, of sympathetic activities could be responsible for the corresponding depression and elevation, respectively, of re ing metabolic rates after fasting and overfeeding (Young and Landsberg, 1979).

If the reduction in maximum thermogenesis after fasting can be interpreted as due to a reduction of maximum sympathetic activity in the fasted animal, then supplementing the endogenous sympathetic activity with exogenous sympathomimetics should restore the level of maximum thermogenesis in fasted rats to the fed level. Experimental evidence indicates that this is not the case, however. Injection of NE (10-40 ug(rat)$^{-1}$) or Isoproterenol (a β-adrenergic agonist; 2-40 ug(rat)$^{-1}$) in fasted rats, failed to restore the depressed maximum thermogenesis to the fed level (Wang, 1982). This seems to suggest that the adrenergic receptors have been fully saturated by endogenous catecholamines released by sympathetic neurons during severe cold exposure, rendering exogenous sympathomimetics redundant. If this interpretation is correct the reduction

of maximum thermogenesis after fasting could be due to a reduction in adrenergic receptor activity, either in receptor sites or drug-receptor affinity, or due to modifications of post-receptor events leading to substrate mobilization.

Beta-adrenergic agonists such as NE exert their effects through the β-adrenergic receptor - adenylate cyclase system by activating the production of cAMP. Cyclic AMP acts intracellularly as a second messenger to elicit the physiological responses, e.g. lipolysis, glycogenolysis and gluconeogenesis (Robison et al., 1971a). The intracellular concentration of cAMP is regulated by phosphodiesterase (Robison et al., 1971b), which degrades cAMP to 5'-AMP. Therefore, if the reduction in the level of maximum thermogenesis after fasting is due to a reduction in receptor activity, then remedial measures aimed at increasing intracellular cAMP concentration and thus increased substrate mobilization should restore the fed level of maximum thermogenesis in fasted rats. Addressing this point, Wang (1982) injected rats (i.p.) with aminophylline (85% Theophylline, 15% Ethylenediamine), a potent phosphodiesterase inhibitor (Robison et al., 1971b). The result was a significant increase in the maximum heat production of both fasted and well-fed rats above that of their saline injected values. Consequently, the onset of hypothermia could either be prevented or its magnitude attenuated (Wang, 1982). This is further evidence that

neither the respiratory-cardiovascular functions nor the cellular oxidative capabilities are exhausted prior to the limitation of maximum thermogenesis imposed by substrates. Moreover, it seems to indicate that the observed depression of maximum thermogenesis in the fasted rat is possibly due to a reduction in the expression of the adrenergic signal between its reception at the cell membrane and its final biochemical manifestation, namely, substrate mobilization..

II. RATIONALE

Since aminophylline inhibits phosphodiesterase, it is possible that aminophylline-stimulated thermogenesis could be via prolongation of cAMP's action on substrate mobilization. This and the inability of exogenous sympathomimetics to restore depressed maximum thermogenesis after fasting suggests that this depressed thermogenesis could be due to a decrease in adrenergic agonist efficacy after fasting. This decrease in efficacy could be due to a decrease in adrenergic receptor activities, either in number or affinity, a decrease in adenylate cyclase activity, or to an increase in phosphodiesterase activity. The specific aim of this study was to provide preliminary information as to which of these areas might underlie the observed modulation of thermogenesis by fasting.

To demonstrate, in vivo, a reduced efficacy of adrenergic stimulation on thermogenesis after fasting, a specific experimental model is required. Since adrenergic stimulation of metabolic rate is a β-receptor effect (Bukowicki et al., 1980; Cannon et al., 1981; Cannon and Nedergaard, 1983), an in vivo β-receptor assay, involving the stimulation of nonshivering thermogenesis (NST) by NE in cold-acclimated rats was utilized.

The NST, which is found as a major source of heat production in many newborn mammals, hibernators and some adult non-hibernators (ie. mice, rats, rabbits, guinea-

pigs etc.) that have been cold-acclimated, is regulated by the sympathetic nervous system, via the neurotransmitter, NE (Hsieh and Carlson, 1957; Carlson, 1960; Depocas, 1960; also Himms-Hagen, 1967 and Jansky 1972 for reviews). The primary site of NST is BAT (Foster and Frydman, 1978b). This NE-stimulated NST is mediated by the β-adrenergic receptor (Bukowicki et al., 1980) adenylate cyclase - cyclic AMP system (Reed and Fain, 1968; Fain and Reed, 1970), and leads to the activation of BAT lipase. The acyl-CoA produced following lipolysis competitively binds a membrane bound H^+ channel protein (the 32,000 dalton GDP binding protein, or Thermogenin, Cannon et.al., 1981, 1982; Cannon and Nedergaard, 1983) which is unique to BAT, and results in the dissipation of the proton electrochemical gradient and the uncoupling of oxidative phosphorylation (Locke and Nicholls, 1981; Cannon et al., 1981, 1982). Consequently, energy released due to β-oxidation of fatty acids in BAT is in the form of heat (Nicholls, 1975, 1979; Cannon et al. 1978, 1982) for thermoregulation during cold exposure.

Foster and Frydman (1979) have provided quantitative estimations as to the possible contribution of NST and shivering in warm- and cold-acclimated rats. In the warm-acclimated rat exposed to severe cold (-19°C), 61% of the cardiac output (CO) is directed to tissues which produce heat by shivering or muscular work (ie. skeletal muscle, heart, ribcage and diaphragm) and only 29% is directed to BAT. However, in the cold-acclimated rat at -19°C, 61% of

the CO is directed to BAT and only 28% is directed to tissues involved in shivering. Although the contribution to heat production made by these various tissues cannot be calculated due to an absence of tissue-specific oxygen extraction coefficients, these data do indicate a shift from shivering thermogenesis in the warm-acclimated rat to NST in the cold-acclimated rat. Based on blood flow measurements, using radioactive microspheres, and the oxygen extraction coefficient for BAT, it was estimated that the contribution to NE- or cold-stimulated NST by BAT ranged between 60% (Foster and Frydman, 1978) and 80% (Foster and Frydman, 1979), respectively in the cold-acclimated rat. There is little question that in small rodents, NE-stimulated NST plays a paramount role in the maintenance of normal body temperature under seasonal cold exposure (Wickler, 1980, 1981; Bockler and Heldmaier, 1981; Feist and Morrison, 1982).

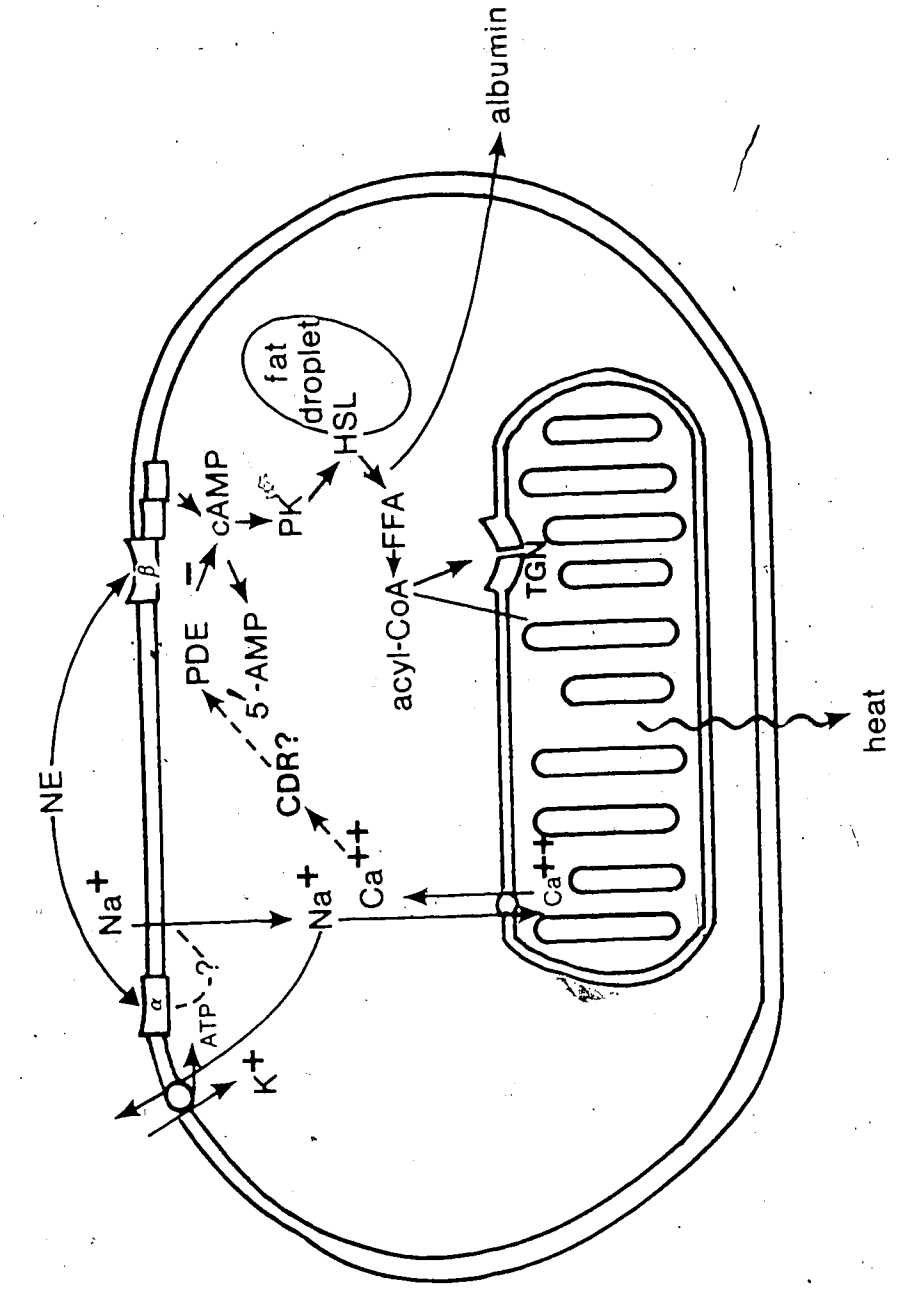
To ascertain that there is a decrease in the efficacy of adrenergic stimulation of thermogenesis after fasting, a dose-response study of NE-stimulated NST was carried out on cold-acclimated rats under fed and fasted conditions. Analysis of the pharmaco-kinetic parameters associated with this β-receptor assay should provide insights to any change in drug-receptor affinity and/or drug efficacy following fasting.

To further test whether the fasting-induced decrease in efficacy of NE-stimulated NST is a post-receptor event, due

to either a decreased production of cAMP and/or increased phosphodiesterase activity, aminophylline (AMPY) was used to enhance intracellular cAMP concentration. The subsequent influences on NE-stimulated NST were assessed in rats under fed, 1-day and 2-day fasted conditions. The 2-day fasted rats were used to provide preliminary information on the possibility of a progressive increase in phosphodiesterase activity with the prolongation of fasting.

A schematic representation illustrating the relationships between adrenergic receptors, cAMP production, phosphodiesterase action and substrate mobilization following NE stimulation is shown in Figure 1.

Figure 1. Schematic representation of β-adrenergic mediated heat production and a hypothetical α-adrenergic inhibition of heat production in the brown adipocyte. PK = protein kinase, PDE = phosphodiesterase, HSL = hormone-sensitive lipase, FFA = free fatty acids, CDR = calcium dependent regulator protein (calmodulin), TGN = thermogenin. Modified from Cannon et.al., 1981.


albumin
fat droplet
HSL
FFA
PK
cAMP
PDE
5'-AMP
CDR?
NE
Na+
Na+
Ca++
Ca++
ATP
?
K+
α
β
acyl-CoA
TG
heat

III. MATERIALS and METHODS

A. ANIMALS

Adult male Sprague-Daweley rats weighing 375-425 gm were used for all experiments. The rats were individually housed in shoe box type cages (45 x 25 x 20 cm) and acclimated to 5±1.5°C under 12/12 L-D photoperiod in a walk-in enviromental chamber for at least 3 weeks prior to experimentation. Food (Vitamite cubes, 25% protein, 5% fat, 6% fiber, salts and vitamins; gross energy approximately 4 $kcal(g)^{-1}$; North West Feeds, Edmonton) was rationed to maintain a constant body weight throughout the experimental period. Food was provided at 1530h daily, water was available at all times. For the experimental condition of "1-day fasted", the ration was removed overnight; for "2-day fasted", the ration was removed two days before the experiment; for the "well-fed" condition food was provided ad libitum overnight. Since the rats generally ate all of their ration within 2-3 h the 1-day fasted rats were approximately 36h and the 2-day fasted rats approximately 60h postprandial prior to experimentation. Each rat served as its own control under at least 2 of the feeding protocols; i.e. well-fed and 1 day fasted and/or 2 day fasted. A minimum of 7 days was allowed between successive experiments in the same rat.

B. CANNULATION

Since these experiments require a self-control design, it was thought necessary that the cannula used for NE infusion remain patent for at least 3 weeks. A great deal of time and effort was spent on this problem prior to arriving at the proper technique. This involved the placing of a silastic cannula (0.50mm ID, 0.92mm OD) 3 cm into the right carotid artery. The cannula was then drawn under the skin to the back of the head where it was exteriorized by passing it through a headset. The headset consisted of a 4 cm piece of 16 gauge stainless steel tubing bent to a J-shape. To this is attached (with dental acrylic) a 1 cm^2 piece of polyethylene mesh (1000 um mesh; Small Animal Parts, Miami, Florida). The headset was mounted loosely under the skin on top of the head where future tissue growth and the skin held it firmly in place. When not in use, the cannula was stored in a small pocket under the skin on the back of the neck and the headset capped with a small piece of tygon tubing, heat sealed at one end. This completely protected the cannula and yet allowed easy access to it by the investigator. The cannula was flushed with heparinized saline (100 $U(ml)^{-1}$) once every 3-4 days. Patency could be maintained for at least 3 weeks. Chronically cannulated rats were allowed to recover overnight at room temperature and then returned to the cold room for one week prior to experimentation. Due to problems associated with the

maintenance of the chronic cannula, such as the prevention of infection, etc., this technique was discontinued at the end of the NE dose-response study and an acute cannulation of the jugular vein was developed and tested.

Cannulation of the jugular vein involved inserting a polyethylene cannula (PE-10, 0.28mm ID, 0.61mm OD) approximately 3 cm into the right jugular near the heart. This cannula was removed at the end of each experiment. It was found that the right jugular could, with care, be cannulated this way 4-6 times without any complications. The effectiveness of the technique was tested gainst chronic cannulation of the jugular by infusing the rat with NE immediatly after cannulation (the acute response) and again one week later. The responses to the two infusions were essentially the same, indicating that the acute surgical procedure did not interfere with the rat's metabolic response to NE. This technique was, therefore, used in the aminophylline experiments.

For all cannulations, the surgical procedures were carried out while the rat was under halothane (Halocarbon, Ontario) anaesthesia (0.5-1.5%). Although strict aseptic procedures are not necessary for rats, every effort was made to ensure optimum aseptic conditions prevailed during surgery. Anti-biotics (Derapen-C; Ayerst, Montreal) were given intramuscularly (0.5ml) following surgery.

C. SEDATION of EXPERIMENTAL ANIMALS

In the present studies, it was desirable to reduce spontaneous activity so that small changes in metabolic response subsequent to NE or aminophylline administration could be discerned without being masked by activity-induced metabolic effects. This is particularly necessary in experiments involving aminophylline since methylxanthines are known to increase spontaneous activity in unanesthetized rats through central nervous system stimulation (Strubelt and Sieger, 1969). This enhanced spontaneous activity is depressed by barbital sedation. The peripheral effects of methylxanthines on cAMP accumulation, however, are still apparent (Foster et al., 1977). My initial attempts using pentobarbital (Somnotal; M.T.C. Pharmaceuticals, Hamilton) as a sedative were unsatisfactory due to its relatively short duration of action, particularly under the agumented metabolic state of NE stimulation. Although supplementary doses were given periodically, this tended to suppress respiration and therefore the response to NE. Furthermore, a stable, reproducible resting state was quite difficult to obtain with this drug. Subsequently, phenobarbital sodium (Abbott, Montreal) was chosen as it has essentially the same mode of action as pentobarbital but is longer lasting (Harvey, 1980). With the appropriate dose (50-100 $mg(kg)^{-1}$) the animal could be sedated to the point where spontaneous activity was essentially eliminated for the 8-10 h which

were required for each experiment, without any significant depression of respiration. It should be noted that while spontaneous activity was eliminated, the animal was quite awake and responded readily to toe pinching, noise and other external stimuli.

D. PROTOCOL

DOSE-RESPONSE NE

Overnight well-fed or fasted rats were sedated with phenobarbital one half hour before being placed in a plexiglass metabolic chamber. The chamber was equipped with a grid of copper tubing at the bottom so that cold or warm water could be circulated to maintain the animal's body temperature between 36-39°C. The metabolic chamber was housed in a constant temperature cabinet maintained at 25-27°C, within the thermal neutral zone of rats. A rectal thermocouple (30 ga copper-constantan) inserted to a depth of 5 cm and taped to the tail was used to monitor body temperature. Room air to the metabolic chamber was regulated at 1.5 $l(min)^{-1}$ (STP) by a Matheson electronic mass flow controller. The exhaust air from the animal chamber was analyzed for O_2 consumption (Applied Chemistry S-3 O_2 analyzer) and CO_2 production (Applied Chemistry CD-3 CO_2 analyzer). A computer based (MacSym 2) data acquisition system was used to calculate O_2 consumption, CO_2 production, R.Q. and for measurement of body and ambient temperatures. Heat production was calculated from O_2 consumption and R.Q. using Kleiber's (1975) equation:

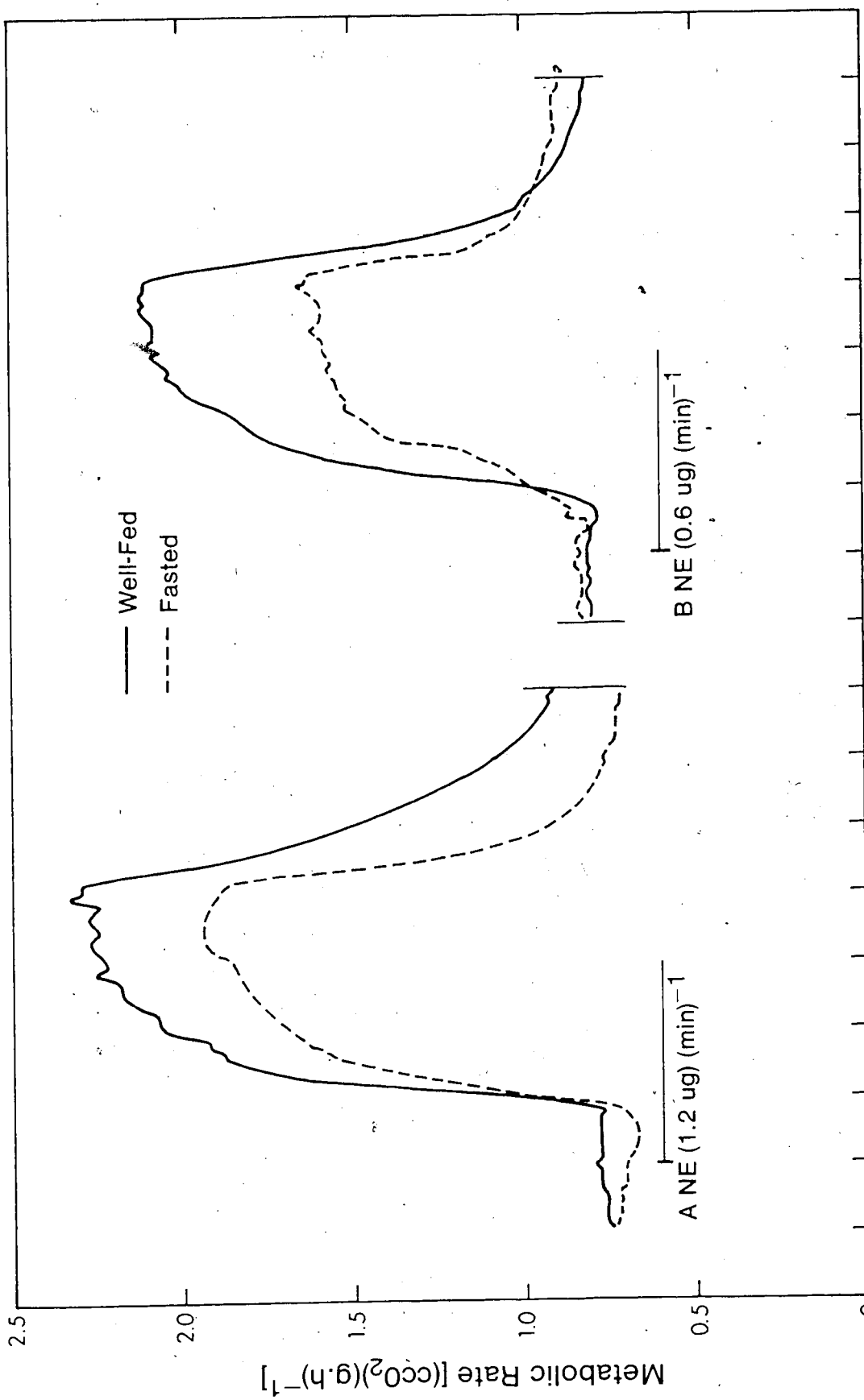
$$HP\ (Kcal) = VO2 \times [(87 + 27RQ)/22.4]$$

The animal was allowed to stabilize for 10-20 min before the first infusion of NE. Norepinephrine (Levoped, 1 mg base$(ml)^{-1}$) was diluted to a stock solution of 0.5 $mg(ml)^{-1}$ with sterile vehicle (Ascorbic acid (1 $mg(ml)^{-1}$, sterilized by filtration through a 0.22 um Millex-GS filter, Millipore Co., Bedford, Ma.) prior to each experiment. This stock was diluted to the appropriate infusion concentration just prior to the start of the infusion. A Gilson Minipuls 2 peristaltic pump was used to infuse NE at a constant rate of 0.5 $ml(h)^{-1}$. This infusion rate was verified daily prior to experimentation. Norepinephrine was infused at a rate of 0.30-4.0 $ug(min)^{-1}$ (i.e. 0.6-8.0 $ug(Kg^{.75} \cdot min)^{-1}$, based on a standard weight of 400 gm) for 30 min. Each infusion of NE was preceded and followed by 10 min infusions of vehicle and a 30 min rest period. During any one experiment each rat received a maximum of five doses of NE (0.30, 0.60, 1.2, 2.4 and 3.2 or 4.0 $ug(min)^{-1}$). Since a similar minimum metabolic rate was always attained between successive NE infusions (Fig. 2), it is indicative that little if any accumulative effects of NE on metabolism results from this protocol.

AMINOPHYLLINE (AMPY)

The protocol for the AMPY experiments is essentially the same as that for the NE dose-response experiments. Briefly, overnight well-fed, 1 day fasted or 2 day fasted rats were cannulated in the right jugular as well as in the

Figure 2. Typical time course of metabolic response to two doses of norepinephrine for both well-fed and fasted states in the same rat. Black line under the curves indicate the duration of NE infusion. Ambient temperature = 26±1°C


Metabolic Rate [(ccO_2)(g.h)$^{-1}$]
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140
160
Time (min)
Well-Fed
Fasted
A NE (1.2 ug) (min)$^{-1}$
B NE (0.6 ug) (min)$^{-1}$

peritoneal cavity just prior to experimentation. The peritoneal cannula facilitated the injection of AMPY without disturbing the animal. Following cannulation the rat was given an i.p. injection of phenobarbital and placed in the metabolic chamber. Once the animal had stabilized (20-30 min) the first of 2 infusions of NE (0.8 or 1.6 ug(min)$^{-1}$) was started. NE was infused for 40-60 min or until a stable plateau had been reached. The rat was then allowed to rest for 1 h before starting the secound infusion. Ten min prior to the start of the second NE infusion each rat recieved (through the i.p. cannula) an injection of AMPY (Abbott, Montreal; 15 or 20 mg(rat)$^{-1}$). This procedure allowed both the effect of AMPY (Foster et al., 1977) and NE (personal observation) to peak at about the same time. Due to the duration (3-5 h; Foster et al., 1977) of AMPY's effect on the animal only these two infusions were used in any one experiment. Data collection and evaluation were as described for NE dose-response studies.

E. DATA ANALYSIS

Since the weight of all animals was maintained constant at approximately 400 gm throughout the experimental period, drug doses were calculated and expressed as total drug per animal (AMPY) or per min (NE). Experimental results are expressed as weight-specific metabolic rates based on $W^{.75}$ (W in Kg) (Kleiber, 1975) to reflect the physiologically relevant metabolic size. Maximum heat production was calculated from the 10 min period which exibited the highest total VO_2 during NE infusion. Total heat production was calculated using 4 ten minute periods, starting with minute 11 and ending with minute 50. Statistical analysis was by the Wilcoxons's Signed Rank Test and Students t test (Snedecor and Cochran, 1968; Zar, 1974). Significance was at $p<0.05$, unless otherwise stated.

IV. RESULTS

A. DOSE-RESPONSE NE

All rats in both states (well-fed and fasted) exhibited the typical dose-response relationship between log dose of NE and heat production. Increasing doses of NE elicited greater metabolic responses until a maximum was reached (Fig. 3). Figure 4 shows the log dose-response relationship of an individual to two control (well-fed) and one fasted trials. The minor difference between the two well-fed trials indicates that there is little, if any, anticipatory response to repeated exposure to this experimental protocol. The mean maximum heat production in well-fed and fasted states in response to NE is summarized in Figure 3 and Table 1.

The mean resting metabolic rates for rats in the well-fed and 1-day fasted states (2.01±0.16 and 1.81±0.04 Kcal($Kg^{.75} \cdot h)^{-1}$, respectively) were not significantly different. In both states, the metabolic responses to all doses of NE were significantly greater than the resting values. Within each state, there was no significant difference in heat production at doses of 2.4 $ug(min)^{-1}$ or higher indicating a maximal plateau had been reached. In the fasted state, the metabolic response was significantly less than that in the well-fed state at all doses (Fig. 3). The difference between the maximum responses of well-fed and

Figure 3. Dose-response relationship between log dose NE and maximum NE-stimulated heat production. Means±1 SE for both well-fed and 1-day fasted states. The number of individuals used to obtain each mean are shown in parentheses at each point.

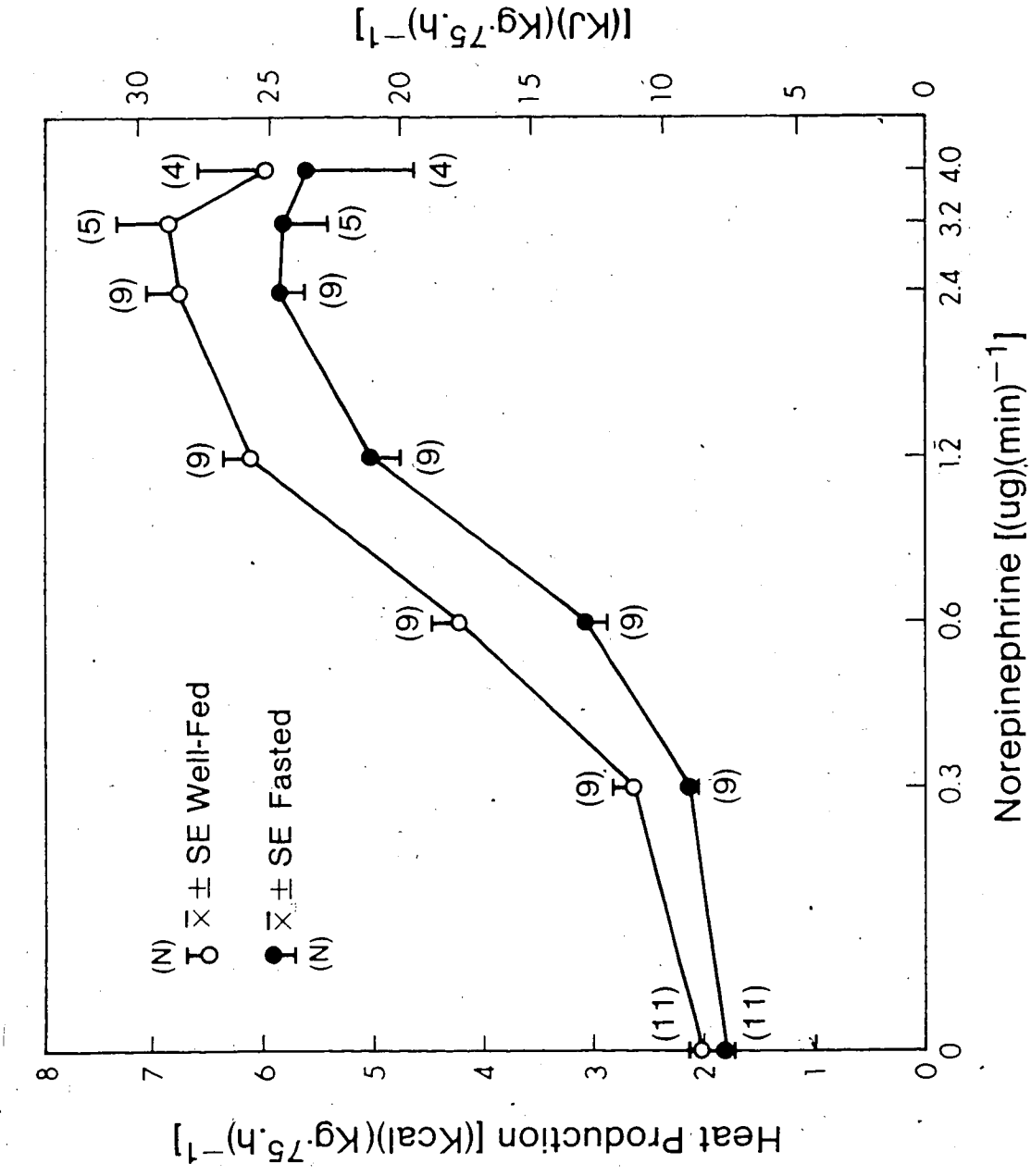

Heat Production [(Kcal)(Kg·75.h)⁻¹]
[(KJ)(Kg·75.h)⁻¹]
Norepinephrine [(ug)(min)⁻¹]
0
0.3
0.6
1.2
2.4
3.2
4.0
1
2
3
4
5
6
7
8
5
10
15
20
25
30
(11)
(11)
(9)
(9)
(6)
(6)
(9)
(9)
(6)
(6)
(9)
(9)
(5)
(5)
(4)
(4)
(N) ō x̄ ± SE Well-Fed
(N) ● x̄ ± SE Fasted

TABLE I

Mean maximum heat production of rats to different doses of norepinephrine. Means±1SE are indicated in Kcal$(Kg^{.75} \cdot h)^{-1}$. Number of measurements used to determine the means are indicated in parentheses. (WF = well-fed, F1D = fasted 1-day)

Dose NE ug$(min)^{-1}$	State	
	WF	F1D
Resting HP	2.02±.05 (11)	1.81±.04 (11)
0.3	2.65±.16 (9)	2.14±.05 (9)
0.6	4.22±.26 (9)	3.09±.18 (9)
1.2	6.15±.19 (9)	5.02±.26 (9)
2.4	6.80±.25 (9)	5.88±.20 (9)
3.2	6.87±.47 (5)	5.81±.39 (5)
4.0	5.99±.31 (4)	5.62±.49 (4)

1-day fasted states at a dose of 3.2 ug(min)$^{-1}$ was 18.2% (6.87±.47 vs 5.81±.39 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$).

The Ke, which is the dose at 1/2 maximum response, was not significantly different (Ke well-fed = 0.43, 1-day fasted = 0.55 ug(min)$^{-1}$) between the two states (Fig. 3).

In 2-day fasted rats the maximum metabolic response (4.59±0.14 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$), which could be elicited by a slightly lower dose of NE (1.6 ug(min)$^{-1}$), was significantly lower than that of 1-day fasted rats (5.32± 0.11 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$) at the same dose (Fig. 5).

B. AMINOPHYLLINE

The maximum and total heat production for well-fed rats with NE alone at 0.8 ug(min)$^{-1}$ was 4.70±0.21 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$ and 2.68±0.13 Kcal(Kg$\cdot^{75}\cdot$40min)$^{-1}$ respectively, for the 1-day fasted rats these values were 3.91±0.11 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$ and 2.22 ±0.07 Kcal(Kg$\cdot^{75}\cdot$40min)$^{-1}$. At 1.6 ug(min)$^{-1}$ the maximum and total heat production values were 6.07±0.10 and 5.32±0.11 Kcal(Kg$\cdot^{75}\cdot$h)$^{-1}$ and 3.15±0.06 and 3.10±0.06 Kcal(Kg$\cdot^{75}\cdot$40min)$^{-1}$ for well-fed and 1-day fasted rats respectively (Fig. 5). These values which were obtained using acute cannulation techniques (see methods) were comparable to those obtained under similar dosages but using a chronical cannulation preparation as described in the NE dose response study.

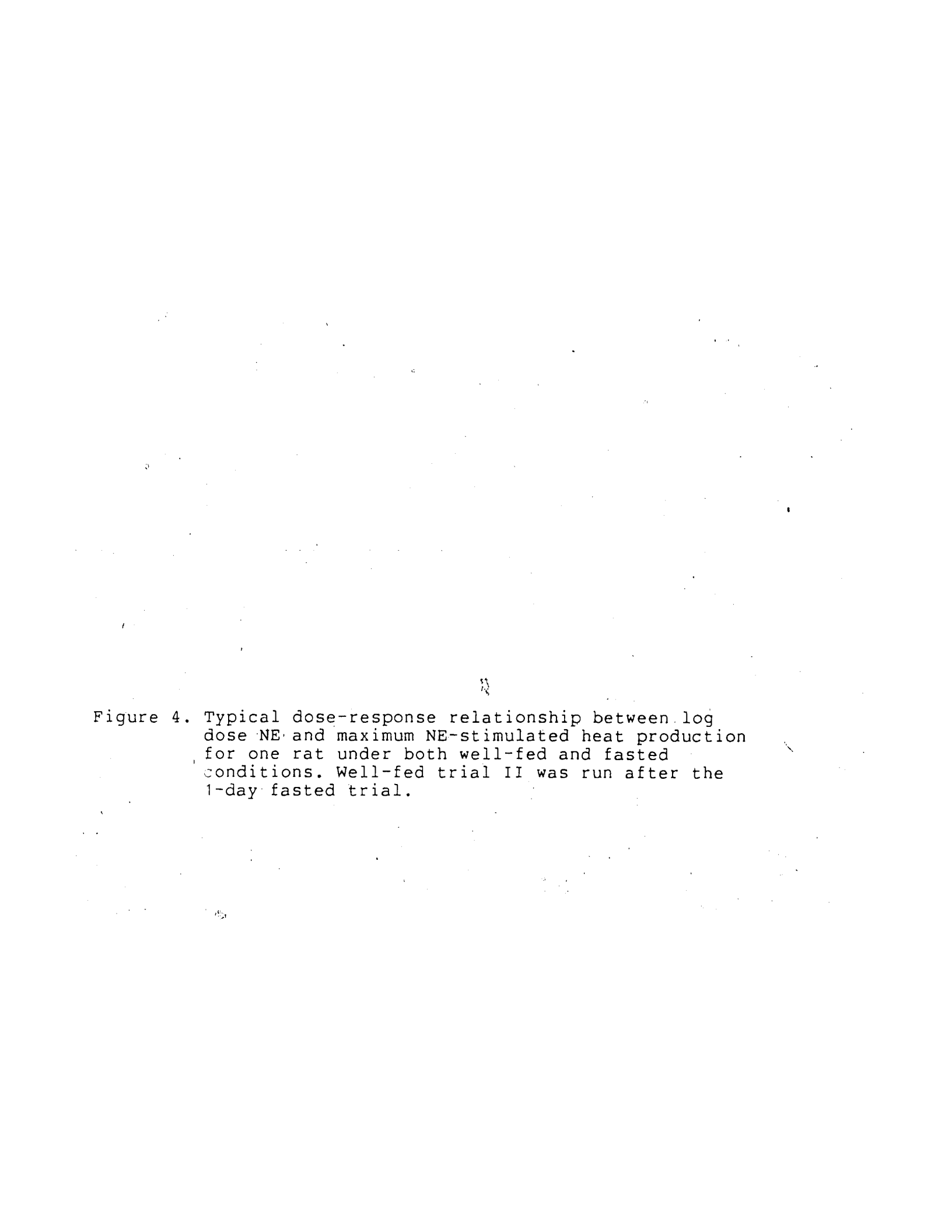
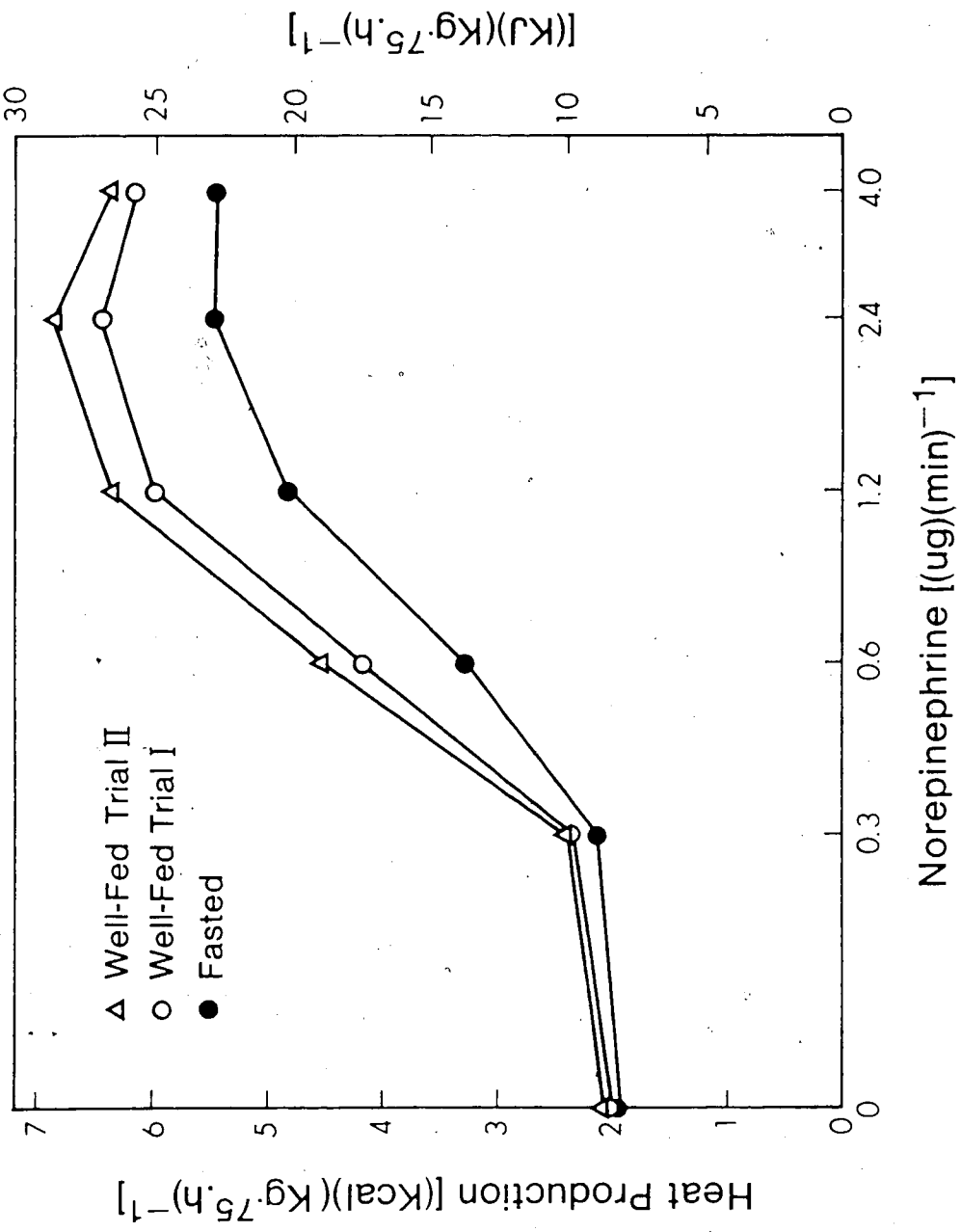
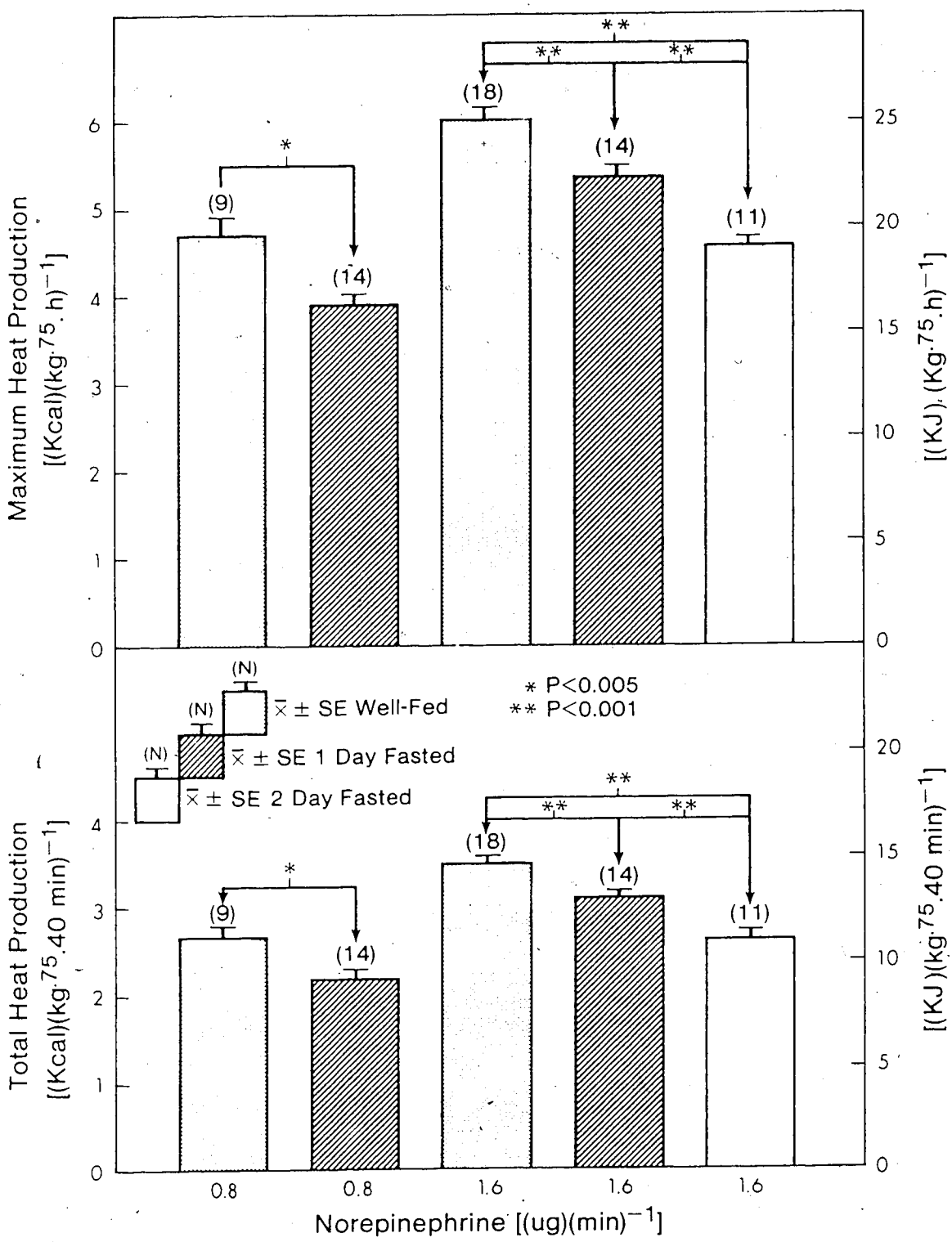


Figure 4. Typical dose-response relationship between log dose NE and maximum NE-stimulated heat production for one rat under both well-fed and fasted conditions. Well-fed trial II was run after the 1-day fasted trial.


Heat Production [(Kcal)(Kg·75.h)⁻¹]
[(KJ)(Kg·75.h)⁻¹]
Norepinephrine [(ug)(min)⁻¹]
Δ Well-Fed Trial II
○ Well-Fed Trial I
● Fasted
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
0.3
0.6
1.2
2.4
4.0

The typical metabolic responses of an individual to NE and NE with AMPY pretreatment are shown in Fig. 6. The metabolic response to NE after AMPY pretreatment was significantly greater under all feeding conditions (Figs. 7 & 8). In well fed animals, AMPY pretreatment (15-20 mg) produced a 9% increase in NE-stimulated heat production (Fig. 9). In 1-day fasted animals the increase after AMPY was 10% (Fig. 9). This 10% increase elevated maximum and total NE-stimulated heat production to levels which were not significantly different from those of well-fed animals receiving NE alone (Figs. 7 & 8). In 2-day fasted animals the NE-stimulated increase after AMPY was 18% (Fig. 9) which is twice the increase observed in well-fed rats treated with AMPY. This increase elevated heat production to levels which were not significantly different from those of 1-day fasted rats treated with AMPY (Figs. 7 & 8).

Figure 5. Effects of norepinephrine on maximum and total thermogenesis in well-fed, 1-day fasted and 2-day fasted rats. Means±1 SE are represented. The number of animals used to determine the means are shown in parentheses.


Maximum Heat Production
[(Kcal)(kg·75 .h)−1]
[(KJ). (Kg.75.h)−1]
Total Heat Production
[(Kcal)(kg·75.40 min)−1]
[(KJ)(kg.75.40 min)−1]
(N) X̄ ± SE Well-Fed
(N) X̄ ± SE 1 Day Fasted
(N) X̄ ± SE 2 Day Fasted
* P<0.005
** P<0.001
(9)
(14)
(18)
(14)
(11)
0.8
0.8
1.6
1.6
1.6
Norepinephrine [(ug)(min)−1]

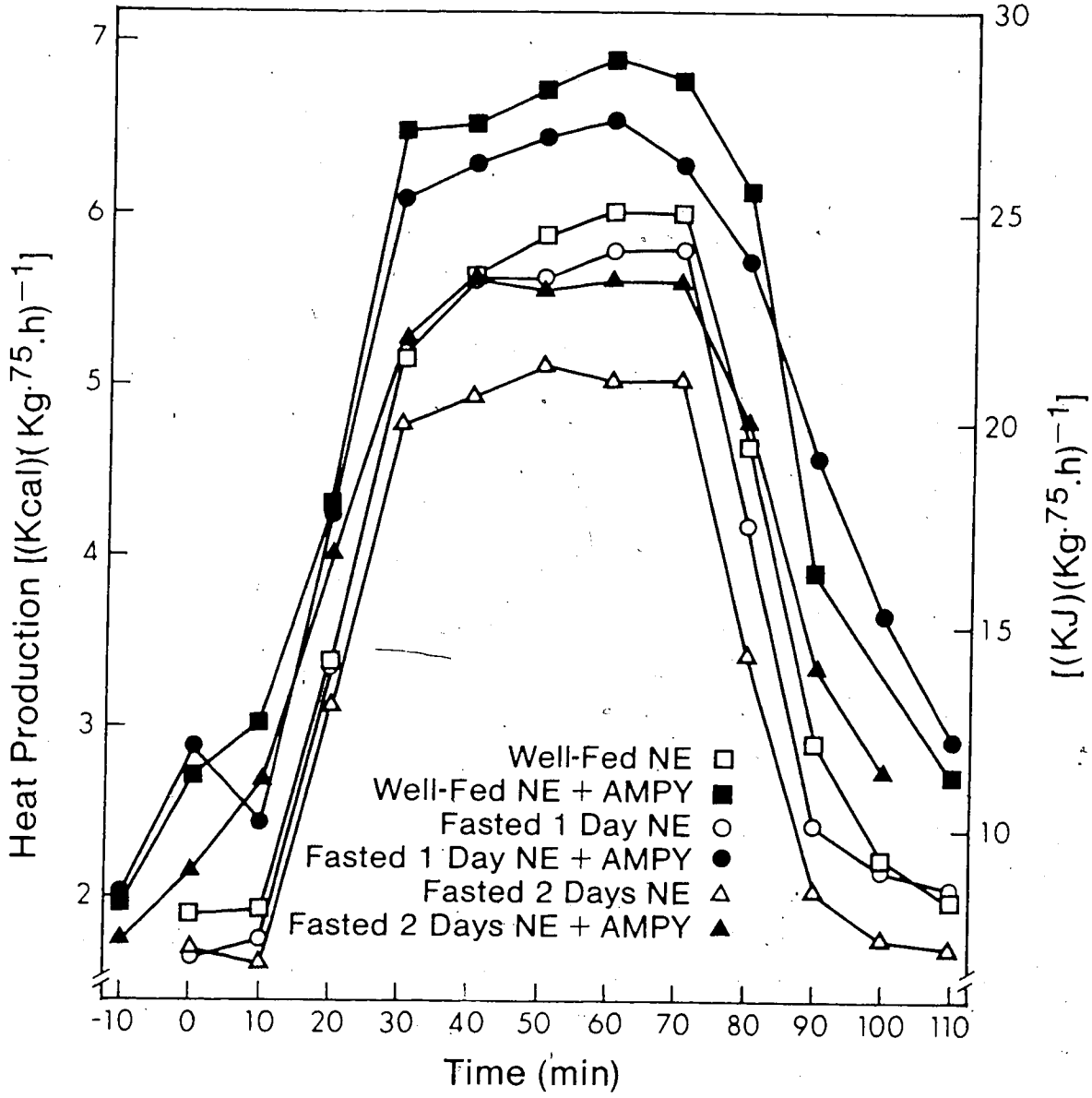
V. DISCUSSION

A. DOSE-RESPONSE NE

The concept of drugs interacting with specific receptors to exert their actions is well established in pharmacology. The first attempt to quantify the relationship between drug dose and response using this concept is credited to Clark in 1937. Presently, the magnitude of response is thought to be a function of 2 properties: 1) the affinity of the drug for the receptor and 2) the efficacy of the drug, a term which describes the biological activity of the drug and includes changes in receptor numbers as well as biochemical changes at post receptor sites (Levine, 1973; Goldstein, 1974).

The validity of using NE-stimulated thermogenesis as an in vivo dose-response assay of adrenergic receptor activity in the present study was based on the study of Depocas et.al.(1978). They found that the concentrations of NE which stimulated thermogenesis in vivo were in the same range (10^{-9} to 10^{-7}M) as those reported (Seydoux and Girardier, 1977) to stimulate O_2 consumption in isolated brown adipocytes. Further, Depocas et al.(1980), studying NE-stimulated NST in cold-acclimated rats in vivo, and Bukowieki et al.(1980), studying β-receptor mediated heat production of brown adipocytes from cold-acclimated rats in vitro, demonstrated that the concentration of NE required

Figure 6. Typical metabolic responses of an individual to norepinephrine (NE, 1.6ug(min)$^{-1}$) and norepinephrine after aminophylline (AMPY, 15mg) pretreatment under well-fed, 1-day fasted and 2-day fasted conditions. The duration of NE infusion was 60 min beginning at time 0. Aminophylline was injected i.p. 10 min prior to the start of NE infusion.

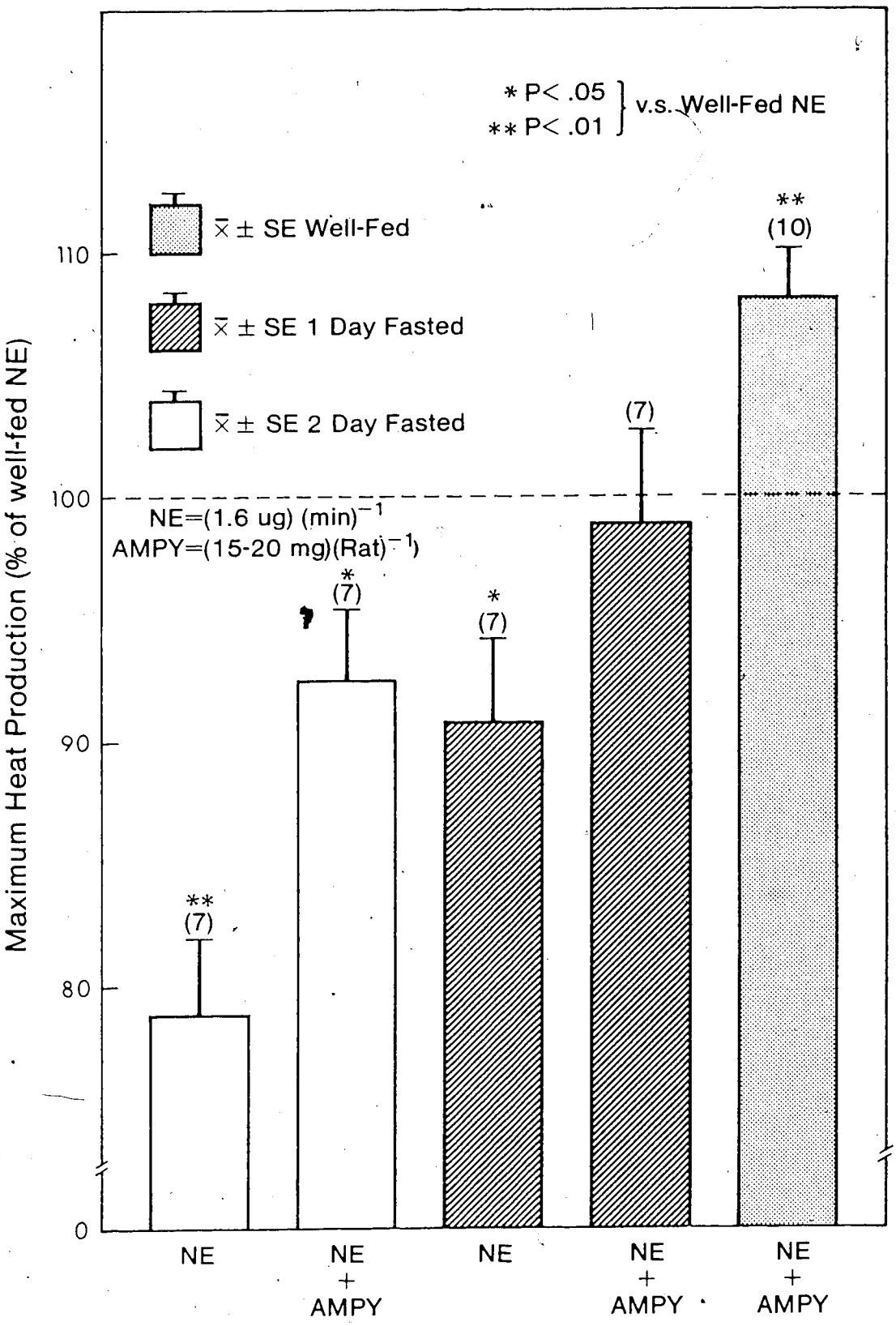

Heat Production [(Kcal)(Kg·75.h)−1]
[(KJ)(Kg·75.h)−1]
Time (min)
Well-Fed NE
Well-Fed NE + AMPY
Fasted 1 Day NE
Fasted 1 Day NE + AMPY
Fasted 2 Days NE
Fasted 2 Days NE + AMPY
2
3
4
5
6
7
10
15
20
25
30
-10
0
10
20
30
40
50
60
70
80
90
100
110

for 1/2 maximal stimulation in vivo was the same as for 1/2 maximal stimulation in vitro. Since NE-stimulated heat production in brown adipocytes is β-adrenergic receptor mediated (Bukowieki et al., 1980; Cannon et al., 1983), and in cold-acclimated rats more than 80% of NST is due to brown adipocyte metabolism (Foster and Frydman, 1979), the in vivo assay of NE-stimulated NST employed in the present study is therefore an estimation of β-adrenergic receptor activity of brown adipocytes.

It is well known that changes in drug-receptor binding affinity manifest themselves by shifts of the dose-response curve to the right or left (Endrenyi, 1975; Levine, 1973; Goldstein et al., 1974). The results of this study show only a slight but non-significant shift of this type after fasting (Fig. 3). Therefore, the observed changes in the dose-response relationships do not appear to suggest any changes in affinity associated with fasting. This is in concert with in vitro studies on both hepatocytes and white adipocytes, in which no change in β-adrenergic receptor affinity was observed after fasting Guidicelli et al., 1981; El-Refai and Chan, 1981).

Interpretation of the reduced response of NE-stimulated heat production in fasted rats as due to reduced receptor numbers is contradicted by in vitro studies. Beta-receptor binding studies on white adipocyte membrane fractions (Guidicelli et al., 1981) and isolated hepatocytes (El-Refai and Chan, 1982) from 72 and 24 h fasted rats show a doubling

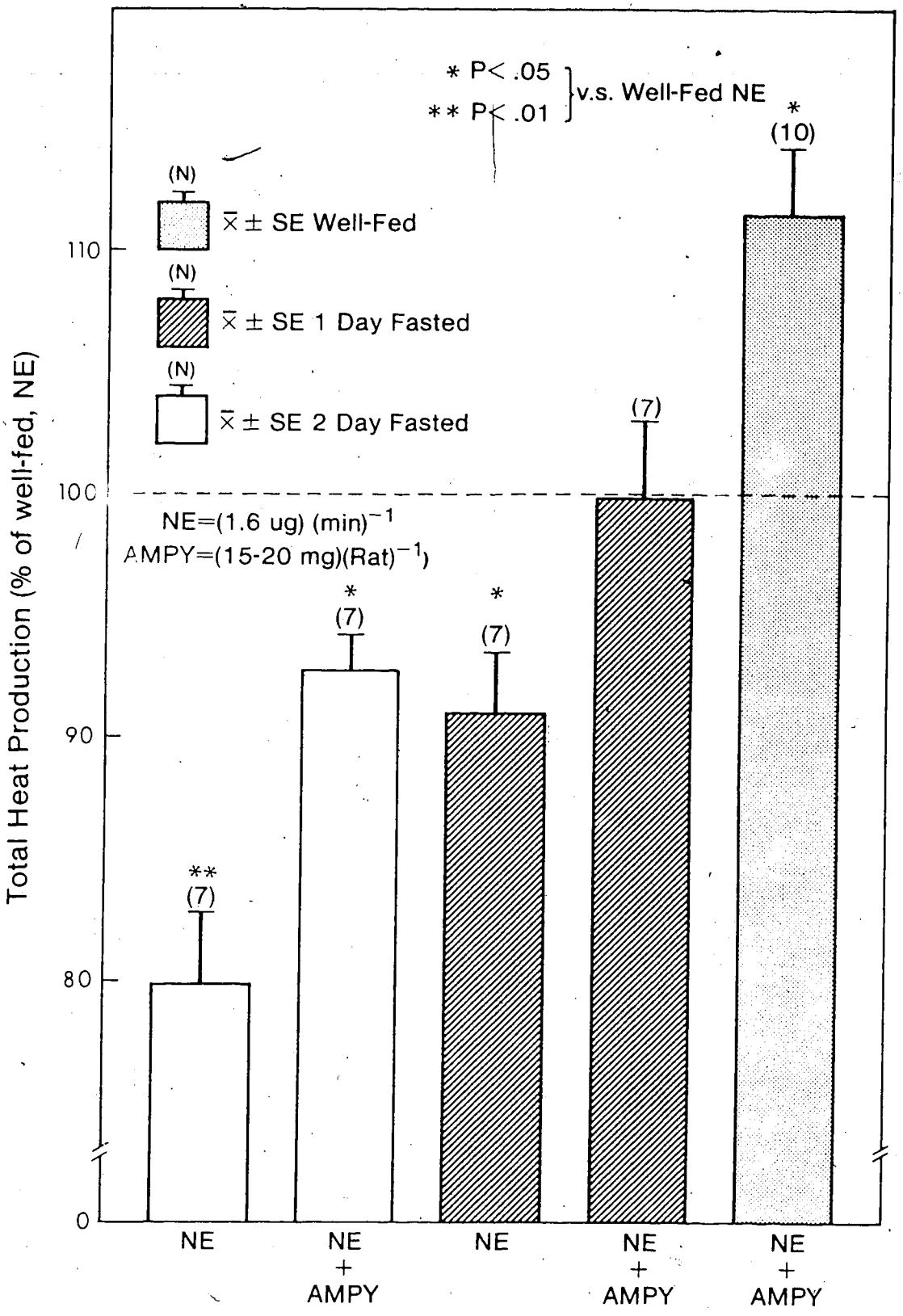
Figure 7. Effects of norepinephrine (NE, 1.6ug(min)$^{-1}$) and NE after aminophylline (AMPY, 15mg) pretreatment (NE+AMPY) on maximum thermogenesis in well-fed, 1-day fasted and 2-day fasted rats. Data are presented as percent of the well-fed NE alone value (horizontal dashed line) in the same rat. The number of animals used to determine each mean is given in parentheses.


* P< .05
** P< .01
v.s. Well-Fed NE
x̄ ± SE Well-Fed
x̄ ± SE 1 Day Fasted
x̄ ± SE 2 Day Fasted
NE=(1.6 ug) (min)⁻¹
AMPY=(15-20 mg)(Rat)⁻¹
Maximum Heat Production (% of well-fed NE)
110
100
90
80
0
**
(7)
*
(7)
*
(7)
(7)
**
(10)
NE
NE + AMPY
NE
NE + AMPY
NE + AMPY

of β-receptor numbers. Dax et al.(1981) on the other hand report no change in the β-receptor parameters, numbers or affinity, of white adipocyte membrane fractions from their 72 h fasted rats. Taken together, it appears that the observed decrease in the efficacy of NE in stimulating heat production in the fasted rat is not due to a reduction of β-receptor numbers, but possibly due to biochemical changes at post receptor sites. The reduction of cAMP production by the adenylate cyclase system also does not appear to be a likely event since Guidicelli et al.(1981) and Dax et al.(1981) report an increase in both adenylate cyclase activity and sensitivity to adrenergic stimulation after fasting. Isoproterenol-stimulated lipolysis, however, is severely depressed (Guidicelli et.al., 1981). Thus, in spite of increases in β-receptor numbers and adenylate cyclase activity after fasting, lipolysis (Guidicelli et al., 1981), cold- (Wang, 1980) and NE-stimulated thermogenesis are depressed.

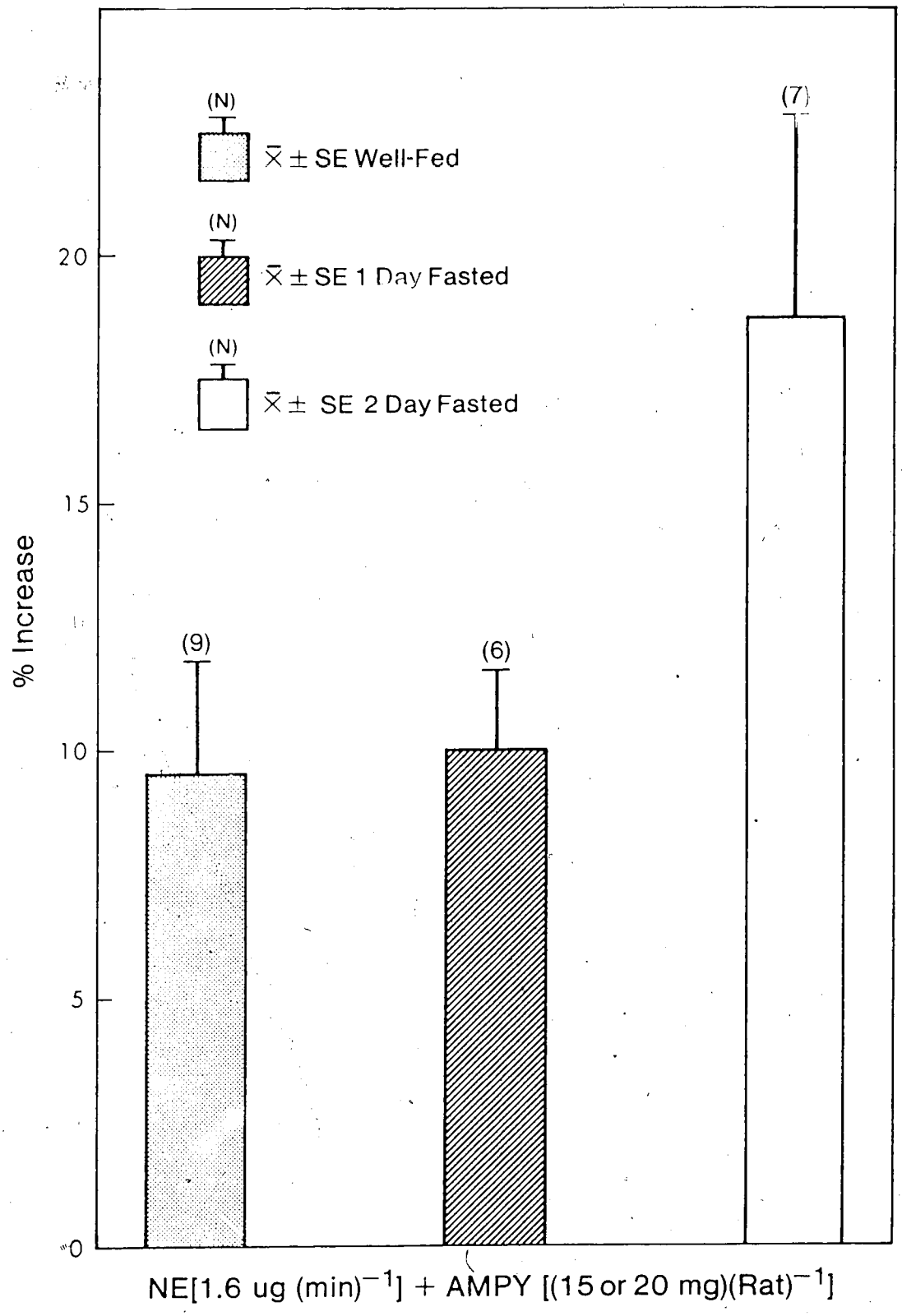
Since NE has almost equal affinity for α- and β-adrenergic receptors, and α-adrenergic receptors are present in both white (Lifkowitz and Hoffman, 1980; Exton, 1982) and brown (Svartengren et al., 1980) adipocytes, it is possible that, as Burns et al.(1979) suggested, there is a shift in the ratio of α- to β-receptor numbers associated with fasting. While both subtypes of α-receptors have been demonstrated in adipose tissue (Exton, 1982) the α_2-receptor does not appear to be a likely site for the fasting

Figure 8. Effects of norepinephrine (NE, 1.6ug(min)$^{-1}$) and NE after aminophylline (AMPY, 15mg) pretreatment (NE+AMPY) on total thermogenesis (total heat production for 40 min) in well-fed, 1-day fasted and 2-day fasted rats. Data are presented as percent of well-fed NE alone (horizontal dashed line) in the same rat. The number of animals used to determine the means is shown in parentheses.


* P< .05
** P< .01
v.s. Well-Fed NE
(N)
x̄ ± SE Well-Fed
(N)
x̄ ± SE 1 Day Fasted
(N)
x̄ ± SE 2 Day Fasted
NE=(1.6 ug) (min)⁻¹
AMPY=(15-20 mg)(Rat)⁻¹
Total Heat Production (% of well-fed, NE)
110
100
90
80
0
**
(7)
*
(7)
*
(7)
(7)
*
(10)
NE
NE + AMPY
NE
NE + AMPY
NE + AMPY

depression of thermogenesis as its antagonism of lipolysis is expressed through inhibition of adenylate cyclase and adenylate cyclase activity has been shown to increase after fasting (Dax et al., 1981; Guidicelli et al., 1981). Alpha$_1$-adrenergic activation, on the other hand, appears to involve an increase in membrane permeability (Horowitz et al., 1971; Exton, 1982; Cannon et al., 1981) resulting in an increase in cytosolic Na^+(Fig. 1). Si[illegible] Na^+ promotes the release of mitochondrial Ca^{++} (Nedergaard et al., 1979; Nedergaard and Cannon, 1980) an increase in cytosolic Ca^{++} concentration results. The activity of several enzymes has been shown to be regulated by intracellular Ca^{++}. One such enzyme is the membrane-bound phosphodiesterase (MPDE). Clayberger et al.(1981) demonstrated that depletion of intracellular Ca^{++} prevents the activation of MPDE brought about by chronic (1h) isoproterenol treatment. The activation of MPDE appears to be mediated by translocation of a calcium-dependent regulator protein (calmodulin) from the cytosol to the plasma membrane (Clayberger et. al., 1981). That this enzyme (MPDE) is an important regulator of cAMP metabolism is demonstrated by the fact that incubation of isolated, intact rat erythrocytes with isoproterenol and the phosphodiesterase inhibitor Ro24-1724 elicited a sevenfold increase in cAMP concentration, whereas incubation with isoproterenol alone yielded only a three to fourfold increase (Clayberger et al., 1981). Therefore, since aminophylline enhances maximum thermogenesis in fasted rats

Figure 9. Effects of aminophylline (AMPY) on norepinephrine (NE)-stimulated heat production in well-fed, 1-day fasted and 2-day fasted rats. Data are presented as percent increase over NE alone in the same rat. The number of animals used to determine the means is shown in parentheses.


(N)
$\bar{X} \pm$ SE Well-Fed
(N)
$\bar{X} \pm$ SE 1 Day Fasted
(N)
$\bar{X} \pm$ SE 2 Day Fasted
% Increase
0
5
10
15
20
(9)
(6)
(7)
NE[1.6 ug (min)$^{-1}$] + AMPY [(15 or 20 mg)(Rat)$^{-1}$]

(Wang, 1981, 1982), it is possible that phosphodiesterase activity is increased after fasting, possibly through increased α_1-adrenergic activity after fasting. Further, since fasting-depressed maximum thermogenesis can be restored to the fed level by aminophylline, possibly through enhanced intracellular cAMP concentration, it is evident that other biochemical mechanisms which are involved with substrate mobilization and heat production but are downstream from cAMP production (eg. protein kinases, etc.) are not directly affected by fasting. However, speculation such as this based on indirect evidence is at best inconclusive. Further studies are required to elucidate the changes responsible for the observed depression of NE-stimulated thermogenesis after fasting.

B. AMINOPHYLLINE

The methylxanthines are alkaloid derivatives of dioxypurine (Xanthine) and are structurally related to uric acid. These compounds have a low solubility which is enhanced by complexing them with salts and various other compounds. The most notable and widely used complex is that of theophylline (1,3-dimethyl-xanthine, 85%) and ethylenediamine (15%), (aminophylline). The general pharmacological properties of the methylxanthines include CNS and cardiac muscle stimulation, stimulation of diuresis

in the kidney (Rall, 1980; Goldstein et al., 1974) and relaxation of smooth muscle, most notably in the bronchus (Swinyard, 1980).

The actions of methylxanthines on the CNS and cardiovascular system may not be due to direct activity of the xanthines but rather as an indirect result of increased monamine turnover (Karasawa et al., 1976). Much of the CNS activity of xanthines is inhibited by sedatives such as the barbiturates, which inhibit turnover of dopamine, NE and serotonin. Leduc (1974), using unanesthetized, cold-acclimated rats, found that theophylline (>50mg$(Kg)^{-1}$) increased the metabolic rate about as much as did NE. However, Foster et al.(1977), using barbital-sedated cold-acclimated rats, found theophylline (100 mg$(Kg)^{-1}$) was less than half as effective as an optimum dose of NE in stimulating thermogenesis. Preliminary work in this study in which we found little or no calorigenic response to AMPY, at doses up to 100 mg$(Kg)^{-1}$ i.v. or i.p., in phenobarbital sedated cold-acclimated rats is in concert with this observation. This seems to suggest that the greater calorigenic effect of theophylline in the unanesthetized animal is due to centrally stimulated enhanced motility as suggested by Strubelt and Siegers (1969). Since barbital sedation effectively eliminates the central effects of theophylline on spontaneous activity, use of sedated animals should accurately reflect the peripheral effects of theophylline.

Pharmaceutically Aminophylline has been used as a bronchodilator because of its' smooth muscle relaxing effects. It is doubtful, however, that the observed stimulatory effects on thermogenesis are due to bronchodilation, as isoproterenol and epinephrine which are both potent bronchodilators do not elicit a similar thermogenic response (Wang and Anholt, 1982). Therefore, it seems likely that the observed increase in NE-stimulated thermogenesis, after pretreatment with AMPY, is due to AMPY's effects on substrate mobilization through action on cAMP metabolism.

All actions of the methylxanthines appear to be underlain by three basic cellular actions. These are: 1) increased translocation of intracellular calcium. 2) increased intracellular cyclic AMP. 3) blockade of adenosine receptors. The primary importance of the actions described in 1) above appear to be in the actions of methylxanthines on skeletal and cardiac muscle, where the permeability of the endoplasmic reticulum to ca^{++} is increased (Bianchi, 1968). Thus, at high concentrations, caffeine, another common methylxanthine, increases the twitch response to motor nerve stimulation and at very high concentrations can produce contraction without nerve stimulation. Theophylline is reported to have low potency in this area (Axelsson and Thesleff, 1958; Bianchi, 1975), and therefore, this action on muscle is not likely to play an important role in the present studies. The actions listed in 2) and 3) above

appear to be more relevant to the work at hand. Both actions result generally in an increase in intracellular cAMP concentration, one through the inhibition of cAMP degradation by blocking phosphodiesterase activity and the other through the blockage of adenosine's inhibition of cAMP production (a general but not universal action of adenosine, Rall, 1980). Since the intracellular concentration of metabolically active cAMP underlies the control of glucose (Exton et al., 1972) and free fatty acid (Carlson and Butcher, 1972) mobilization and since the depressed efficacy of a β-adrenergic agonist (NE), which acts through the stimulation of cAMP production, can be restored by pretreatment with AMPY (Figs. 7 & 8), it seems plausible that reduced intracellular cAMP concentration is responsible for the observed depression in maximum heat production after fasting.

Foster et al.(1977) showed that doses of NE and theophylline which stimulate calorigenesis result in changes in plasma cAMP which are directionally correlated with changes in calorigenesis. Further, these changes in plasma cAMP levels corresponded with the established effects of theophylline on increasing intracellular cAMP (Foster et al., 1977; Robison et al., 1971a). Adenosine at micromolar concentrations has been shown to suppress the cAMP accumulation due to NE in isolated fat cells (Fain, 1973); enzymatic removal of adenosine with adenosine deaminase markedly potentiates the sensitivity of cAMP accumulation

and lipolysis to NE (Fain and Weiser, 1975). However, this adenosine-regulated change in NE sensitivity appears to be mediated through regulation of adenylate cyclase activity (Fain and Malbon, 1979). Since the activity and sensitivity of adenylate cyclase to NE have been shown to increase rather than decrease after fasting (Guidicelli et al., 1981; Dax et al., 1981), the observed depression of NE-stimulated thermogenesis after fasting does not appear to be due to alterations in the adenosine regulated adenylate cyclase locus.

Phosphodiesterase (PDE) is present in cells in two primary forms: low Km membrane bound PDE (MPDE) and high Km soluable PDE (SPDE) (Van Inwegen et al., 1975). Of these two forms MPDE appears to be the primary regulator of intracellular cAMP concentration (Clayberger et al., 1981; Morgan et al., 1982; Van Inwegen et al., 1975). Lipson et al.(1979) and Capito and Hedeskov (1974) report no change in PDE activity in unstimulated isolated islets from fasted rats and mice. Capito and Hedeskov (1974), however, show a 400% increase in the concentration of cAMP in pancreatic islets from fasted mice as compared to a 250% increase in islets from fed mice, when the islets were incubated with glucose and 3-isobutyl-1-methylxanthine (the most potent of the methylxanthines; Beavo, 1970). Clayberger et al.(1981) demonstrated a 3-fold increase in the Vmax of MPDE from isoproterenol-stimulated erythrocytes, over control (unstimulated) cells. Two points seem clear from these

studies: a) the importance of using an activated (stimulated) system for PDE activity studies, as differences in the active state may not be apparent in the resting state; and b) fasting appears to increase PDE activity since a greater increase in cAMP is observed after inhibition of PDE. These points are consistent with the observations that basal lipolysis is unchanged, but under adrenergic stimulation, lipolysis (Guidicelli et al., 1981) and maximum thermogenesis (Wang, 1978) are significantly depressed after fasting. Methylxanthines have been shown to cross cell membranes readily (Bianchi, 1962). A close correlation between their PDE inhibitory activities and their enhancement of lipolytic activities has also been demonstrated (Beavo, 1970). In view of the significant increases in both cold-stimulated (Wang, 1982) and NE-stimulated heat production after pretreatment with AMPY (Figs. 7 & 8) and the apparent increase in sensitivity to AMPY in 2-day fasted rats (Fig. 9), it seems likely that the observed depression of maximum thermogenesis after fasting could be due to increased PDE activity. Again, conclusions based on indirect evidence are at best tentative and further studies at the biochemical level are necessary before the mechanisms underlying fasting-depressed maximum thermogenesis can be fully elucidated.

VI. CONCLUSIONS AND PERSPECTIVES

The current results indicate a significant depression of NE-stimulated heat production after overnight fasting. This is in concert with previous observations that fasting depresses maximum thermogenesis in severe cold (Wang, 1978).

The lack of difference in Ke's between the fed and fasted states (Fig. 3) suggests that fasting does not affect the NE-receptor binding affinity but decreases the efficacy of NE in stimulating heat production. This interpretation is supported by available in vitro β-adrenergic and adenylate cyclase studies using white adipocytes from 72-h fasted rats. Guidicelli et al.(1981) report an increase in β-receptor number and adenylate cyclase activity, but no change in Ke, in membrane fractions treated with isoproterenol. Isoproterenol-stimulated lipolysis, however, was severely depressed. These observations indicate a decreased efficacy of isoproterenol on lipolysis in spite of the increase in β-receptor numbers and unchanged drug-receptor binding affinity. Although the mechanisms remain unknown, it is possible that the phosphodiesterase (PDE) activity may be increased by fasting.

The enzyme, PDE, particularly the membrane bound form (MPDE) has been shown to be an important factor in the control of cellular cAMP concentration (Clayberger et al., 1981; Van Inwegen, et al., 1975). In our study there is an increase in NE-stimulated heat production in all animals

after inhibition of PDE with AMPY pretreatment (Figs. 7 & 8). Further, there is an increased sensitivity to AMPY in 2 day fasted animals (Fig. 9). This seems to indicate that PDE activity does increase with prolonged fasting and such changes are manifested by depressed NE-stimulated thermogenesis. If this interpretation is correct, then increased PDE activity could effectively counteract any increases in receptor number or adenylate cyclase activity, resulting in the observed decreased lipolysis in vitro and thermogenesis in vivo.

Lipon et al.(1979) and Capito and Hedeskov (1974) report no change in PDE activity in unstimulated pancreatic islet cells from 48-h fasted mice and rats. However, under adrenergic stimulation, MPDE activity is increased up to 3-fold (Clayberger et al.; 1981). Thus, while unstimulated lipolysis (Guidicelli, et al., 1981) may be unchanged in the fasted state, the maximal levels of lipolysis during adrenergic stimulation may be significantly depressed due to an increase in PDE activity after fasting. This decreased lipolysis (substrate mobilization) under adrenergic stimulation could explain the observed reduction of maximum thermogenesis induced by either NE or cold in the fasted rat.

It is apparent that in vitro biochemical studies ,of α- and β-adrenergic receptors, adenylate cyclase, and PDE activities, are required to further elucidate the biochemical mechanisms underlying the observed depression of

maximum thermogenesis after fasting.

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