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# Contribution made by parabiosis to the understanding of energy balance regulation

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## Abstract

Parabiosis is a chronic preparation that allows exchange of whole blood between two animals. It has been used extensively to test for involvement of circulating factors in feedback regulation of physiological systems. The total blood volume of each animal exchanges approximately ten times each day, therefore, factors that are rapidly cleared from the circulation do not reach equilibrium across the parabiotic union whereas those with a long half-life achieve a uniform concentration and bioactivity in both members of a pair. Involvement of a circulating factor in the regulation of energy balance was first demonstrated when one member of a pair of parabiosed rats became hyperphagic and obese following bilateral lesioning of the ventromedial hypothalamus. The non-lesioned partner stopped eating, lost a large amount of weight and appeared to be responding to a circulating "satiety" factor released by the obese rat. These results were confirmed using different techniques to induce obesity in one member of a pair. Studies with phenotypically similar ob/ob obese and db/db diabetic mice indicated that the obese mouse lacked a circulating signal that regulated energy balance, whereas the diabetic mouse appeared insensitive to such a signal. Positional cloning studies identified leptin as the circulating factor and subsequent parabiosis studies confirmed leptin's ability to exchange effectively between parabionts. These studies also suggest the presence of additional unidentified factors that influence body composition.

### Keywords

Humoral factors; parabiotic disharmony; obesity; hypothalamus; leptin

## 1.1 Introduction

Parabiosis is the surgical union of two animals to produce an experimental model that has been likened to naturally occurring conjoined twins. The procedure was initially developed at the end of the nineteenth century to test the viability of skin grafts [1]. Its utility as a model for investigating the role of circulating factors in the regulation of physiologic responses was soon recognized (see [2]) and parabiosis has since been used in the study of many different systems including reproduction [3–4], diabetes [5], aging [6], pituitary function [7], immune function [8] and stem cell biology [9].



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The first half of this review focuses on the surgical techniques that have been used and the limitations of the procedure in relation to the rate of blood exchange between partners. The second half of the review focuses on the work initiated by Hervey in 1959 [10] using parabiosis to determine whether there was a humoral satiety signal produced by obese rats that inhibited food intake. Subsequent parabiosis studies by different investigators clearly demonstrated the presence of a circulating negative feedback signal in the regulation of energy balance [11–12] [13] and this evidence ultimately led to the discovery of leptin [14].

## 2.1 Parabiosis Techniques and Limitations

### 2.1.1 Surgical Procedures

Although a variety of methods have been used to produce a cross-circulation in species such as mosquitoes [15], cockroaches [16], rabbits [17] and dogs [18], this review will focus on the work done with rodents because rats and mice are predominant in both past and present literature on parabiosis.

The use of parabiosis was initiated in the 1860's by the French biologist Bert [1] who was testing the viability of allografts. He joined two rats by attaching flaps of skin from one animal onto another, but also extended his work to other species and reportedly attached a rat to a cat and maintained them for several months, producing a successful xenograft (see Allen [19]). Bert [1] demonstrated that a viable cross-circulation was established between the two members of a pair by injecting various substances into one animal and observing their appearance in the partner. In addition he reported that post-mortem examination revealed the presence of newly formed capillaries in the skin union. Based on this work a number of other scientists recognized the potential of parabiosis to test for the involvement of circulating factors in physiological systems. In the early 1900's Sauerbruch and Heyde [20] modified the surgical procedure to involve the union of skin flaps covering a celio-anastomosis between members of a pair of rats. The opening of the peritoneal cavity and celio-anastomosis reportedly increased the exchange of humoral factors, but a large number of animals died soon after surgery due to infection [21]. In addition the skin union stretched and became twisted over time [21]. Drawings of rats with this type of surgery and the associated complications are included in a review by Schmidt [22].

The procedure of Sauerbruch and Hyde was modified several times to extend the union along the flank of the animals and to join the abdominal incision with a single suture without celio-anastomosis [23]. In 1933 Bunster and Meyer [21] described a technique that made the union more substantial by joining muscle in addition to the abdominal wall and skin. This remains the most commonly used parabiosis procedure today and an illustrated step-by-step description of the surgery has recently been provided by Sung et al [24]. Although the abdominal cavity of each animal is open the peritoneal walls are joined without performing a celio-anastomosis, reducing the possibility of gut strangulation caused by intestines from one animal shifting into the body cavity of its partner. The union is given more stability by suturing through the muscle of the shoulder blades of the rats. Shallow sutures are made along the thoracic wall to close the pocket between the rats and in older animals iliac bones may be sutured to provide additional strength to the union [21]. Because the union in these animals is predominantly skin, with connective tissue forming between muscle, several investigators have successfully separated partners after a period of time to determine the longevity of responses that are initiated by blood exchange while the animals are joined [25] [24] [26].

In 1959 Hervey [10] published a study investigating the role of circulating factors in the regulation of energy balance. In this experiment the parabiosis technique of Bunster and Mayer [21] was gradually refined such that the peritoneal cavity was not opened, but the

union was made between bone and skin. The scapulae were sutured together after the surface muscle had been removed and the bone was exposed. The femora were tied together after the bone was separated from surrounding muscles and the thorax and peritoneal muscles were joined by shallow suture to prevent pockets forming between the bodies of the rats. The procedure has been described in more detail by Harris [13]. In this preparation, the bones of the rats fuse together and muscle blocks are held together by the formation of connective tissue. Blood exchange appears to be dependent upon fusion of the bones and the rate of exchange is greater than in the other surgical preparations [10] [27]. If the bones do not fuse, then the skin incision gradually heals and the animals start to separate [28].

In all of the preparations described above parabiosis is achieved by straight-forward suturing the tissues of two animals together to facilitate the growth of capillaries. By contrast, the parabiosis studies of Koopmans [29] were designed to test the importance of delivery of nutrients into the small intestine in the control of food intake and required a much more complex surgery [30]. In Koopmans model rats were initially joined as parabionts, suturing together skin and muscle. After several weeks when the rats were stabilized they were subjected to a second surgery that connected a section of the lower duodenum and upper jejunum of the “donor” parabiont to the duodenum of its “recipient” partner. Food consumed by the recipient rat would travel through its own stomach and upper duodenum before passing into the segment of intestine that belonged to its partner and remained situated in the peritoneal cavity of the donor before traveling back into the lower duodenum of the recipient rat. The factors absorbed from the transferred intestinal segment would enter the blood stream of the donor rat. Clearly this surgery is much more complicated and elegant than the standard procedures that are used by other investigators.

### 2.1.2 Parabiotic Disharmony

Because parabiosis involves the continuous exchange of fluids and large cells between partners there is a high risk of one animal rejecting its partner due to immune incompatibility [31]. This is referred to as parabiotic disharmony or intoxication and develops soon after significant blood exchange is established within a pair, usually 9–12 days after surgery [32]. One member of a pair will develop hyperemia evident by reddening and dilation of blood vessels of the feet, ears and tail. Meanwhile its partner becomes anemic, stops eating, loses weight and dies within a few days [31]. If the members of the pair are separated as soon as the signs of disharmony become apparent, then it is possible that both of the individual animals will survive [33] and, occasionally, a pair that displays disharmony will survive and the symptoms will recede (unpublished observations).

Although disharmony was initially attributed to an imbalance in blood pressure between partners [34], this possibility was excluded when it was observed that there were no differences in blood pressure until after the symptoms of disharmony had developed [35]. It was clear that disharmony was much less frequent when the members of a pair were litter mates or came from an inbred strain of rats or mice than when the parabiotic partners were from outbred strains of rats or mice [35–36]. The incidence of disharmony can be as high as 65% of pairs made from outbred strains [32], whereas disharmony may be non-existent when inbred strains of rats [10] or mice [37] are used. There have been many studies evaluating the immunologic basis of the disharmony [8] and although the rejection has now been clearly associated with intolerance of the major histocompatibility system the mechanism still is not fully resolved.

### 2.1.3 Blood Exchange Between Parabiosed Rats and Mice

The objective of a majority of parabiosis experiments is to determine whether a circulating factor plays a critical role in a regulatory system. Therefore, with each parabiosis procedure

it is essential that blood exchange be established and that the rate of blood exchange between partners is measured. In the early 1900's there was some disagreement as to whether whole blood could exchange between parabiosed animals, or whether there was a "parabiotic barrier" (see review by Huff et al [38]). Starting with the initial studies by Bert [1] a number of investigators identified common blood vessels in parabiosed pairs of rats and Suaerbruch and Heyde [20] recorded the exchange of many different substances including bacteria and concluded that there was anastomosis of capillaries that allowed large cells to exchange between animals. Other investigators, however, failed to see equilibrium in the concentration of specific factors in the two members of a pair, or observed a limited physiologic response to administration of a toxin or a hormone in the partner of a treated animal [see [38]]. This led to the conclusion that there was a "parabiotic barrier" which limited transport of certain substances and it was suggested that the exchange of factors between parabiotic partners was dependent on the lymphatic system and exchange of body fluids in the common peritoneal cavity (see [2]).

In 1947 Van Dyke et al [39] measured the exchange of radioactive $Fe^{59}$ labeled red blood cells in parabiosed pairs that were joined by different surgical procedures and reasoned that if red blood cells could exchange between partners, then this would provide irrefutable evidence of common blood vessels between animals. Exchange of red blood cells started within minutes of one animal being injected, but total mixing between the pairs of rats took an average of almost 4 hours. They found no difference in the rate of blood exchange established by surgical procedures that joined skin alone (n=1), included opening of the abdominal cavity (n=3) or involved the joining of muscles according to the methods of Bunster and Mayer (n=1). In all preparations they reported that 0.64% of an animal's total blood volume was exchanged each minute. This translates to the total blood volume of each animal exchanging about 10 times each day. Van Dyke et al also reported that effective blood exchange occurred within two days of surgery, but that the maximal rate of exchange was not established until 4 days after surgery. The ability of red blood cells to cross the parabiotic union confirms that the exchange of soluble factors between animals is mediated by the development of common blood vessels rather than by simple diffusion of body fluids between the two animals.

Huff et al [38] provided a much more detailed examination of the nature of blood exchange between parabiosed rats, calculating the exchange rate for substances that were limited to the circulation and for those that could diffuse into other tissue spaces. In addition they created mathematical models to account for the rate of clearance of factors from a pair, assuming that both animals contribute to the removal of a substance from the circulation. Based on these calculations it was concluded that the "parabiotic barrier" existed only for substances that had a high turnover or clearance rate. More recently the early work with radiolabeled red blood cells has been repeated using mice in which nucleated blood cells were tagged with green fluorescent protein [40]. Using the surgical techniques of Bunster and Mayer these investigators calculated a much slower rate of blood exchange of 0.66% per hour than the 0.64% per minute that had been reported for the rats. In pairs in which one partner expressed GFP and the other did not, the proportion of leukocytes and lymphocytes that expressed GFP reached equilibrium with non-tagged cells within 14, but not 7 days after surgery [40].

Although there has not been a careful examination of the dynamics of blood exchange in parabiosed pairs made using the procedure described by Hervey that includes fusion of bones [10], the rate of exchange measured with Evans Blue dye indicates that 1.0 – 2.0% of each animal's total blood volume exchanges each minute in both rats [28] [41] and mice [42]. These data suggest the development of a more extensive network of capillaries when bones fuse than when only skin and muscle are joined. Despite the faster rate of blood

exchange parabiotic disharmony is not apparent until post-surgical day 9 [43] or later [13], which is the same as for other parabiotic preparations.

### 2.1.4 Clearance Rate of Circulating Substances

The rate of clearance of factors from the circulation is a critical consideration when designing parabiosis studies to test for the involvement of a circulating factor in a regulatory system. A failure to appreciate that some proteins are cleared from the circulation faster than they can exchange initially led to the misconception that there was a “parabiotic barrier” that prevented some factors from reaching the untreated partner in a parabiotic pair. In other instances the failure to see a physiological response in a parabiosed animal in response to a disturbance in its partner was misinterpreted as the lack of involvement of a humoral component in a control system. For example, Kawai [44] concluded that there was no humoral exchange between members of parabiotic pairs because one of the partners died of starvation 5 days after it’s lips were sutured together so that it could not eat. Several experiments have since shown that a glucose concentration gradient exists between parabionts that are members of pairs in which blood exchange has been confirmed [11] [41]. In order for a factor to be present at significant concentrations in the circulation of the recipient member of a pair the rate of exchange has to be faster than the rate of clearance in the two partners combined. Because the rate of blood exchange between parabiosed rats and mice is in the range of only 1% of blood volume per minute factors that are rapidly cleared from the circulation, due to degradation or transfer out of the circulation, will not be detectable in the recipient.

Huff et al [38] performed several experiments to show that if clearance of a substance was reduced, then it was possible to change the outcome of a parabiosis experiment. They reported that it was impossible to anesthetize the partner of a rat that was injected with sodium pentobarbital, but if they removed the kidney of the non-injected rat, then it was easily anesthetized by injection into its partner. Because clearance was slow in the nephrectomized animal it also remained sedated longer than the injected rat. In a second experiment with intact and castrate rats they were able to induce the effects of testosterone in the castrate animal only if the portal circulation was diverted away from the liver to inhibit clearance of testosterone from the circulation.

Van Dyke et al [45] were some of the first to clearly demonstrate the impact of the half-life of a hormone in the circulation on the outcome of parabiosis experiments. They compared the adrenal weights of hypophysectomized rats parabiosed to normal rats. The experiments were conducted before the advent of radioimmunoassays or ELISAs, but they used a bioassay that determined the weight of adrenal glands in hypophysectomized rats that received daily injections of ACTH. From this they calculated that ACTH had a half-life in the circulation of approximately 5.5 min, and because of this rapid clearance rate it was distributed in a ratio of 85:1 between the normal and hypophysectomized rats. By contrast, when they measured the relative concentrations of growth hormone, using a cartilage growth bioassay, they determined that growth hormone had a half-life in the circulation of 26 minutes and was distributed in a ratio of 2.7:1 between the normal and hypophysectomized partners. They also noted that the combined growth of the two rats in the pair was equivalent to the growth of a single normal rat, suggesting that diluting circulating concentrations of growth hormone between two animals did not provide any feedback regulation of pituitary growth hormone secretion. In a more recent study we found very similar results when examining the exchange of leptin in parabiosed mice. It was determined that leptin had a half-life in the circulation of 36 minutes [37] and was distributed in a 3: 1 ratio between hyper-leptinemic *db/db* mice and their leptin-deficient *ob/ob* parabiotic partners [42]

The rate of blood exchange in parabiosis models is relatively slow and, as discussed, limits the effective exchange of circulating factors that have a short half-life in the circulation. Acute cross circulation studies with larger animals, such as dogs and pigs, are achieved by directly connecting large blood vessels of the two animals using shunts [46] [47] [48]. Because of the high rate of exchange in this type of preparation there is complete mixing of blood in less than 10 minutes [46] and it is much more likely that factors that are rapidly cleared from the circulation will be present at high enough concentrations to be active in the recipient animal.

## 3.1 Parabiosis in the Investigation of the Regulation of Energy Balance

### 3.1.1 Background

In the 1940's and 1950's it was generally accepted that energy balance was achieved by controlling food intake to compensate for variations in daily energy expenditure. This was supported by studies in which rats would increase their food intake if the energy density of the diet was diluted [49–50], or if there had been an enforced increase in energy expenditure [51]. There also was an increasing amount of evidence that damage to the hypothalamus would disrupt energy balance and the control of food intake [52–55]. Because it seemed unlikely that the hypothalamus could directly measure the amount of energy that was expended during any given period it was proposed that the hypothalamus could monitor some indices of energy status and respond to correct for an energy imbalance. A number of hypotheses had been proposed for the feedback signal that was monitored by the hypothalamus to achieve this control, including the glucostatic [56] and thermostatic [57] theories.

In 1953 Kennedy [58] argued that although circulating concentrations of glucose and body temperature may influence consumption of meals they could not provide any "memory" of previous disruptions in energy balance. He put forward a lipostatic hypothesis for the regulation of energy balance, proposing that the hypothalamus could sense humoral information on the degree of adiposity in an animal, rather than monitor absolute food intake. This mechanism had the advantage that body fat would integrate the outcome of previous disruptions of energy balance because the energy stored in fat allows an individual to buffer short-term imbalances between energy intake and expenditure. Without endogenous energy stores animals would have to constantly adjust energy intake and expenditure to be equal across both and long and short time intervals.

### 3.1.2 Parabiosis Using Rat Models of Hypothalamic Obesity

In 1959 Hervey [10] performed a parabiosis study to provide a direct demonstration that the control of food intake involved a feedback system. Young rats were parabiosed at 4 weeks of age and four months later one member of each experimental pair received bilateral lesions of the ventromedial hypothalamus (VMH). Pairs without lesions provided controls and there also were lesioned and unlesioned single, non-parabiosed rats. Single and parabiosed lesioned rats immediately started to overeat and gain weight whereas the partner of lesioned rats appeared to lose interest in food and lost weight. The pairs were maintained for 8 – 12 weeks and post-mortem inspection showed little visible fat and atrophied organs in the partners of the obese lesioned animals. Because food was freely available to the partners of lesioned rats, but they did not show any interest in eating, Hervey concluded that the hypothalmi of the non-lesioned partners were processing information on the energy balance status of the obese rats. It had previously been demonstrated that large changes in blood glucose in one animal did not influence the circulating glucose concentrations in their parabiotic partner [56], and because it was difficult to visualize how a signal related to body temperature could be transmitted between parabionts, Hervey [10] concluded that the results

of this study were most easily explained by Kennedy's lipostatic theory [58]. He proposed that the signal that was monitored was related to the body fat content of the obese animal, rather than food intake specifically because the food intake of the lesioned rats gradually returned to normal and their weight stabilized, but this did not restore the food intake of the partner. Additional evidence that fat was being monitored was provided by observations that members of control parabiotic pairs had approximately half as much carcass fat as that found in single rats. Thus it appeared that intact hypothalami monitored the total amount of fat present in a parabiosed pair of rats due to a relatively stable factor released into the circulation in proportion to the size of fat depots.

Although two subsequent parabiosis experiments using VMH lesions to induce hyperphagia and obesity failed to confirm the drop in food intake and loss of fat from partners of lesioned rats [59] [60], possibly due to the surgical technique used to pair the animals or the housing conditions used for feeding [59], other studies have confirmed the outcome and interpretation of the experiment described by Hervey [10]. Nishizawa and Bray [61] determined that the body fat content of partners of VMH lesioned rats was significantly reduced within 9 days of the partner being lesioned. They also demonstrated that if one member of a pair was overfed by gastric intubation for three weeks, then the food intake of its partner tended to be below control levels and body fat was lower than that of members of control pairs [61]. Parameswaran et al [11] induced one member of a pair of rats to overeat by electrically stimulating the lateral hypothalamus (LH) for three 30 minute sessions on each of 15 days. Because this rat only ate during the periods of stimulation it was possible to measure the food intake of the individual members of the pair. The intake of the stimulated rats increased dramatically on the days of stimulation and plateaued at a level four-times that of a control parabiont. By contrast, the voluntary intake of the non-stimulated partner gradually declined to almost nothing by the end of the study. The authors reported that although these rats were malnourished they also actively tried to pull their partners away from the food dish during the periods of stimulation, confirming Hervey's [10] observations that the low food intake of partners of obese rats was due to a lack of hunger and not because the obese partner interfered with access to food. At the end of the study the body fat mass of partners of stimulated rats was reduced by about 40% compared with control parabionts. The loss of fat and lean tissue from the partners of obese rats combined with measurements of serum glucose confirmed that the hypophagia of the non-stimulated partner could not be attributed to the transfer of significant quantities of energy substrates between the partners. Parameswaran et al [11] also excluded insulin and glucagon as potential "satiety" signals because glucagon did not change and only small amounts of insulin exchanged successfully between the parabionts.

### 3.1.3 Parabiosis Using Rat Models Of Overfeeding

Although the previous rat studies using pairs in which one animal was made obese by either VMH lesions [10] or electrical stimulation of the LH [11] had clearly demonstrated the presence of a circulating factor that inhibited food intake and caused a substantial loss of fat and lean tissue in the untreated partner, little was known about the identity of the factor, the details of the conditions that induced release of the factor or its metabolic impact in the partner.

Starting in 1984 [13] we published a series of studies in which one member of a pair of rats was made obese by overfeeding by gavage. The out-bred strain of Sprague Dawley rats was used in all of these experiments because this was the strain most commonly used for studies on nutrition and obesity, but it also resulted in a significant loss of pairs due to the development of parabiotic disharmony [13, 62]. Rats weighing approximately 50 g were joined in parabiosis and were used in experiments starting six to eight weeks later. Blood exchange was confirmed in all of the pairs using Evans Blue dye and was found to be in the

range of 1–2% of each rat's blood volume exchanging per minute. During the experimental period the pairs had free access to a powdered diet in the cage. Overfed rats were fed with the same diet in liquid form. In experimental groups one member of a pair was tube-fed three meals a day and intake was increased from 100% to 200% [13] or 250% [62] of voluntary intake over a period of 7 to 10 days. The duration of tube-feeding varied from 25 to 60 days in the different experiments. Both members of control pairs ate ad libitum throughout the experiments and, because the overfed rats did not eat any dry food, it was possible to measure the food intake of the ad libitum fed partners of the overfed rats.

The results from these experiments have many similarities to those of Hervey [10] and Parameswaran et al. [11], but also show some striking differences. Measurements of daily food intake showed a small, but non-significant decline in the voluntary food intake of partners of overfed rats compared with members of control pairs [13]. The possibility that the intake of the ad libitum rats was overestimated due to the tube-fed rat continuing to eat dry diet was excluded by putting chromic oxide in the dry diet that was available in the cage. The feces of the ad libitum partner turned green, but the feces of the overfed rat did not show any sign of coloration [13]. Although the decrease in food intake of the partners of obese rats was not significant, the energy deficit compared with controls was 96 kcal over 45 days, whereas loss of carcass fat from these rats during the same period represented 84 kcal. A subsequent experiment [41] confirmed that this small change in daily energy intake was a critical aspect of the response that reduced body fat mass. Partners of overfed rats were tube-fed the same intake as ad libitum control parabionts were consuming and this represented an increase in their daily food intake of only 0.5 g. After 40 days the body fat of ad libitum partners of overfed rats was reduced by 60% compared with members of control pairs in which both animals ate ad libitum, whereas the carcass fat of the tube-fed partners of overfed rats was not different from members of pairs in which both rats were tubefed a normal daily food intake [41]. These observations were similar to those of Hervey [10] and of Parameswaran et al [11] in that the partners of obese rats lost fat due to a suppression of food intake, however the magnitude of the feeding response was greatly exaggerated when obesity was induced by hypothalamic lesions or stimulation.

Another difference between the experiments was that the ad libitum partners of overfed rats had the same lean body mass as controls [13], whereas the partners of hypothalamic obese rats lost lean tissue in addition to fat [11] and it is likely that this was secondary to the substantial energy deficit experienced by the partners that were not eating. The reason for this difference in response has not been investigated, but it is possible that it is simply due to the degree of overeating. Parameswaran et al [11] reported that the food intake of LH stimulated rats increased approximately four-fold compared with the doubling of intake in the tube-fed rats. Alternatively, it is possible that manipulation of the hypothalamus leads to the release of multiple circulating factors, one or more of which have a relatively long half-life in the circulation and also have a strong inhibitory effect on food intake.

The parabiosis preparation in which one member of a pair was made obese by overfeeding was also used to investigate the metabolic basis of the apparently specific depletion of body fat in the ad libitum fed partner [27]. A time course study demonstrated that body fat did not change until between 23 and 30 days of overfeeding. The carcass fat content of the overfed rat was already increased 5-fold over that of the members of control pairs by day 23, indicating that a substantial change in fat mass was required for the circulating factor produced by the obese rat to be bioactive in its partner [27]. Measurements of tissue glucose and fatty acid utilization indicated that the loss of fat from the partners of obese rats was due to an inhibition of adipose tissue fatty acid esterification, which was present by 23 days of overfeeding and thus preceded the loss of fat in the partners of overfed rats. Adipose tissue

lipolysis was not increased, but both lipolysis and hepatic fatty acid esterification decreased over time as a secondary response to loss of fat [27].

Additional studies with the overfed model showed that the change in body composition of partners of overfed obese rats was independent of diet composition [62–63] and was not associated with a change in insulin sensitivity [41] even though the overfed rats were hyperinsulinemic and insulin resistant. The partners of obese rats showed an interesting phenotype in that they were metabolically similar to food restricted animals and, although these rats were maintaining an almost normal food intake, they were not making any attempt to overeat to restore a normal body composition [62]. It was not possible to determine whether there was an expenditure component to the energy imbalance of the individual members of a pair, but because of the very small energy deficit that would be required to account for the loss of fat from the thin partners it is unlikely that calorimetry would be sensitive enough to detect the change that would be biologically relevant.

### 3.1.4 Parabiosis with Genetically Obese Mice

The earliest description of parabiosis with genetically obese mice is an abstract by Hausberger [64] who reported obese ob/ob mice were parabiosed to non-obese littermates the weight gain of the ob/ob partner was inhibited. If the members of the pairs were separated after 4 months, then the ob/ob mice rapidly gained weight and the author concluded that the obesity was caused by “the lack of a factor which can be transmitted by successful parabiosis”. In a second study Chlouverikas [65] parabiosed ob/ob mice with normal mice to test for the involvement of circulating factor in the development of insulin resistance. He found that after 50 days of parabiosis there was a small inhibition of growth in the lean partners of ob/ob mice compared with their controls, but their food intake and activity appeared normal. By contrast, the weight gain of ob/ob partners of lean mice was almost totally inhibited and this was accompanied by a partial reversal of hyperglycemia, hyperinsulinemia and insulin sensitivity and a normalization of serum triglycerides. In another study Haessler and Crawford [66] parabiosed lean and ob/ob mice to determine whether there was a circulating factor that either promoted or inhibited fat mobilization. In contrast to the two previous studies, they found weight loss in the lean partner and described the ob/ob mouse as ”living parasitically” off the lean animal. In this experiment the ob/ob mice were food restricted before surgery to the same weight as the lean partner, but following surgery they were allowed free access to food and, in retrospect, it is not surprising that the ob/ob mouse gained more weight than its lean littermate.

In 1969 and 1973 Coleman and Hummel published two parabiosis studies using obese (ob/ob) mice and diabetes (db/db) mice. The diabetes mice were known to have a spontaneous single autosomal recessive gene mutation that resulted in early onset Type II diabetes [67] and the objective of the first experiment was to determine whether hyperinsulinemia was due to a deficit in a factor that normally inhibited insulin release. In this study [68] db/db mice destined to be parabiotic partners of wild type mice were food restricted for an average of 45 days (25–93 days) before the surgery in order to minimize obesity and hyperinsulinemia. A majority of the pairs died between 8 and 49 days after surgery with the wild type partner dying first. In this experiment it was determined that blood glucose of the db/db partner normalized while that of its partner continuously decreased to approximately 60 mg/dL, which is typical of blood glucose concentrations found in a starved animal and lethal if maintained. There also was a great disparity in the circulating insulin concentrations of the partners and there were associated changes in the histology of the pancreas. Not only were there few and small islets in the wild type partners, but the acinar cells which secrete digestive enzymes were atrophied and similar to those found in a mouse that died of starvation. It was not possible to measure food intake of individual members of the pairs of mice, but total intake of the pair was only slightly more than would be consumed by one

member of a db/db pair and it was concluded that the wild type mouse ate very little food. This was confirmed with post-mortem observations that these mice had empty stomachs, small livers and almost no body fat. Coleman [68] likened the outcome of this experiment to that of Hervey's [10] and concluded that "a defective hypothalamus in the diabetic mouse causes death by starvation of the normal parabiont".

In light of the previous work with ob/ob mice [64] and knowing that ob/ob mice and db/db mice each had a single gene mutation that were expressed on different chromosomes [69], but resulted in identical phenotypes that could only be distinguished in the outcome of parabiosis studies, Coleman [12] performed the experiment that has received a lot of recent attention in that it provided justification for the positional cloning that led to the discovery of leptin. Experimental groups of parabiosed pairs of mice included ob/ob mice joined to lean mice, ob/ob mice joined with ob/ob mice and lean mice joined with lean mice. All of these pairs were viable until they were killed 4 to 6 months after surgery. By contrast, pairs in which ob/ob mice were joined to db/db mice had an average survival time of only 26 days. In these pairs the ob/ob partner appeared to eat very little, become hypoglycemic and lost significant amounts of body fat. Meanwhile the db/db partner continued to overeat, remained hyperglycemic and had large fat depots. Coleman [12] concluded that the ob/ob mice had normal satiety centers and were able to respond to a humoral factor produced by normal mice and by db/db mice. The concentrations of the humoral factor were relatively low in the lean mice and therefore food intake of ob/ob partners of lean mice was normalized. By contrast, it was hypothesized that concentrations of the factor were elevated in the db/db mice and this resulted in high levels of the satiety factor reaching the ob/ob partners of db/db mice, which caused an almost total inhibition of food intake and starvation in the ob/ob mice. Evidence that ob/ob mice were unable to produce the satiety factor whereas db/db mice appeared to produce, but did not respond to, the factor explained why mutations of two unrelated genes produced identical phenotypes of overeating, obesity and diabetes [69].

## 4.1 Identification of Leptin as the Parabiotic Satiety Signal

Although the parabiosis studies had identified the theoretical cause of obesity in db/db and ob/ob mice, it was difficult to discern the primary defect in the animals due to the complex nature of their phenotypes and interactions with background strain [70]. In 1990 the laboratories of Leibel and Friedman published their first paper on the use of "reverse genetics" to identify the db gene [71], reasoning that cloning would locate the gene without having to identify the primary physiological defect in the mouse. Subsequently, they also mapped the fa mutation in fatty Zucker rats and found it to be homologous to the db mouse gene [72]. In 1994 Zhang [14] reported the locus of the ob gene and the protein product was named leptin. Soon after the discovery of leptin it was also confirmed that db and fa genes were associated with mutation of the leptin receptor [73]. Based on the parabiosis studies with ob/ob mice [12, 64] it had to be assumed that leptin would fulfill the role of a feedback signal in the regulation of energy balance. Many studies have since confirmed that replacement of leptin in ob/ob mice [74–76] will rapidly normalize many aspects of their obesity syndrome whereas db/db mice are unresponsive [75–76]. In normal mice leptin will suppress food intake, inhibit weight gain and reduce body fat mass [77], however, obesity [78–79] and/or hyperleptinemia [80] induce a condition of "leptin resistance" that is defined as a failure of leptin to modify energy balance.

Following the identification of leptin we tested whether it met the criteria for the circulating factor that was detected in the earlier parabiosis studies. Injections of recombinant leptin into one member of pairs of ob/ob-ob/ob mice demonstrated that leptin could exchange between parabiotic partners, but did not reach equilibrium [37] (see Section 1.1.4). Because

the concentrations of leptin were higher in the injected mice than their partners, it was also possible to show that lower doses of leptin were required to normalize insulin than to correct body temperature and food intake or to reduce body fat mass in the ob/ob mice. Two additional studies essentially replicated the experiments reported by Coleman [12, 68], examining the phenotype of genetically obese and diabetic mice parabiosed either to each other [42] or to lean littermates[43]. The results of these experiments confirmed the earlier observations that ob/ob partners of db/db mice had a dramatically reduced food intake, lost large amounts of body fat but normalized body temperature, blood glucose and insulin and retained lean body mass [42]. Surprisingly, lean partners of db/db mice did not show a significant reduction in food intake, measured as gut content, but lost both lean and fat tissue, implying a stimulation of thermogenesis even though body temperature did not change [42]. These mice also were hypoglycemic and hypoinsulinemic, similar to the results from Coleman's study [68].

Beyond confirming the results from Coleman's experiments [12, 68] these parabiosis studies also provided some new information on the effect of blood exchange between obese and diabetic mice and suggested that leptin in ob/ob mice induced release of an additional factor that could influence body composition. When ob/ob mice were partnered with db/db mice the db/db partners experienced a small loss of body fat, but also increased lean mass by 30% during the 25 days of the experiment [42]. When lean mice were partnered with ob/ob mice they lost 37% of their body fat over a period of 50 days and had a lower body temperature than controls, but did not show any change in lean tissue mass [43]. Despite the loss of fat in these mice serum leptin and adipose tissue leptin mRNA expression were unchanged. It is possible that activation of leptin receptors in ob/ob mice caused release of a circulating factor that selectively reduced body fat and protected lean tissue. This factor would already be present in wild type mice that express leptin and functional leptin receptors, but it is possible that increased concentrations of the factor were present in lean partners of ob/ob mice because ob/ob mice are hyper-responsive to leptin. If release of the factor is dependent upon activation of leptin receptors, then it would not normally be present in db/db mice and this may explain why lean mass when they shared a cross-circulation with ob/ob mice that could produce the factor.

## 5.1 Conclusion

Parabiosis is an experimental technique that allows demonstration of the involvement of humoral factors in the regulation of various physiologic systems. When using parabiosis it is important to measure the rate of blood exchange between partners as this will determine which factors exchange in sufficient amounts to produce a physiologic response in their partner. Proteins that are cleared from the circulation of the two animals at a higher rate than they can exchange between the individual animals will not show any bioactivity in the recipient partner [38]. Parabiosis between lean and obese rodents has been used to successfully demonstrate the presence of a humoral feedback signal in the regulation of energy balance. The methods used to induce obesity in one member of a pair may influence the specifics of the response in their lean partner. Hypothalamic obesity appears to cause a significant inhibition of food intake which results in loss of fat and lean mass from their partners [10–11], where as partners of rats made obese by overfeeding show a much slower and selective loss of body fat [13]. The difference in response may be due to the release of circulating "satiety" factors in the hypothalamic obese rats caused either by hypothalamic manipulation or simply by the excessive nature of their hyperphagia. Studies with genetically obese mice [12] provided the motivation for positional cloning studies that led to the identification of leptin as a humoral feedback signal in regulation of energy balance [14], but also indicate the presence of additional unidentified factors that influence body composition.

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## Highlights

- Parabiosis produces a chronic cross circulation between two animals.
- Only factors with a long half-life in the circulation exchange between parabionts
- Different obesity models demonstrated the presence of a circulating “satiety” factor
- Positional cloning studies identified this factor as leptin
- Leptin has been shown to exchange effectively in parabiosied mice