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Research Article

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Morphology and Metabolism of an Aortic Intima-Media Preparation in Which an Intact Endothelium is Preserved

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A B S T R A C T An in vitro preparation of rabbit aortic "intima-media" previously shown to exhibit stable rates of respiration and glucose metabolism and the high rate of aerobic glycolysis considered characteristic of the metabolism of this tissue was subjected to electron microscopic examination. In samples examined immediately after the aortae were dissected free of adipose tissue and adventitia, under conditions similar to those now in common use, marked and widespread alterations in endothelial cell structure were present, including loss of cell integrity. The vascular smooth muscle cells retained a normal electron microscopic (EM) appearance. During subsequent incubation with 5 mM glucose in Krebs-Ringer bicarbonate (KRB), pH 7.4, under the conditions usually employed in studies of this preparation, large zones of the luminal surface were rapidly denuded of endothelium, and the remaining endothelial cells exhibited a wide range of ultrastructural alterations. The smooth muscle cells, however, continued to maintain a normal EM appearance.

A method was developed to prepare segments of rabbit aortic intima-media which retained an intact layer of endothelium resembling that observed in tissue fixed *in situ*. During a 1-h incubation with 5 mM glucose in KRB, pH 7.4, gas phase 5% CO_2/95% O_2, containing 6% bovine serum albumin, the intact aortic intima-media preparation retains an essentially unmodified EM appearance and exhibits linear rates of respiration. Under these conditions the intact aortic intima-media preparation exhibits significantly higher rates of O_2 uptake and glucose uptake than those observed in our previous prep-

aration or in other reported aortic intima-media preparations. The intact aortic intima-media does not exhibit the high rate of aerobic glycolysis during in vitro incubation that has been considered characteristic of the metabolism of rabbit, rat, and swine aortic intima-media. In addition, the magnitude of the Pasteur effect was far greater than that observed in other aortic intima-media preparations. The data suggest that component cells of the aortic intima-media may derive a major fraction of their energy requirements from respiration; they raise further questions concerning the significance of the high rate of aerobic glycolysis observed when aortic intima-media preparations are incubated in vitro, and they suggest that documentation of the EM appearance of the endothelium in such preparations is desirable.

INTRODUCTION

Preparations of aortic "intima-media" from rabbits, rats, and swine have been extensively employed for in vitro incubation and perfusion studies designed to provide information concerning the composite metabolism of the inner arterial wall (1–18). Despite differences in the techniques employed to prepare aortic intima-media for study, there are certain consistent features. The adventitia and adherent adipose tissue are removed by dissection or, less commonly, the aorta is opened and the luminal surface is stripped off to provide tissue for study. With rare exceptions (15–18) these procedures have been carried out in cold Krebs-Ringer bicarbonate buffer (KRB)[1] or cold buffered saline solutions with varying degrees of attention to the O_2 requirements of the tissue. In the reported studies the dissected aortae have been

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; EM, electron microscopic; KRB, Krebs-Ringer bicarbonate.

perfused, or for incubation they have been cut into thin rings or larger segments, or opened and cut into pieces of varying sizes. Rabbit thoracic aorta has been the most common source of tissue for such studies, and most of the data presently available concerning the metabolism of glucose, lipids, and extracellular macromolecules in the arterial wall are based upon studies of rabbit aortic intima-media preparations. These data form an essential portion of the basis for current speculations concerning the pathogenesis of arterial lesions.

We previously made extensive use of a preparation of tubular segments of rabbit thoracic aorta free of adventitia and adipose tissue (preparation I) for in vitro studies (1–5). Preparation I provides two pooled samples from the same rabbit which exhibit comparable metabolic activities; this permits the use of paired controls in studies of the effects of hormones and substrate concentrations on various aspects of aortic metabolism. Like most of the reported rabbit thoracic aortic intima-media preparations, preparation I exhibits a linear rate of O_2 uptake for 2–3 h when incubated in KRB containing a physiological glucose concentration (5 mM); similarly, the rates of glucose uptake, lactate production, and the conversion of [U-^{14}C]glucose to $^{14}CO_2$ remain constant over the same time interval. The pattern of glucose metabolism and respiration observed in preparation I conforms to that presently attributed to aortic intima-media based not only on data derived from rabbit aortic intima-media but on observations made in tissue from rats and swine (7, 13, 17). This pattern of metabolism was well recognized when Lehninger reviewed the metabolism of the arterial wall in 1959 (14) and has not been significantly modified in the interim. Previously reported rabbit thoracic aortic intima-media preparations consistently exhibit an O_2 uptake during incubation with a physiological glucose concentration in KRB equilibrated with 5% CO_2/air or 5% CO_2/95% O_2 which is very low when compared with other tissues on a wet weight or dry weight basis (9). Under these conditions lactate production by preparation I accounts for approximately 45% of the glucose uptake (1), and other workers have observed that lactate production consistently accounts for 50–80% of the glucose uptake of rabbit aortic intima-media (10, 18). These observations are in agreement with data derived from studies of rat and swine aortae and conform to the presently accepted view that aortic intima-media exhibits a high rate of aerobic glycolysis under physiological conditions (9, 10). In accordance with this view, the magnitude of the Pasteur effect observed in aortic intima-media preparations of rabbit and swine aorta (although quite variable) has in general been quite small when compared with that observed in tissues that normally derive the major fraction of their

energy requirements from reactions linked to respiration (9, 19).

When studies with preparation I were first reported more than ten years ago (2), it was subjected to extensive light microscopic studies to confirm the absence of adventitia and adipose tissue and the preservation of a normal light microscopic appearance after incubation. The limitations of light microscopic examination with regard to endothelial cell morphology and intimal structure are now obvious, but this is the extent to which most of the aortic intima-media preparations now in common use have been characterized. The relative stability of the reported preparations, as assessed by oxygen uptake or other measurements of metabolic activity, and the considerable agreement in the results reported by different laboratories has undoubtedly contributed to the neglect of the status of the endothelial cells in these preparations.

The studies that form the basis of this report demonstrate that striking alterations in the transmission electron microscopic (EM) appearance of the aortic endothelial cells occur during the preparation of rabbit aortic intima-media for incubation, and that an intact endothelial cell layer is rapidly lost during the initial period of subsequent incubation under conditions similar to those now in common use.

Conditions have been defined which permit the preparation of intact rabbit thoracic aortic intima-media for incubation (preparation II) and for the maintenance of an essentially intact endothelial cell layer during incubation for periods as long as 1 h. The intact aortic intima-media preparation has been found to exhibit a pattern of respiration and glucose metabolism that differs significantly from that presently attributed to the inner arterial wall.

METHODS

Young, male, white, New Zealand rabbits (1.5–2.5 kg) fed freely on Wayne rabbit ration (Allied Mills, Inc., Chicago, Ill.) plus carrots and lettuce.

Preparation I. The method for the preparation of paired pooled samples of tubular segments of rabbit thoracic aorta free of adventitia and adipose tissue previously described (1, 2) involved the following procedures. The rabbits were sacrificed by decapitation; the descending thoracic aorta was quickly removed and rinsed in a large volume of cold (4°C) KRB buffer, pH 7.4, continuously bubbled with 5% CO_2/air. The aorta was dissected free of adipose tissue and adventitia with watchmaker's forceps while immersed in cold fresh buffer of the same composition in a large Petri dish under an atmosphere of 5% CO_2/air; the tissue was transferred to fresh buffer at least three times during the dissection. The ends of the aorta (approximately 3 mm) were cut off and the remaining tissue was gently placed on a disk of filter paper and cut into six equal segments approximately 1 cm in length; the segments were designated "1" through "6," according to proximal to distal anatomical position. The odd and even numbered segments were then

pooled to provide two pooled samples, each weighing approximately 100 mg. In any series of paired experiments the odd and even numbered samples were alternatively assigned to each of the two test conditions examined. The freshly prepared aortic intima-media segments were then fixed immediately for examination by transmission electron microscopy as described below or quickly weighed on a torsion balance and transferred to an incubation vessel. This consisted of a 25-ml Erlenmeyer flask containing 5.0 ml of KRB, pH 7.4, gas phase 5% CO_2/air, and 5 mM glucose which had been preequilibrated at 37°C in a Dubnoff metabolic shaker. The flask was shaken at 88 cycles per min and gassed with 5% CO_2/air for an additional 5 min; the flask was then sealed with a rubber cap and incubation continued for the times indicated in the text. The total time required from sacrifice to the point at which the tissue was added to the incubation flask was carefully monitored; it was approximately 7–8 min in most instances, and all samples which required more than 10 min for preparation were routinely discarded without further study.

Preparation II: intact aortic intima-media. The rabbits were sedated with diazepam (2.0 mg/kg) i.m. 90 min before sacrifice, and given phentolamine (0.5 mg/kg) i.m. 30 min before decapitation. The descending thoracic aorta was quickly removed and rinsed in a large volume of warm (37°C) KRB buffer containing 6% dialyzed bovine serum albumin (BSA) and 5 mM glucose; the pH of the solution was maintained at 7.4 by continuous gassing with 5% CO_2/95% O_2. The aorta was transferred to a large Petri dish containing medium of the same composition and temperature, and dissected free of adventitia and adipose tissue; the medium was changed at least three times during the procedure. The dissected aorta was cut into six equal segments while still immersed in the warm medium, and segments pooled as described for preparation I to provide two paired samples from each aorta. The samples were then quickly drained of medium on a piece of filter paper and immediately fixed for EM examination, or quickly weighed on a torsion balance and transferred to a 25-ml Erlenmeyer flask for incubation. Under the *standard conditions* the flask contained 5.0 ml of KRB, pH 7.4, gas phase 5% CO_2/95% O_2, containing 5 mM glucose and 6% dialyzed BSA; the medium was preequilibrated to 37°C. The details of the incubation procedure were otherwise as described for preparation I. The time required from the sacrifice of the rabbit to the transfer of the tissue to the incubation medium was approximately 7–8 min and was never permitted to exceed 10 min.

BSA, fraction V powder (Sigma Chemical Co., St. Louis, Mo.) was dissolved in KRB, 5% CO_2/95% O_2, pH 7.4, and dialyzed against 100 times the volume of the same buffer for 24 h at 4°C with two changes of buffer; the FFA concentration of each batch of dissection or incubation medium containing 6% dialyzed BSA (g/v) was determined by the method of Novak (20) and was consistently less than 0.200 meq/liter. As noted in the text, in specific instances the BSA employed was a preparation essentially free of FFA prepared from fraction V, by the same supplier.

Oxygen uptake was determined by means of a model 53, biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio) and recorder. For these determinations the tissue was rapidly transferred from the dissection or incubation flask to a chamber of the monitor system containing medium of the same composition which had been preequilibrated with the same gas phase at 37°C. The methods for the determination of glucose uptake, aortic lactate, pyruvate, and glycogen concentrations, and medium lactate and pyruvate concentrations were previously described (1, 21).

To examine the utilization of [U-^{14}C]glucose by the intact aortic intima-media preparation, samples of tissue were incubated under the standard conditions described above in medium containing 5 mM [U-^{14}C]glucose (New England Nuclear, Boston, Mass.) with a specific activity of 0.10 mCi/mmol. For the collection of $^{14}CO_2$ the incubation flask was sealed with a rubber cap fitted with a plastic rod supporting a well (Kontes Glass Co., Vineland, N. J.); the incubation was stopped by the addition of 0.40 ml of 4 N H_2SO_4 to the medium with a syringe and needle, and in a similar fashion 0.30 ml of hydroxide of hyamine was added to the well. The evolved $^{14}CO_2$ was collected for a period of 1 h at 37°C, the well then cut from its supporting rod and transferred to a scintillation vial containing 15 ml of Bray's solution (22), and counted in a liquid scintillation spectrometer, with an internal standard (Teledyne Isotopes, Westwood, N. J.). Control flasks without added tissue were run with every experiment.

The incorporation of [U-^{14}C]glucose into glycogen was examined in intact aortic intima-media samples after a 1-h incubation under the conditions described above. In these experiments the aortic tissue was removed at the end of the incubation, drained of medium, and quickly homogenized in 5.0 ml of 6% trichloroacetic acid at 4°C; the supernate was recovered after centrifugation and glycogen precipitated by the addition of 95% ethanol to a final concentration of 66% at 4°C; the glycogen was redissolved in water and reprecipitated. This procedure was carried out three times. The final precipitate was then dissolved in 0.20 ml of water and quantitatively transferred to a spot on a disk of Whatman no. 5 filter paper and further purified and prepared for liquid scintillation counting as described by Thomas et al. (23). To determine the incorporation of [U-^{14}C]glucose into the total lipid fraction after a 1-h incubation under the standard conditions, the aortic intima-media samples were quickly removed, blotted to remove excess medium, and homogenized in 20 vol of chloroform-methanol (2 : 1, vol/vol); 3.3 vol of 0.29% NaCl was added to the homogenate and the mixture was allowed to separate into two phases. The lower phase was washed five times with 5.0 ml of the theoretical upper phase by the method of Folch et al. (24). One-half of the washed lower phase was evaporated to dryness in a liquid scintillation vial, Bray's solution (22) was added, and the sample counted with the use of an internal standard.

As indicated in the text, paired tissue samples were employed in these experiments, and the differences between the paired samples were analyzed for significance by the *t* test for paired comparisons (25). However, in some cases the mean and the standard error of the mean for groups were also computed to permit comparisons with data obtained in other series of experiments.

For examination by transmission electron microscopy the tubular segments of rabbit aorta were quickly drained of dissection or incubation medium and exposed to 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, at 4°C for 5 min. (All of the subsequent procedures were carried out at 4°C until the start of the dehydration procedure when the temperature was increased to 21°C.) The aortic segments were then cut in half along their longitudinal axis and transferred to fresh 2% glutaraldehyde in phosphate buffer and fixed for an additional 4 h. The tissue was postfixed in 2% osmium tetroxide in 0.02 M phosphate buffer, pH 7.4, for 2 h, and then washed three times for 10 min with water. The tissue was then stained en bloc with 2% uranyl acetate

in water for 2 h, washed three times with distilled water, dehydrated by exposure to graded concentrations of ethyl alcohol and then of propylene oxide. The tissue was embedded in Epon 812 (Shell Chemical Co., Div. of Shell Oil Co., New York) and cured for 48 h at 60°C. Thin sections were cut with an LKB microtome equipped with a diamond knife (LKB Instruments, Inc., Rockville, Md.); the sections were mounted on uncoated grids and sequentially stained with alcoholic uranyl acetate for 5 min, and with lead citrate for 12 min, and examined with a Siemens Elmskop I electron microscope (Siemens Corp., Iselin, N. J.).

RESULTS

Preparation I. Tubular segments of rabbit thoracic aorta free of adventitia and adipose tissue were prepared by the technique previously described and immediately fixed for electron microscopy. In these freshly prepared samples of preparation I, marked and widespread alterations in the structure of the endothelial cells, including loss of cell integrity, were consistently observed (Fig. 1). In contrast, no significant alterations were present in the EM appearance of the vascular smooth muscle cells (Fig. 1).

Samples of preparation I were incubated for 2 h with 5 mM glucose in KRB, gas phase 5% CO_2/air, pH 7.4, at 37°C as in the previously reported studies of this preparation and then fixed for EM examination. Large zones of the luminal surface were found to be denuded of endothelium, and the endothelial cells present in other areas exhibited a wide spectrum of ultrastructural alterations (Fig. 2). In contrast, the vascular smooth muscle cells retained a normal EM appearance (Fig. 2). The loss of an intact endothelium appears to occur rapidly after the initiation of incubation, for large areas denuded of endothelium were consistently present in samples incubated for 30 min.

The biochemical parameters commonly employed to assess the viability and stability of aortic intima-media preparations gave no intimation of the marked alterations in endothelial cell structure present in preparation I at the outset or of their progression during subsequent incubation. Under the conditions employed in these experiments, O_2 uptake by preparation I is linear when initially determined after a 15-min equilibration period and remains unaltered during a 2-h incubation (1). Glucose uptake, lactate production, and $^{14}CO_2$ production from [U-^{14}C]glucose also remain linear for periods of 2–3 h (1–4).

Preparation II: intact aortic intima-media. The method for preparing segments of intact rabbit aortic intima-media (preparation II) was developed from a systematic study of modifications of our previous technique; it is detailed in Methods. Freshly prepared samples of preparation II contain an intact endothelial cell layer whose cells retain the EM appearance observed in samples of aorta fixed *in situ* (Fig. 3). The EM appear-

ance of the vascular smooth muscle cells in freshly prepared samples of preparation II is also unaltered (Fig. 3).

The modifications of our previous technique incorporated into the method for preparation II had the following origins. We initially focussed upon the conditions under which the tissue was dissected since EM examination of aortae fixed without prior dissection demonstrated the presence of endothelial cells which resembled those observed in tissue fixed *in situ*. At the outset, glucose (5 mM) was added to the dissection buffer to obviate possible substrate depletion, and, to minimize possible mechanical injury, the aorta was cut into segments while still immersed in buffer. These modifications did not alter the EM appearance of freshly dissected samples; they were, however, retained as potential safeguards in all subsequent studies.

The addition of 6% BSA to the cold dissection buffer resulted in significant improvement in the EM appearance of the endothelial cells, but their appearance still differed significantly from that found in tissue fixed *in situ*. Exposure to cold buffer does not appear to be a primary factor in inducing endothelial cell injury during the dissection procedure for the endothelial cells in samples dissected in KRB at 37°C without added BSA showed marked alterations indistinguishable from those present in preparation I. However, some additive improvement in the appearance of the endothelial cells was observed in samples dissected in KRB containing 6% BSA at 37°C when compared with samples dissected in the same buffer at 4°C. We concluded that both the addition of 6% BSA and maintaining the temperature at 37°C were desirable modifications for our purpose; however, the endothelial cells in the resulting preparations still differed from those in tissue fixed *in situ* with regard to variable degrees of cell swelling and alterations in the appearance of their organelles. Increasing the BSA concentration to 8% or the substitution of 6% dialyzed rabbit serum did not obviate these differences.

We recognized that in the course of handling and decapitating unanesthetized rabbits, we might induce alterations in circulation, in the blood levels of vasoactive substances and other parameters which might adversely affect the ability of the endothelium to adapt to an artificial environment and to withstand the mechanical trauma of the dissection. We found that sedating the rabbits with diazepam (see Methods) resulted in significant additive improvement in the EM appearance of endothelial cells in tissue dissected in KRB-6% BSA at 37°C. These cells differed from those observed in tissue fixed *in situ* primarily by the presence of minor degrees of cell swelling in most of the samples examined. Pretreatment with diazepam was incorporated into the method on this basis. To minimize the possible effects of

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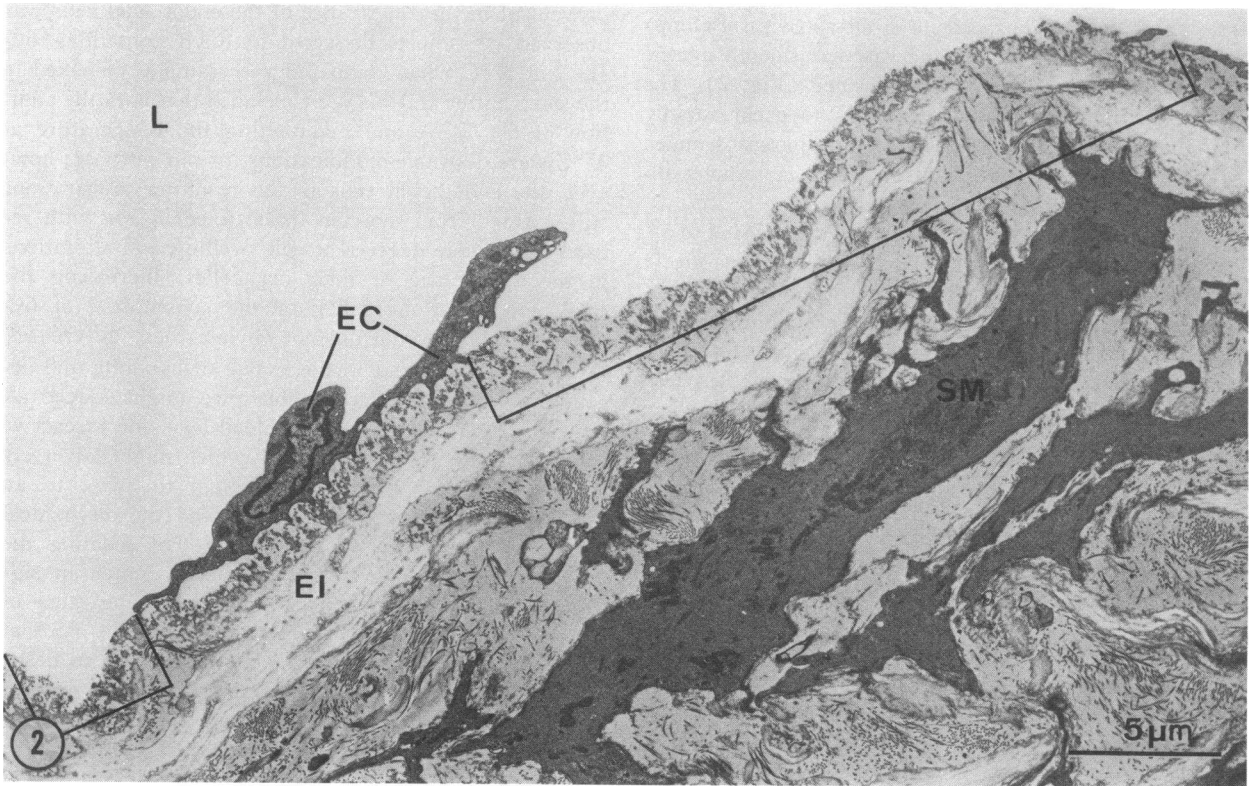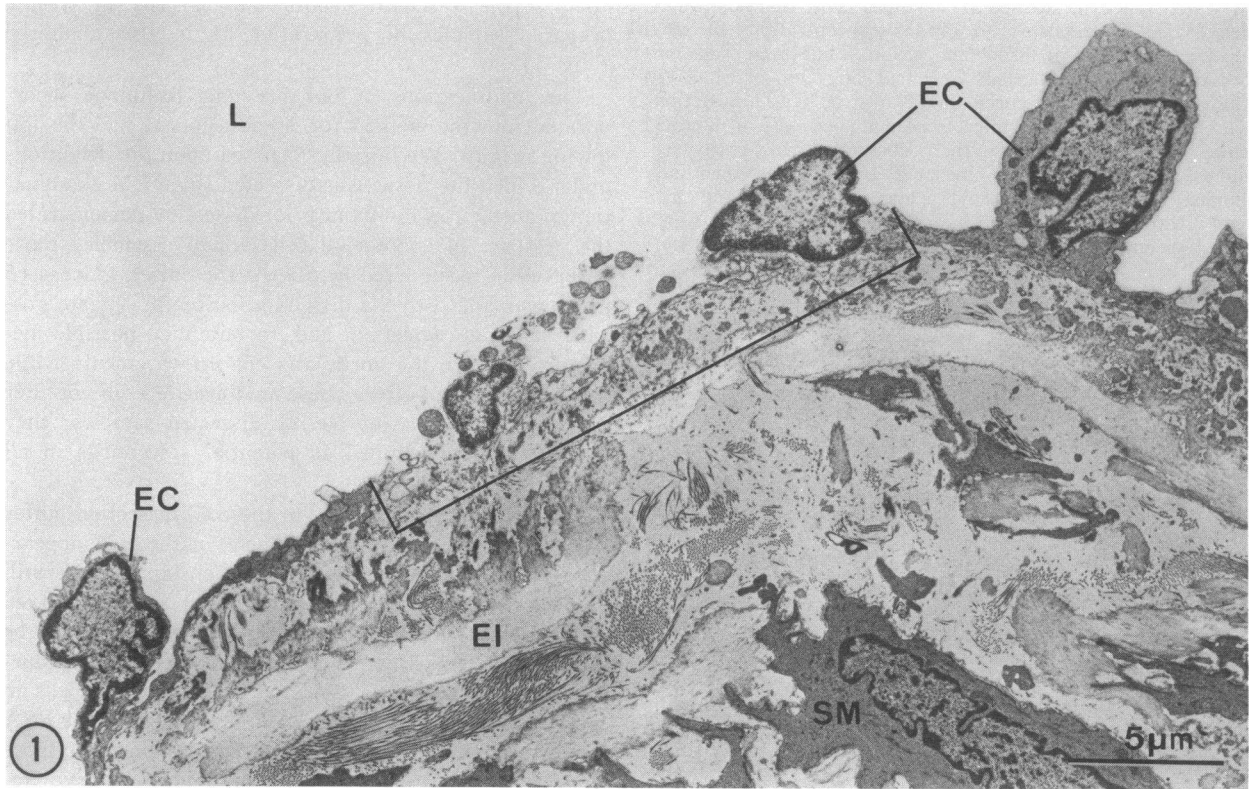
α-adrenergic stimulation at the time of sacrifice we examined the effects of pretreating the rabbits with phentolamine and diazepam before dissection in KRB-6% BSA at 37°C; the resulting preparations consistently contained an intact endothelium whose EM appearance was essentially indistinguishable from that in tissue fixed *in situ.*

As the result of our initial studies of the O_2 uptake of preparations containing an intact endothelial cell layer we introduced the routine use of 5% CO_2/95% O_2 as the gas phase instead of 5% CO_2/air; this modification is not required to preserve an intact endothelium in freshly prepared samples but was considered an appropriate safeguard in view of the relatively high respiratory rate of the tissue *(vide infra)*. The method for preparation II given in Methods has consistently yielded samples of aortic intima-media whose EM appearance resembles that illustrated in Fig. 3 when reexamined at intervals during a 1-yr period.[a]

When freshly prepared samples of preparation II were incubated for 1 h with 5 mM glucose in KRB containing 6% dialyzed BSA equilibrated with 5% CO_2/95% O_2 at

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[a] Before preparing this report we reexamined the value of the major modifications introduced into the method which we had employed for the studies included. We reconfirmed all of our previous observations with one exception. The additive effect of diazepam pretreatment on the EM appearance of tissue dissected in KRB containing 6% BSA at 37°C was still easily demonstrable; however, these preparations very closely resembled those of tissue fixed *in situ,* and the minor degree of cell swelling previously observed was present in only rare instances. Under these conditions we could not consistently distinguish between the appearance of the endothelium in tissue from rabbits pretreated with diazepam alone or with diazepam plus phentolamine in samples prepared on the same day. While the technique given in Methods includes phentolamine pretreatment, and has consistently given a preparation with the desired EM appearance during a prolonged trial, we cannot say that phentolamine pretreatment is necessary. We have been unable to identify the source of this improvement in the quality of the preparation from rabbits pretreated with diazepam alone and it does not appear to be related to the use of 5% CO_2/95% O_2 as the gas phase, but it may be related to the greater experience of the workers handling the tissue.

37°C and then fixed for EM examination, we consistently observed the preservation of an intact endothelial cell layer; only in isolated instances were discontinuities observed. The aortic endothelial cells retained an essentially normal appearance (Fig. 4). The vascular smooth muscle cells also retained a normal EM appearance after the 1-h incubation, and there were no significant alterations in other aspects of the EM appearance of the inner aortic wall (Fig. 4).

Respiration and glucose utilization of intact aortic intima-media. To examine these aspects of the metabolism of preparation II, paired, pooled samples were incubated for periods not exceeding 1 h under a set of standard conditions shown to be associated with an intact intima-media throughout the period of study. The incubations were carried out at 37°C in 25-ml Erlenmeyer flasks containing 5.0 ml of KRB, pH 7.4, gas phase 5% CO_2/95% O_2. The KRB contained 5.0 mM glucose and 6% dialyzed BSA. Tissue weight was not permitted to exceed 100 mg per flask. Under these standard conditions preparation II exhibited a linear rate of O_2 uptake over a period of 1 h, and there was no significant difference in the O_2 uptake of paired samples from the same rabbit. The O_2 uptake of preparation II incubated under the standard conditions for 30 min averaged 196±16 μl O_2/g wet wt per h, and the O_2 uptake of paired samples incubated for 60 min averaged 194±16 μl O_2/g wet wt per h; the mean Δ in O_2 uptake of paired samples from the same rabbit examined after 60 and 30 min of incubation was −2±7 μl/g wet wt per h (P = NS; $n = 6$).

The O_2 uptake of preparation II during incubation with 5 mM glucose under the standard conditions was approximately 50% higher than that previously observed when preparation I was incubated with 5 mM glucose; the O_2 uptake of preparation I averaged 129±7 μl O_2/g wet wt per h in KRB equilibrated with 5% CO_2/air and the rate was similar with 5% CO_2/95% O_2 (1). (The incubation conditions employed in previous studies of preparation I are given in Methods.) Samples of preparation I were incubated with 5 mM glucose under the standard conditions; after a 30-min incubation O_2 uptake

FIGURE 1   Electron micrograph of the intimal and subintimal regions of a tubular segment of rabbit thoracic aorta which had been dissected free of adventitia and adipose tissue in cold KRB buffer, pH 7.4, gas phase 5% CO_2/air. The tissue was fixed 7 min after the sacrifice of the rabbit at the point at which preparation I is ready for transfer to the incubation medium. In some endothelial cells (EC) there is breakage of cell membranes with leakage of organelles (underlined area). The subjacent smooth muscle cells (SM) appear unaffected. EI, elastica interna; L, aortic lumen. Magnification × 4,000. Line equals 5 μm.

FIGURE 2   Electron micrograph of the intimal and subintimal regions of a tubular segment of rabbit thoracic aorta prepared by the technique employed in preparation I which was fixed after a 2-h incubation at 37°C in KRB, pH 7.4, gas phase 5% CO_2/air, containing 5 mM glucose. The endothelium has disappeared (underlined area) exposing the elastica interna (EI) to the luminal surface (L) except for one area in which an intact endothelial cell (EC) is seen. The smooth muscle cells (SM) appear unaltered. Magnification × 4,000. Line equals 5 μm.

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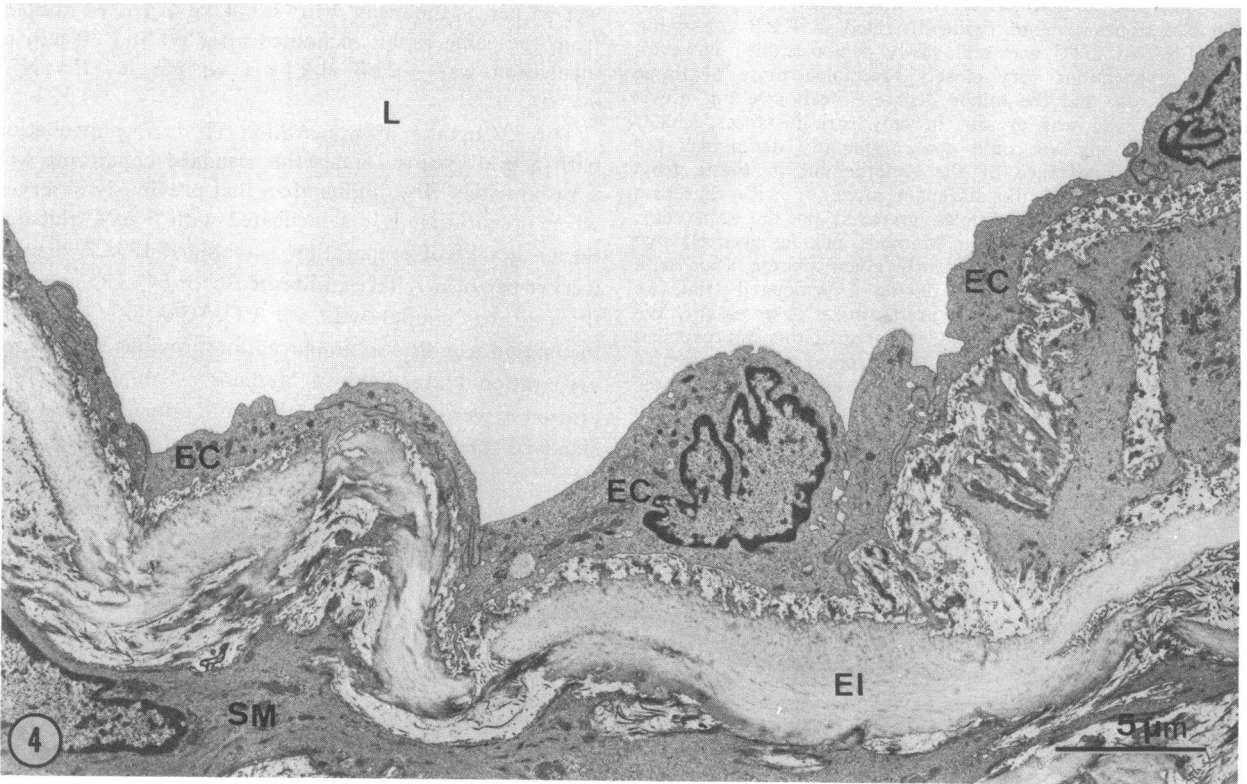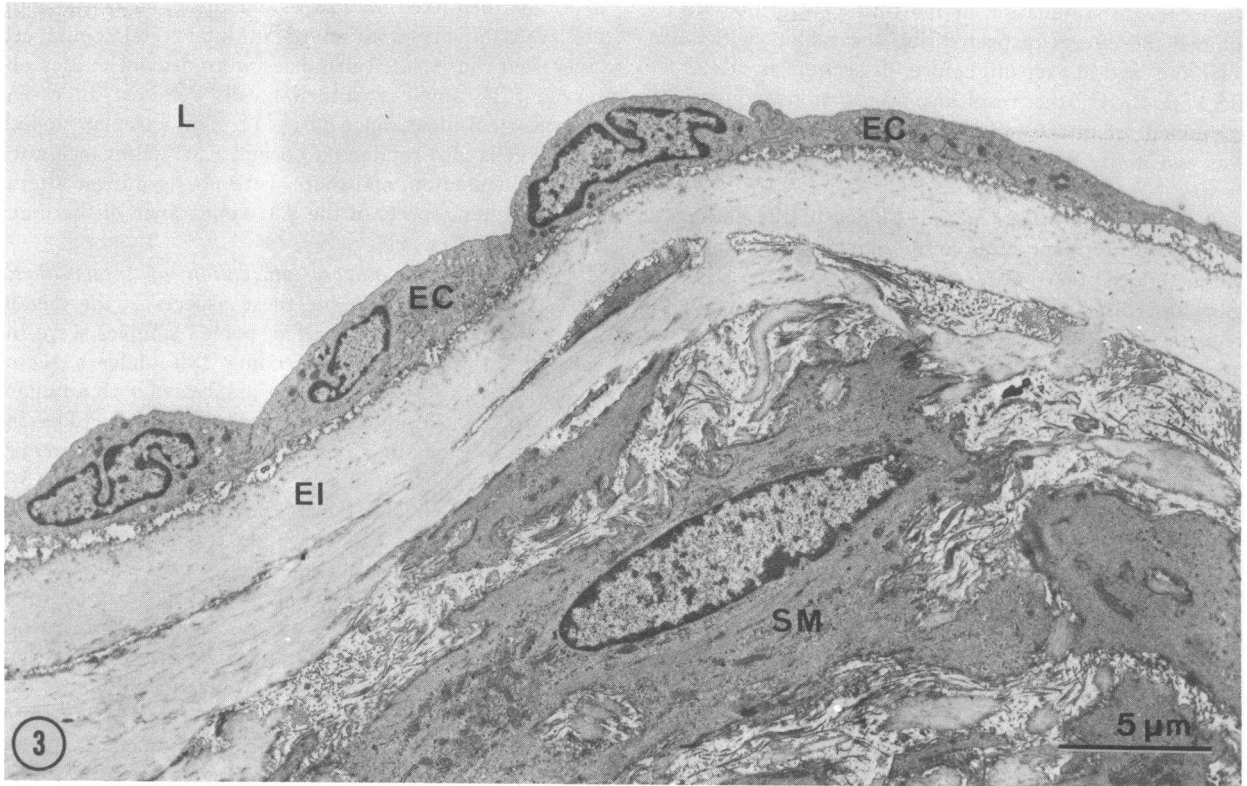
averaged 123±9 μl O₂/g wet wt per h (n = 6), and there was no significant difference in the O₂ uptake of paired samples examined after a 1-h incubation. The differences in incubation conditions do not, therefore, appear to account for the higher O₂ uptake of preparation II.

To exclude the possibility that the higher respiratory rate of preparation II is primarily a consequence of the pharmacologic agents given to the rabbits before sacrifice, rabbits were pretreated with diazepam and phentolamine but their aortae then dissected under the conditions described for preparation I. On EM examination there were marked alterations in the endothelial cells in these preparations, and when the samples were examined after incubation under the standard conditions for preparation II large zones were denuded of endothelium. These samples exhibited a linear rate of O₂ uptake that averaged 126±4 μl O₂/g wet wt per h after a 30-min incubation under the standard conditions and 124±7 in paired samples incubated for 1 h (n = 6). These values are significantly lower than those observed in the intact aortic intima-media preparation. After the recent recognition that pretreatment with phentolamine is no longer consistently required in our hands to obtain an intact aortic intima-media preparation, the possible contribution of phentolamine pretreatment to the high O₂ uptake of preparation II was reexamined. Samples of preparation II were prepared but without the use of phentolamine; the presence of an intact endothelium similar to that of tissue fixed *in situ* was documented. After a 1-h incubation under the standard conditions the O₂ uptake of these samples averaged 210±7 μl O₂/g wet wt per h (n = 6). This rate is not significantly different from that observed when samples of preparation II are incubated under the same conditions (*vide supra*).

The O₂ uptake of samples of preparation II incubated under the standard conditions but with the substitution of "essentially FFA-free BSA" for the BSA normally employed averaged 192±19 μl O₂/wet wt per h after 30

min, and 197±13 in paired samples incubated for 1 h (n = 6). These values are virtually identical to the O₂ uptake of preparation II in medium containing the BSA normally employed; the latter consistently resulted in FFA concentrations of less than 0.200 meq/liter.

The relatively high O₂ requirements of preparation II require careful attention when modifications in the standard incubation conditions are attempted. When paired samples of preparation II were incubated for 30 min under the standard conditions but with one sample from each pair in medium equilibrated with 5% CO₂/air rather than with 5% CO₂/95% O₂, and the O₂ uptake then determined in fresh medium equilibrated with the higher oxygen tension, the samples incubated with 5% CO₂/air consistently exhibited a linear O₂ uptake that was significantly lower than that of its paired sample; mean Δ of six paired samples was −30±3 μl O₂/g wet wt/h (P < 0.001). This suggests that inadequate provision of the O₂ requirements during incubation of the tissue may impair the integrity of the tissue. It is possible to preserve a linear O₂ uptake for 1 h in samples of preparation II incubated in the standard incubation medium but equilibrated with 5% CO₂/air; however, this requires that the medium to tissue ratio be increased to 25 ml/100 mg of tissue and that the incubation be carried out in a 125-ml Erlenmeyer flask. Under these conditions the O₂ uptake determined in fresh medium with the same gas phase averaged 192±7 μl O₂/g wet wt per h (n = 8) after a 1-h incubation, a value that is virtually identical to that observed in preparation II after a 1-h incubation under the standard conditions, in which 5% CO₂/95% O₂ is the gas phase.

Glucose uptake by preparation II during a 1-h incubation with 5 mM glucose under the standard conditions (Table I) was more than twice that previously observed when samples of preparation I were incubated with 5 mM glucose in KRB equilibrated with 5% CO₂/air; the latter rate averaged 11.6±0.7 μmol/g wet wt during a 2-h incubation (1).

FIGURE 3 Electron micrograph of the intimal and subintimal regions of a tubular segment of rabbit thoracic aorta dissected free of adventitia and adipose tissue under the conditions described for the preparation of intact aortic intima-media (preparation II). The rabbits were sedated with diazepam and given phentolamine i.m. before sacrifice; the dissection was carried out at 37°C in KRB buffer, pH 7.4, gas phase 5% CO₂/95% O₂, containing 5% dialyzed BSA and 5 mM glucose. The tissue was fixed 7 min after the rabbit was sacrificed at the time that preparation II is ready for transfer to the incubation medium. The aortic endothelial layer is intact and the endothelial cells (EC) are well preserved. The subintimal space and smooth muscle cells (SM) are normal in appearance. EI, elastica interna; L, aortic lumen. Magnification ×4,000. Line equals 5 μm.

FIGURE 4 Electron micrograph of tubular segment of intact rabbit aortic intima-media preparation (preparation II) fixed after a 1-h incubation at 37°C in KRB buffer, pH 7.4, gas phase 5% CO₂/95% O₂, containing 6% dialyzed BSA and 5 mM glucose. The endothelial cells (EC) retain an essentially normal appearance. The endothelial cell layer is intact (only in isolated instances were some discontinuities in the endothelial lining observed). The smooth muscle cells (SM) are normal in appearance. EI, elastica interna; L, aortic lumen. Magnification × 4,000. Line equals 5 μm.

TABLE I

Glucose Utilization by Intact Aortic Intima-Media

Glucose uptake	(16)	13.6 ± 1.3 μmol/g wet wt per h
Lactate production	(16)	3.44 ± 0.24 μmol/g wet wt per h
% of glucose uptake accounted for by lactate production	(16)	$14.4 \pm 1.6\%$
$^{14}CO_2$ production from [U-^{14}C]glucose*	(12)	0.077 ± 0.006 μmol glucose/g wet wt per 30 min
	(12)	0.177 ± 0.013 μmol glucose/g wet wt per h
Incorporation of [U-^{14}C]glucose into total lipid fraction	(6)	0.010 ± 0.002 μmol glucose/g wet wt per h
Incorporation of [U-^{14}C]glucose into glycogen	(6)	0.076 ± 0.009 μmol glucose/g wet wt per h

Intact aortic intima-media samples were incubated with 5 mM glucose in KRB, pH 7.4, gas phase 5% CO_2/95% O_2 containing 6% dialyzed BSA; all incubations were for 1 h except for the experiments (*) in which paired samples were incubated for 30 min and 1 h to examine $^{14}CO_2$ production. The specific activity of the medium glucose when [U-^{14}C]-glucose was present was 0.10 mCi/mmol.

Lactate production by preparation II during a 1-h incubation with 5 mM glucose under standard conditions (Table I) accounted for only $14.4 \pm 1.6\%$ of the glucose uptake by the same tissue samples; thus, the intact aortic intima-media preparation does not exhibit a high rate of aerobic glycolysis during incubation under conditions in which it retains a normal EM appearance. In contrast, lactate production accounted for approximately 45% of the glucose uptake by samples of preparation I incubated with 5 mM glucose in KRB 5% CO_2/air (1), and other workers have reported that lactate production consistently accounts for 50–80% of the glucose uptake by rabbit aortic intima-media preparations under similar conditions (10, 17).

Intact aortic intima-media samples contained glycogen in concentrations equivalent to 2.56 ± 0.21 μmol of glucose/g wet wt ($n = 16$). Consequently, the data in Table I on the recovery of ^{14}C from [U-^{14}C]glucose in CO_2, total lipid, and glycogen after a 1-h incubation with 5 mM glucose under the standard conditions must be considered minimal estimates. The rate of $^{14}CO_2$ production was essentially linear over a 1-h period (Table I). Despite the restrictions on the quantitative interpretation of the ^{14}C data in Table I, they suggest that glucose oxidation to CO_2 and incorporation into total lipid and glycogen account for only a small fraction of the glucose uptake in intact aortic intima-media from normal fed rabbits under the standard incubation conditions.

A marked Pasteur effect has not been consistently observed in the previously reported aortic intima-media preparations whether assessed by changes in glucose uptake or lactate production (9). We were previously unable to demonstrate any significant increase in glucose uptake by samples of preparation I when incubated with 5 mM glucose in KRB in an atmosphere of 5% CO_2/N$_2$ as compared with samples incubated in an atmosphere of 5% CO_2/air. Scott et al. (15) reported that anaerobic conditions resulted in a 50% increase in lactate production by a swine aortic intima-media preparation during

a 40-min incubation with 8.3 mM glucose; the demonstration of this effect required both the avoidance of chilled buffers and the initiation of incubation within 10 min of sacrifice or the effect was not observed.

When preparation I is incubated with elevated glucose concentrations (20–50 mM) for 2 h in KRB 5% CO_2/air, there is an approximately 50% increase in lactate production associated with a decrease in O_2 uptake; under these conditions O_2 diffusion appears to be limiting for respiration (1). As shown in Table II, the intact aortic intima-media preparation exhibited a 36% increase in glucose uptake and a 380% increase in lactate production when exposed to an atmosphere of 5% CO_2/95% N$_2$ during a 1-h incubation with 5 mM glucose under otherwise standard incubation conditions. This significant increase in glucose uptake and marked increase in lactate production in response to anoxia is in marked contrast to the effects reported in other mammalian aortic intima-media preparations under comparable conditions (9).

DISCUSSION

Our previous aortic intima-media preparation met the biochemical criteria usually employed to assess the viability and stability of such in vitro tissue systems, and its metabolic activities conformed to the pattern considered characteristic of rabbit, rat, and swine aortic intima-media. It is, however, clearly unsuitable for in vitro studies of aortic metabolism since the aortic endothelial cells appear to be subjected to marked and potentially irreversible injury during the preparation of the tissue, and large areas are rapidly denuded of endothelium during the subsequent incubation. The discrepancy between the stable rates of respiration and glucose metabolism in preparation I and these striking morphological alterations cannot be explained with certainty. The contribution of the endothelium to the composite metabolism of the tissue may normally be so small that its loss is undetectable alternatively, the endothelium may have ceased

TABLE II

Effect of Anaerobiosis on Glucose Uptake, Glycolysis, and
Tissue Lactate and Pyruvate of Aortic Tissue
Incubated for 1 h in 5 mM Glucose

5% CO_2/O_2	5% CO_2/N_2	Mean Δ of paired experiments $\pm$SE	P
Glucose uptake, $\mu mol/g$ wet wt per h			
11.82$\pm$1.14	16.11$\pm$1.61	+4.30$\pm$1.68	<0.05
Lactate production, $\mu mol/g$ wet wt per h			
3.60$\pm$0.48	13.81$\pm$2.54	+10.21$\pm$2.45	0.005
Pyruvate production, $\mu mol/g$ wet wt per h			
1.12$\pm$0.14	0.88$\pm$0.13	−0.24$\pm$0.13	NS
Tissue lactate, $\mu mol/g$ wet wt			
4.57$\pm$1.57	5.89$\pm$1.50	+1.31$\pm$0.53	<0.05
Tissue pyruvate, $\mu mol/g$ wet wt			
0.14$\pm$0.02	0.08$\pm$0.02	−0.06$\pm$0.01	<0.001

Animals were pretreated and descending thoracic aortae were prepared as described in the text. Eight paired aortic samples were incubated at 37°C for 1 h in KRB-CO_2/O_2 or N_2 containing 6% dialyzed BSA and 5 mM glucose.

to make a detectable contribution by the time that the initial determination of O_2 uptake was made after a 15-min equilibration period. The vascular smooth muscle cells in preparation I retain a normal EM appearance throughout a 2-h incubation; these cells constitute the bulk of the cellular mass of aortic intima-media, and it seems likely that they are primarily responsible for the stable metabolic activities exhibited by preparation I. However, in the face of marked injury to one cellular component of the tissue it is difficult to exclude the possibility that the metabolism of the surviving cells has not been altered.

Preparation I exhibits the high rate of aerobic glycolysis that has been considered a potential artefact of in vitro tissue preparations since the time of Warburg; many tissues that do not normally exhibit a high rate of aerobic glycolysis have been observed to do so when injured or exposed to unphysiological conditions during in vitro studies. As a consequence, it is always difficult to assess the physiological significance of a high rate of aerobic glycolysis by a tissue in vitro for the possibility that this behavior is an artefact is difficult to exclude even when it is observed consistently by different workers (19). In the case of the mammalian retina, this required evidence that the tissue exhibits this pattern of metabolism in situ. For these well recognized reasons Lehninger (14), in reviewing the metabolism of the arterial wall, commented that it is essential to be cautious in evaluating the high rate of aerobic glycolysis of the arterial wall in vitro. The observation that a preparation of aortic intima-media that exhibits the high rate of aerobic glycolysis considered characteristic of this tissue during in vitro incubation shows EM evidence of marked

injury to one of its cellular components raises further questions concerning the physiological significance of this pattern of metabolism.

The intact aortic intima-media preparation (preparation II) appears to provide an appropriate tool for studies of the composite metabolism of the inner aortic wall; the extent to which such observations can be extended to other regions of the arterial system and to other species remains to be examined. The limitations of data derived from in vitro studies of segments of intact aortic intima-media are obvious; the independent contributions of the endothelial and vascular smooth muscle cells are not readily separated, and the preparation is not exposed to pulsatile perfusion. However, preparation II provides a tool to examine the metabolism of this tissue under conditions in which its differentiated cell types retain their usual anatomical relationships; data of this type are required to complement those derived from isolated cell types grown in tissue culture.

During incubation under conditions that preserve an essentially normal EM appearance of its cellular components the intact aortic intima-media has a pattern of metabolism that differs markedly from that presently attributed to this tissue. The O_2 uptake of preparation II is much higher than that observed with preparation I under the same conditions. The high respiratory rate of the intact aortic intima-media appears to be a characteristic of the tissue and not a consequence of the pharmacological agents given to the animal before sacrifice or to the presence of low concentrations of FFA in the incubation medium.

The possibility that the high respiratory rate of the intact aortic intima-media during incubation under the standard conditions is primarily a consequence of unphysiologically high oxygen tensions in regions of the media where a low oxygen tension may be the norm appears to be excluded by the fact that a virtually identical O_2 uptake is observed when the medium is equilibrated with 5% CO_2/air rather than the usual 5% CO_2/95% O_2. In addition, the intact aortic intima-media does not exhibit the high rate of aerobic glycolysis that has been observed in preparation I and other aortic intima-media preparations during in vitro incubation. These observations suggest that rabbit aortic intima-media can derive the major fraction of its energy requirements from respiration. This conclusion is supported by the marked Pasteur effect demonstrable in the intact aortic intima-media preparation; this is in contrast to the relatively modest, and frequently inconsistent, Pasteur effects observed in other rabbit, rat, and swine preparations of the same tissue (9). These observations indicate that a high rate of aerobic glycolysis is not an obligate pattern of metabolism in rabbit aortic intima-media and tends to weaken the case for its physiological significance, since the latter

has rested heavily upon the consistency with which a high rate of aerobic glycolysis had been observed when aortic intima-media preparations were incubated in vitro.

The conditions used to prepare our previous aortic intima-media preparation for incubation and to study its metabolism were similar to those now in common use. The status of the endothelium has not been documented by EM studies in the preparations that have provided most of our present information concerning the metabolism of the aortic wall. Since the commonly used biochemical parameters for the viability and stability of aortic intima-media did not reflect the extensive morphologic alterations in the endothelium found in our previous preparation, it would seem appropriate to suggest that the status of the endothelium in other commonly employed preparations of aortic intima-media should be reexamined. There would appear to be a possibility that much of our present information concerning the metabolism of the aortic wall may have been derived from preparations unsuitable for such studies, and this question merits rapid clarification.

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