The cause of the delay in uricase development is unknown. We hypothesized that it could have resulted from placental transport maintaining a low plasma concentration in the fetus. Birth would remove this mechanism and result in substrate excess and enzyme induction; however, we showed that fetal plasma urate concentrations are similar to those seen in the newborn (14) and higher than maternal values, essentially disproving our hypothesis. Possibly birth itself initiates development as it does microsomal development (3).

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19. This work was supported by New Faculty Grant #H-00047 (FBS).
20. Received for publication July 1, 1982.
21. Accepted for publication December 9, 1982.

0031-3998/83/1709-0716$02.00/0
PEDIATRIC RESEARCH
Vol. 17, No. 9, 1983
Printed in U.S.A.
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Studies on Bile-Salt-Stimulated Lipolytic Activity in Human Milk. II Demonstration of Two Groups of Milk with Different Activities

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Summary

Human milk samples were assayed for bile-salt-stimulated lipolytic activity in the presence of an unpurified and purified preparation of glycoconjugated bile salts and two patterns of activity were obtained. One group of milks (high activity) had similar activities (mean ± 1 S.D.) of 16.9 ± 3.5 (purified bile salts) and 17.2 ± 3.5 μmole•ml^{-1}•h^{-1} (unpurified bile salts) whereas another group (low activity) had a lower activity with the purified (4.2 ± 2.3 μmole•ml^{-1}•h^{-1}) than with the unpurified bile salt preparation (19.9 ± 4.0 μmole•ml^{-1}•h^{-1}).

Activities of the low activity milks (assayed with the purified bile salts) could be increased following storage at −20°C, sonication, or the preparation of an acetone ether powder and, in some cases, reached the maximal activity obtained with the unpurified bile salt preparation. These procedures had no effect on the activity of the high activity milks.

Recombination experiments, whereby the milk serum from a low activity milk was added to the milk fat of a high activity milk and *vice versa*, and also kinetic studies suggest that the differences in activities between the two groups result from differences in availability of the enzyme and are independent of the source of milk fat.

Human milk contains at least two different lipases. First, a lipase that requires added serum for activity and has properties similar to lipoprotein lipase (12) and second, a lipase that is stimulated by bile salts (11). The latter activity appears to be ideally suited for a physiologic role in the small intestine of the newborn. It remains active during passage through the stomach as it is stable above pH 3.5 and only slowly inactivated by pepsin (10). The optimal bile salt concentration for activity is around 2 mmole•liter^{-1} (9, 10), which is within the physiologic range found in the newborn (2, 5) and bile salts also protect the enzyme from tryptic inactivation (10).

In a previous paper (9) we described a procedure for the assay of the bile-salt-stimulated lipase activity using human milk as both the source of enzyme and substrate, and reported some properties of this enzyme and its nutritional implications. We now report some additional studies which suggest that in human milk, the bile-salt-stimulated lipase forms a close relationship not only with its substrate but also with other components of milk that may regulate its activity.

SUBJECTS AND METHODS

Subjects. In total, 59 samples of human milk were collected from 24 women from the 2nd day to the 72nd wk postpartum.

One woman provided 21 samples over this period. The milks were collected and handled as described previously (9).

Methods. The bile-salt-stimulated lipolytic activity of fresh human milk was assayed as described previously (9) and expressed as μmole fatty acid produced·ml milk$^{-1}\cdot$h^{-1}. Some fresh milks were aliquotted, stored at −20°C and included as quality control specimens in each assay run. In general, a milk was divided into six such aliquots and used over a period of approximately 6 wk. The coefficient of variation for the assay was found to be 13.0%.

During the study three preparations of glycoconjugated bile salts were used in the incubation mixture: (*1*) sodium glycocholate (Lot 57-B-0250 from Sigma Chemical Company Limited) was used as supplied (unpurified preparation); (*2*) the Sigma preparation was purified by preparative thin layer chromatography using the solvent system amyl acetate/propionic acid/*n*-propanol/water (4:3:2:1, v/v) (13). There was incomplete separation of the glycocholic acid from the taurodihydroxy bile salts and the preparation was found by gas-liquid chromatography (3) to consist of 73% glycocholate, 21% taurodeoxycholate and 6% taurochenodeoxycholate; and (*3*) a preparation of glycoconjugates (B129 supplied by Difco Laboratories) was purified by the procedure of Gidez (7) and found to consist of 82% glycodihydroxy and 18% taurodihydroxy bile salts. On a number of separate occasions lipolytic activities were compared using two different purified bile salt preparations (*2* and *3*). The results were always similar and, therefore, lipase activities will be referred to as those obtained with either the unpurified or purified bile salt preparation.

Preparations of milks with varying substrate concentration for kinetic studies were prepared as described previously (9). Recombination studies were also performed with the milk serum from one milk and the milk fat from another. The separation and recombination was performed as for the kinetic studies but without altering the amount of milk fat. Control experiments showed that quantitative recoveries of lipase activity, lipid, and protein concentrations were obtained using this procedure.

Some milks were sonicated in ice (Ultrasonic Limited) for 20 sec at maximum power before assay. Others were assayed after storage at −20°C for periods of 1 wk to 8 months.

An acetone ether powder preparation of the enzyme was prepared from whole skimmed milk by the method of Hernell and Olivecrona (12).

Total protein and lipid concentrations were estimated as described by Hall (8). The results are expressed throughout as mean ± 1 S.D.

RESULTS

Lipase activity in the presence of unpurified and purified glycoconjugated bile salts. In our previous communication (9), only data using the unpurified sodium glycocholate preparation, supplied by Sigma Chemical Company Limited, was presented. In general, however, the assays were performed with both the unpurified and purified bile salt preparations, and it became apparent that there were two groups of milks. One group, designated "high activity" milks, had similar activities and no consistent changes in activity with the two bile salt preparations (Fig. 1a). The mean activities of 15 milks were 16.9 ± 3.5 and 17.2 ± 3.5 μmole·ml$^{-1}\cdot$h^{-1} with the purified and unpurified bile salts, respectively. The other group of 24 milks (Fig. 1b), termed "low activity" had a much lower activity with the purified bile salt preparation (4.2 ± 2.3 μmole·ml$^{-1}\cdot$h^{-1}) than with the unpurified bile salts (19.9 ± 4.0 μmole·ml$^{-1}\cdot$h^{-1}). Both groups of milks showed similar activities with the unpurified bile salt preparation and this activity will be termed the "maximal lipase activity." Three of the low activity milks had activities of 1.0 μmole·ml$^{-1}\cdot$h^{-1} or less, which increased by 20–50-fold with the unpurified bile salt preparation. The activities of the other 21 milks increased by a mean factor of 4.9 ± 1.8.

The observation of two distinct groups of milks was not an artefact of the method as different quality control samples (both high and low activity milks) behaved consistently with the different bile salt preparations.

Effects of various physical procedures. A number of low activity milks were assayed with the two bile salt preparations before and after storage at −20°C or sonication. The results are expressed as % of maximal activity (Fig. 2). The six fresh milks assayed before

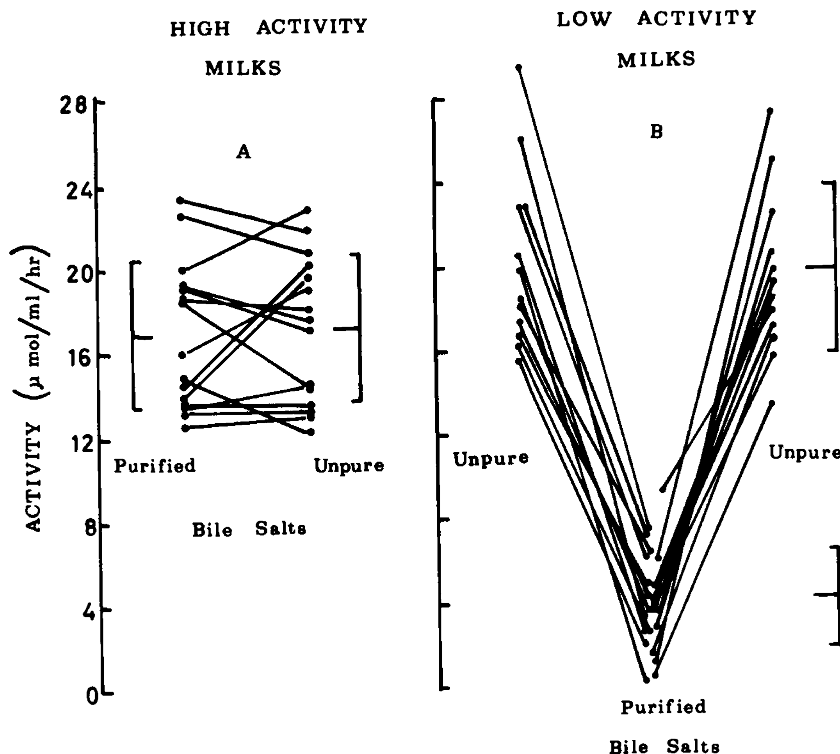

Fig. 1. The activity of the bile salt stimulated lipase in the presence of the purified or unpurified bile salt preparation. Each line joins the same milk assayed with the two bile salt preparations. The bars indicate mean activity ± 1 S.D.

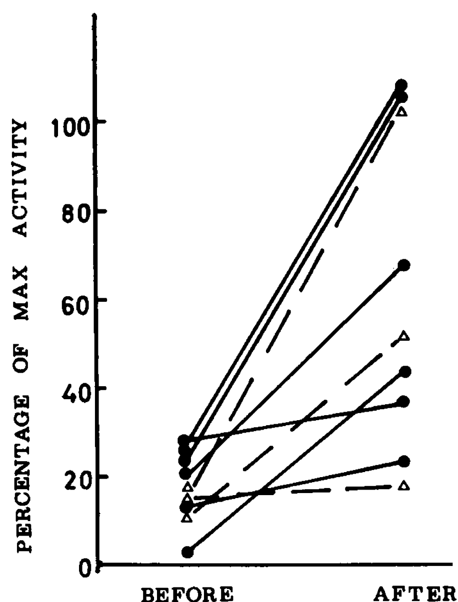

Fig. 2. The effect of storage at −20°C and sonication on the lipolytic activity of low activity milks assayed with the purified bile salt preparation. Each line joins the same milk assayed fresh and after the procedure. (●——●) stored at −20°C and (Δ— —Δ) sonicated. Maximum activity is that obtained with the unpurified bile salt preparation.

storage had a mean % of maximal activity of 18.5 ± 9.1%. After storage, two milks reached their maximal activity (104.5 and 107.5%, respectively), two were markedly stimulated (from 2.8 to 42.6% and 20 to 67.8%) and in two the activities were only marginally increased. A similar pattern was seen with the three milks that were sonicated. The two procedures had no significant effect on the maximal activities of the low activity milks or on the activities of the high activity milks assayed with either bile salt preparation.

An acetone ether powder preparation from a low activity milk gave an activity of 30.2 μmole·ml^{-1}·h^{-1} in the presence of purified bile salts whereas the fresh milk assayed in the routine manner with the same bile salt preparation had an activity of 6.8 μmole·ml^{-1}·h^{-1}.

Recombination experiments. To examine whether the difference in activities of the low and high activity milks resulted from differences in the properties of either the milk fat or milk serum fractions, recombination experiments were carried out with fresh samples of the two types of milks. Two studies were carried out whereby the milk serum from a low activity milk was added to the milk fat of a high activity milk and *vice versa.* On both occasions the activity followed the milk serum fraction and was independent of the source of milk fat. The results from one study assayed with the purified bile salt preparation are shown in Table 1.

Kinetic studies. To further examine the milk serum, kinetic studies were undertaken with both the low and high activity milks, using both bile salt preparations. The low activity milk showed Michaelis Menten type kinetics with both bile salt preparations. The Eadie-Hofstee plots are shown in Figure 3. The apparent Vmax was approximately 4.5 times greater with the unpurified compared to the purified bile salt preparation (18.3 and 4.0 μmole·ml^{-1}·h^{-1}, respectively) whereas the apparent Km's were virtually identical (5.0 and 4.8 μmole·liter^{-1}, respectively) under the two conditions.

The high activity milk gave identical results with the two bile salt preparations but on two separate occasions did not show Michaelis Menten type kinetics above a substrate concentration of 50 mmole·liter^{-1}. The Eadie-Hofstee plot for the high activity milks is shown in Figure 4.

The apparent Vmax and Km calculated from the data up to 50 mmole·liter^{-1} gave results of 26.5 μmole·ml^{-1}·h^{-1} and 9.5 mmole·liter^{-1}, respectively.

Table 1. *The effect on lipolytic activity of recombining the milk serum from a low activity milk and the milk fat from a high activity milk and vice versa*

Milk	Lipase activity (μmole·ml^{-1}·h^{-1}) Purified bile salts	Unpurified bile salts
Low activity milk (A)	7.8	20.7
High activity milk (B)	16.7	15.5
Serum from A + lipid from B	7.0	18.2
Serum from B + lipid from A	15.0	16.7

The occurrence of high and low activity milks during lactation. Of the 59 milks assayed with purified bile salts 24 (40.7%) showed high activity and 35 (59.3%) low activity. The activities of these milks were also examined in relation to the stage of lactation. At the height of lactation (2–26 wk) 17 of 26 milks fell into the high activity group whereas early (first 2 wk) and late (after 26 wk) in lactation, only 2 of 11 and 5 of 22 milks respectively, fell into the high activity group. This trend was noted in milk samples obtained from individual women as well as in the group as a whole.

DISCUSSION

The bile-salt-stimulated lipase activity of human milk was estimated in a system using whole milk as source of both enzyme and substrate, and on the basis of the activities obtained with two different (purified and unpurified) bile salt preparations the milks could be divided into two distinct groups. One group of milks, designated high activity had similar activities with the two bile salt preparations. A second group of milks (designated low activity) had, on average, only 20% of the activity with the purified compared with the unpurified bile salt preparation. The activity obtained with the unpurified bile salt preparation was, however, similar to that found in the high activity milks with both bile salt preparations. Glycoconjugated bile salts were used throughout the study, as we (9) in contrast to others (10) have found that only glycoconjugated and not tauroconjugated bile salts activate the enzyme. Virtually identical activities were obtained with the two different purified bile salt preparations that consisted principally of glycocholic and glycodihydroxy bile salts, respectively and thus the reported observations do not depend on a particular species of glycoconjugate.

Theoretically, the different activities found with the low activity milks could have resulted from the presence of an activator in the unpurified bile salt preparation or an inhibitor in the purified preparation. The latter possibility seemed unlikely as the addition of both bile salt preparations to an assay of a low activity milk gave the maximum activity (22.8 μmole·ml^{-1}·h^{-1}) compared to activities of 22.3 and 3.7 μmole·ml^{-1}·h^{-1} when the unpurified and purified bile salts respectively were used on their own. The results, therefore, suggest that the unpurified bile salt preparation, which was prepared from ox bile, may contain a water soluble component that enables the low activity milks to reach their maximal activity.

Physical procedures such as storage of milk at −20°C and subsequent thawing, sonication and the preparation of an acetone/ether powder of the enzymatic activity also resulted in an increase in activity of the low activity milks when assayed with the purified bile salt preparation; approximately one-third of these milks achieving their maximal activity.

The different activities of the two groups of milks with the purified bile salt preparations could have resulted from differences in the substrate and in particular the membrane, which is known to surround the milk fat globules (15), or from differences in the enzyme, which is present in the milk fraction (11). The recombination studies showed, however, that the activity was related to

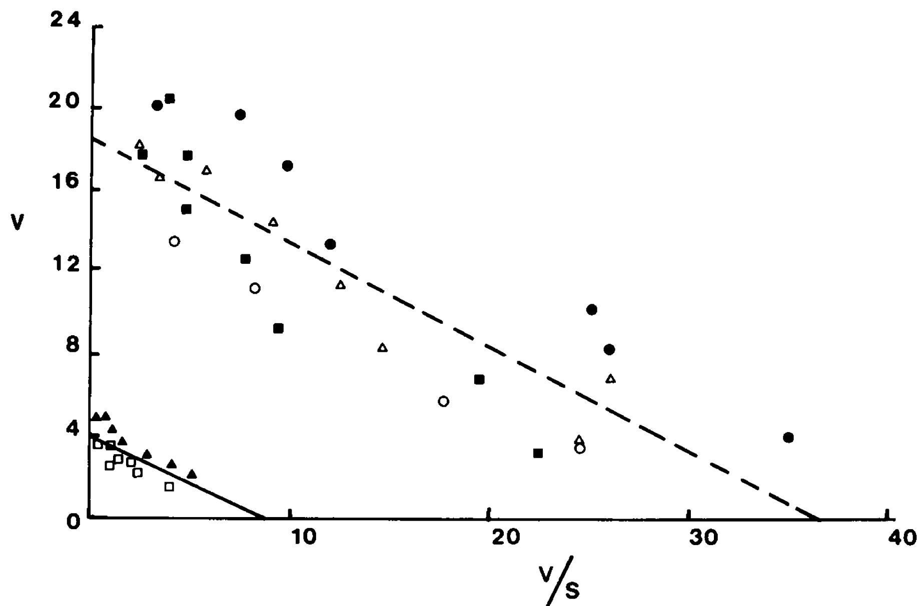

Fig. 3. Eadie-Hofstee plots of lipolytic activity (μmole·ml^{-1}·h^{-1}) as a function of lipolytic activity divided by the substrate concentration (mmol·liter^{-1}), using low activity milks with purified and unpurified bile salt preparations. The lipid concentration was the concentration after reconstitution of the separated lipid and milk serum. (● ○ ■ △ ▲ □) denote different milks. (— —) denotes unpurified bile salt preparation, Vmax = 18.3 μmole·ml^{-1}·h^{-1} and Km 5.0 mmole·liter^{-1}. (——) denotes purified bile salt preparation; Vmax = 4.0 μmole·ml^{-1}·h^{-1} and Km 4.8 mmole·liter^{-1}.

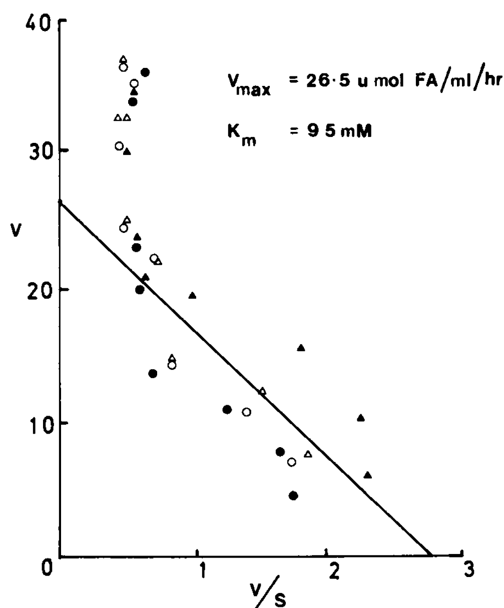

Fig. 4. Eadie-Hofstee plot using two high activity milks with both the purified (△ ▲) and unpurified (○ ●) bile salt preparations. Milk 1 (△ ○), Milk 2 (▲ ●).

the milk serum fraction and was independent of the substrate. This was confirmed by the kinetic studies.

The kinetic parameters obtained with the low activity milks (similar apparent Km's but a 4–5-fold increase in the apparent Vmax with the unpurified compared to the purified bile salts preparation) are typical of classical noncompetitive inhibition. This may suggest that after the addition of the unpurified bile salt preparation or after the various physical procedures more of the enzyme becomes available to the substrate. This hypothesis would be consistent with the recent studies of Blackberg and Hernell (1), who found a single protein band after extensive purification of the bile-salt-stimulated lipase. An understanding of the mechanisms involved must await further studies but one possibility is that a proportion of the lipase of the low activity milks is bound in some way and unavailable for activity. It could, for example, be complexed within casein micelles, a phenomenon which is known to occur in cow's milk (4, 14). Studies using pancreatic lipase have shown that 70% of activity was lost after its addition to whole or skimmed cow's milk and that the enzyme was bound within the casein micelles (4). It is possible, therefore, that a water soluble component of the unpurified bile salt preparation and the various physical procedures may disrupt this complex and release the lipase resulting in an increase in its apparent Vmax. Dimethylformamide has been used to dissociate the lipase of cow's milk from casein micelles (6) and it would, therefore, be of interest to study the effect of this agent on the bile salt stimulated lipase activity of low activity milks in the presence of the purified bile salt preparation.

The high activity milks gave identical values for Km and Vmax with both bile salt preparations suggesting that in these milks the enzyme was freely available. The observation that above 50 mmole·liter^{-1} the high activity milks gave greater activities than would be expected from Michaelis Menten type kinetics was a consistent finding. It may result from a factor present in the lipid fraction of the milk, which only promotes activity above a certain concentration.

The nutritional significance of these observations are uncertain. It could be that the various effectors of lipase activity are present in human bile and/or normal intestinal content and that as a result all milks achieve their maximal potential lipolytic activity *in vivo*. To answer this question it will be necessary to carry out detailed studies in the presence of duodenal contents from the newborn, and milk which has been exposed to gastric contents.

The possible presence of a lipid bound activator in the high activity milks which causes an increase in activity at substrate concentrations greater than 50 mmole·liter^{-1} might have a practical nutritional significance. These studies have shown that at the height of lactation (2–26 wk postpartum) high activity milks predominate; thus, the enzymatic activity would be well adapted to the high concentration of milk fat found during this period of lactation, which reaches a mean concentration of greater than 90 mmole·liter^{-1} at the end of a feed (9).

Further studies are, therefore, necessary before the physiologic significance of these two patterns of lipase activities in human milk are fully understood. It does appear, however, from these *in vitro* studies that the bile-salt-stimulated lipase activity of human milk is closely controlled by components found in both the milk

serum and lipid fractions. This study also emphasizes the importance of using an assay procedure which is as close as practicable to conditions *in vivo.*

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16. We wish to thank Dr. D. Harvey (Queen Charlotte's Maternity Hospital, London W.1.) for allowing us to study the milk from women under his care, the staff of Victoria Ward for their help and cooperation and the breast feeding promotion group of The National Childbirth Trust for putting us in contact with some of the women. We would also like to thank Professors O. H. Wolff and J. T. Harries for their helpful advice during the course of the study. BH is grateful to Farley Health Products Limited for financial support.
17. Requests for reprints should be addressed to: Dr. D. P. R. Muller, Department of Child Health, Institute of Child Health, 30 Guilford St., London, WC1N 1EN, England.
18. Dr. B. Hall thanks Farley Health Products Ltd. for financial support.
19. Received for publication June 1, 1982.
20. Accepted for publication December 9, 1982.

0031-3998/83/1709-0720$02.00/0
PEDIATRIC RESEARCH
Vol. 17, No. 9, 1983
Printed in U.S.A.
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Pulmonary Vascular Effects of Amrinone in Conscious Lambs

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Summary

The direct pulmonary vascular effects of amrinone, a nonglycoside, noncatechole cardiotonic agent were studied in conscious newborn lambs using a double-flow probe preparation that allows separation of direct and indirect pulmonary vascular effects. Amrinone was found to be a direct pulmonary vasodilator with a threshold dose of 0.3 mg/kg. Amrinone also increased cardiac output and decreased aortic pressure at somewhat higher threshold doses (1.0 mg/kg and 3.0 mg/kg, respectively). Amrinone's peak effect on pulmonary resistance appeared greater than its peak systemic effects. These effects were noted in both normoxia and hypoxia, and were not changed by pretreatment with propranolol.

The appearance of right ventricular enlargement and pulmonary arterial hypertension (cor pulmonale) is ominous when associated with chronic lung disease. The ideal therapy for cor pulmonale would be directed at the underlying lung disease, although this is seldom possible. For cardiovascular therapy to be most effective, it should provide positive inotropic effects and pulmonary vasodilitation. Traditional therapy has consisted of digitalis, diuretics, and oxygen. Although oxygen may contribute in lowering pulmonary vascular resistance, diuretics neither improve cardiac output nor dilate the pulmonary vessels. Although digitalis may improve cardiac output, it is a known pulmonary vasoconstrictor (10, 17) and is of questionable benefit in cor pulmonale (10).

An orally active inotropic agent capable of dilating pulmonary vessels might be particularly useful in the therapy of cor pulmonale. Amrinone, (Fig 1), a bipyridine derivative [5-amino-3,4'-bipyridine-6(1H)-one], has been shown recently to be a strong positive inotrope *in vitro* and *in vivo* in both animals (1, 2, 8) and in man (3, 6, 11). It is active both orally (11, 19) and intravenously, and does not produce arrhythmias even at high doses (14); however, its pulmonary vascular effects are unknown.

In the present study, we have investigated the pulmonary vascular effects of amrinone in lambs. Amrinone is known to have effects on cardiac output (3, 14, 19) and systemic vascular resistance (7). Because changes in these variables are known to indirectly alter calculated pulmonary resistance, (5, 9) the experimental design should allow separation of direct from indirect effects of a drug on the pulmonary circulation. The pulmonary vascular effects of amrinone were, therefore, studied using a double-flow probe preparation that allows determination of local pulmonary vascular responses *in vivo.*

MATERIALS AND METHODS

The advantages and limitations of the double-flow probe preparation that we used have been described in detail elsewhere (16).