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The efficacy of density gradients for islet purification: a comparison of seven density gradients

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Abstract. Seven different density gradient-forming materials were compared as to their efficacy for rat islet purification. Continuous density gradients were used in order to determine the bouyant densities of the different pancreatic tissue components. Hand-picked islets served as a control. A significant separation of large numbers of islets from the exocrine tissue band was only seen in the albumin, dextran-40, and metrizamide gradients. Pure islet preparations could not be obtained with any of the gradients studied as none of the gradients completely separated lymph nodes, vessels, and ducts from the islets. Gradients containing sucrose resulted in low islet yields. The islet yields obtained with the other gradients were in the same range as those obtained by hand-picking. Metrizamide gave significantly higher yields than the widely used Ficoll. Judged both in terms of numbers of islets and their in vitro function, the best results were obtained with metrizamide and dextran-40.

Key words: Pancreatic islet purification – Density gradients in pancreatic islet purification

The purity of the islet graft is of the utmost importance for the success of islet transplantation. Exocrine tissue contaminating the islets can impair islet implantation [7], probably due to inflammation around the graft, and cause loss of transplanted endocrine tissue [6]. Suboptimal endocrine mass is thought to be a cause of early loss of graft function after transplantation due to exhaustion of the beta cells [1]. Furthermore, in humans, portal hypertension and disseminated intravascular coagulation have been reported after transplantation of nonpurified pancreatic tissue grafts [17]. Lymph nodes, exocrine tissue [5], and, in humans, also ductal endothelium [4] have been shown to contain MHC class II positive cells. As MHC class II positive cells play an essential role in the initiation of allograft rejection [12], these tissues should also be removed from the islet graft. Islet grafts can only be considered to consist of pure islets when they are depleted of exocrine tissue, lymph nodes, vessels, and ducts.

Purification of large numbers of islets from the collagenase-digested pancreatic tissue suspension cannot, for logistical reasons, be performed by hand-picking of the islets. Various other techniques have been applied [19], but generally purification by density gradient centrifugation is used.

Ficoll density gradients [15, 20] are often used for the separation of large numbers of islets from the collagenase-dissociated pancreatic tissue suspension, but they insufficiently separate lymph nodes, vessels, and ducts from the islets. Several other gradient-forming substances with different physiochemical properties, such as viscosity and osmolarity, have been described [2, 9, 11, 13, 18]. Their suitability for islet purification is usually determined by the number of functionally intact islets obtained, the amount of exocrine tissue contaminating the islet tissue, and islet function in vitro or in vivo after transplantation. However, the capacity of density gradients to separate lymph nodes, vessels, and ducts from the islets is not mentioned.

The present study compares the efficacy of seven different density gradient-forming materials for rat islet purification.

Materials and methods

Experimental design

In each experiment, eight male and fed Wistar rats with a mean body weight of 350 g (range 345–355 g) were used. The pancreases were dissociated using a standard collagenase digestion procedure. All of the digested pancreatic tissue was pooled and suspended in a total volume of 48 ml Krebs' Ringer HEPES (KRH), to which 0.25% bovine serum albumin (BSA, Sigma) was added. The tissue suspension was transferred to a 60-ml syringe. The syringe was continuously tipped to keep the suspension homogeneous. The suspension was divided into eight equal parts by weight so that each aliquot corresponded to the amount of pancreatic tissue obtained from one rat

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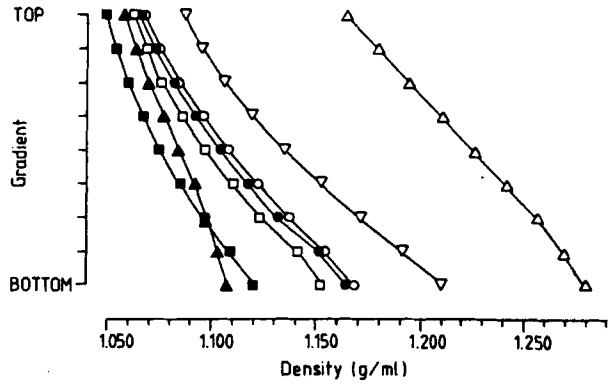

Fig. 1. Density distribution curves of the continuous density gradients as determined by weight per volume (g/ml) at regular intervals in the gradient. □—□, Ficoll; △—△, sucrose; ○—○, Ficoll-sucrose; ▲—▲, Percoll; ■—■, albumin; ●—●, dextran; ▽—▽, metrizamide

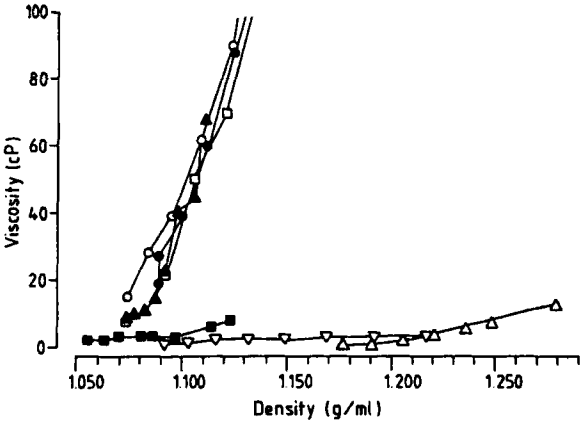

Fig. 2. Relation between the density and the viscosity of the gradients. □—□, Ficoll; △—△, sucrose; ○—○, Ficoll-sucrose; ▲—▲, Percoll; ■—■, albumin; ●—●, dextran; ▽—▽, metrizamide

pancreas after collagenase digestion. The aliquots were randomly allocated to the seven different density gradients or to the hand-picked control in each experiment.

Continuous density gradients were used in order to be able to determine the buoyant densities of the different pancreatic tissue components (i.e., islets, exocrine tissue, lymph nodes, vessels, and ducts). The density range of the gradients was chosen so as to include the buoyant densities of all the tissue components. After centrifugation, the buoyant level of the tissue bands in the density gradients was determined visually and compared to the graduation scale on the centrifuge tubes. The corresponding buoyant densities were read off the density distribution curves. The tissue bands were then carefully aspirated from the centrifuge tubes and inspected under a dissection microscope for confirmation of the previously identified pancreatic tissue components. We have refrained from further identification of the tissue bands by, for instance, dithizone staining or insulin and amylase determination in order not to interfere with the islet counting and the in vitro function tests. The islet band, i.e., islets and gradient material, was gradually diluted with KRH-0.25% BSA in order to avoid osmotic shock in case the gradients were not isoosmotic.

All islets with a mean diameter larger than 100 μm retrieved from the islet band in the gradients and the control aliquot were counted. Islets with smaller diameters were not included since their numbers vary widely with each isolation procedure and since they do not contribute substantially to the total islet mass [23]. Changes in islet function due to the gradient-forming materials was assessed by testing in vitro four aliquots of ten islets obtained from each gradient immediately after the purification procedure and by comparing the results with hand-picked control islets. The remaining islets were cultured overnight for 16 h in RPMI-1640 (Gibco), supplemented with 10% fetal calf serum and penicillin/streptomycin (Flow Laboratories). In order to assess whether the changes in islet function were reversible or permanent, four aliquots of ten islets of each gradient were tested after the culture period and compared with cultured, hand-picked control islets.

The exocrine tissue band was examined to determine the number of islets larger than 100 μm that had not been separated from the exocrine tissue. These islets were not tested in vitro.

Collagenase digestion

The collagenase digestion method applied is a slight modification of a previously described procedure [21]. The abdomen of the rat was opened by a midline incision. The common bile duct was identified and the proximal segment was cannulated. A clip was placed on the entrance of the bile duct into the duodenum, and the pancreas was subsequently distended with 10 ml KRH containing 10% BSA [22]. After pancreatectomy, the pancreases were cut into small pieces with a pair of scissors and washed 4–5 times with KRH-10% BSA. Tissue from two pancreases in a total volume of 8 ml was transferred to a 25-ml Erlenmeyer flask. To each Erlenmeyer flask, 5 ml collagenase solution was added. The collagenase solution was prepared by dissolving 100 mg collagenase (Sigma type XI, 2200 U/mg) in 32 ml KRH-10% BSA. The flasks were stoppered and incubated in a horizontal position at 37 °C in a Dubnoff metabolic incubator and shaken at 200 cycles per minute. After 4 min the flasks were removed from the incubator and vigorously shaken manually for 1 min. The flasks were returned to the incubator and incubated for another 4 min. After a 2nd minute of vigorous manual shaking, the digestion was stopped by adding 10 ml KRH-10% BSA at room temperature to each flask. The contents of the flasks were poured into 100 ml conical vessels and the tissue was washed and sedimented twice with KRH-10% BSA. The volume of tissue sediment and KRH-10% BSA was brought up to 10 ml, transferred to the Erlenmeyer flasks, and 3 ml collagenase solution was added. The tissue was incubated a second time for 4–7 min in the water-bath, followed by 1 min of manual shaking. The digestion was stopped by adding KRH-0.25% BSA and the tissue was washed and sedimented four times.

Density gradients

The gradient materials studied were Ficoll 400 DL (Sigma), sucrose (Merck), Ficoll + 0.25 m*M* sucrose, Percoll (Pharmacia), BSA (Sigma), dextran-40 (Sigma), and metrizamide (Nygard). We always used 40 ml of gradient material in 50 ml graduated conical centrifuge tubes (Falcon 2098). The stock solutions of the gradients were made by dissolving the substances in KRH. The pH was adjusted to 7.4, if necessary. The continuous gradients were made with the aid of a multichannel peristaltic pump (Ismatic mp13 GJ10) by continuous dilution of the stock solution with KRH. The tissue aliquot, suspended one-in-one (v/v) in the lowest density of the gradient, was layered on top of each gradient. The tubes were centrifuged for 20 min at 1500 *g* at 20 °C. At the end of the run, no brakes were applied in order to avoid vortexing in the gradients.

The density distribution in the gradients was determined by weighing samples of the gradient taken with a constriction pipette that had previously been calibrated with distilled water. The density was measured every 5 ml of the graduation on the centrifuge tube (Fig. 1). The viscosity distribution was also determined every 5 ml of the graduation on the centrifuge tube in a viscosity meter (Brabender Rheotron) and expressed as the viscosity for a given density of each gradient. With the results of these determinations, density and viscosity distribution curves for the whole gradient could be drawn (Fig. 2).

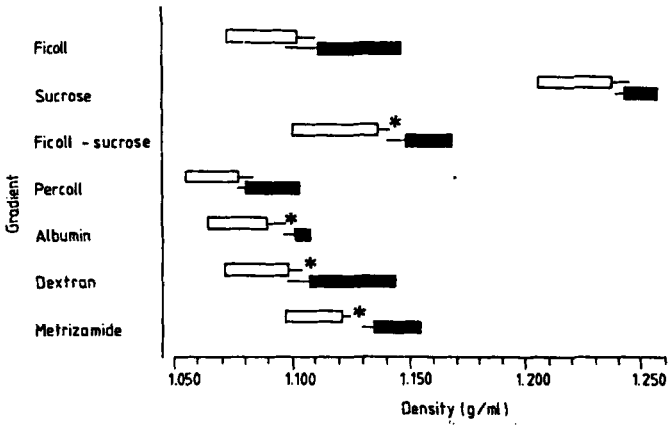

Fig. 3. Buoyant density (g/ml) of rat islets and exocrine tissue in continuous density gradients. Mean ± SEM limits of the islet band (□) and exocrine bands (■). * Significantly different

In vitro function

Determination of the islet function in vitro was performed by testing four aliquots of ten islets each. The islets were preincubated in 2 ml Krebs' Ringer bicarbonate (KRB), gassed with 95% O_2 and 5% CO_2, containing 0.25% BSA and 2.75 m*M* glucose. The in vitro function was then assessed by three consecutive incubations of 45 min each at 2.75 m*M*, 16.5 m*M*, and 2.75 m*M* glucose in KRB, respectively. At the end of each period, the incubation media were removed and frozen for insulin determination by a radioimmunoassay for rat insulin. After completion of the in vitro function tests, the islets were frozen for DNA content determination [10]. Insulin release was expressed as mU insulin/μg DNA/45 min.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using the unpaired Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

Results

The density ranges of each of the gradients were chosen so as to include, within each gradient, the buoyant densities of all of the different pancreatic tissue components. The density distribution of the gradients thus constructed are given in Fig. 1. It is obvious that much higher densities of sucrose were needed than any of the other gradient-forming substances. Both density and viscosity of a specific gradient solution are related to the concentration of the gradient-forming substance in the density gradient. In this study we measured the density and the viscosity simultaneously and expressed the viscosity of gradient in relation to the density distribution (Fig. 2). Two groups of substances can be distinguished by their viscosity characteristics. Solutions made of the high molecular weight materials – i.e., albumin, Ficoll, and dextran-40 – show a sharp increase in viscosity with increasing densities. Gradients made of sucrose and metrizamide, which have a low molecular weight, and of the colloidal Percoll show no more than a slight increase in viscosity with increasing densities.

Tissue bands containing islets, exocrine tissue, lymph nodes, vessels, and ducts could be identified in each gradient after centrifugation of the aliquot of pancreatic tissue. Most of the islets and exocrine tissue were found in two well-defined bands. Smaller amounts of islets and exocrine tissue were found at higher and lower densities, respectively. The upper and lower limits in the gradients where islet or exocrine tissue were found were determined visually. The corresponding densities of the gradient, read off the density distribution curves, are given in Fig. 3. The capacity of a gradient to separate islets from exocrine tissue could be illustrated by determining the difference between the highest density at which islets were found and the lowest density at which exocrine tissue was found. This difference was statistically significant when the gradients were made of Ficoll-sucrose, BSA, dextran-40, or metrizamide (Fig. 3).

Lymph nodes, vessels, and ducts apparently have almost the same density as islets. The mean buoyant density limits of lymph nodes, vessels, and ducts showed a considerable overlap with the density limits of the islet bands (Table 1). An exception to this finding were the Ficoll-sucrose gradients in which the tissue band containing the lymph nodes was sometimes separated from the islet band; the mean density limits of both bands are adjacent.

The number of islets larger than 100 μm in the hand-picked controls was 694 ± 75. The mean number of islets collected from the islet band of the Ficoll, Percoll, albumin, dextran-40, and metrizamide gradients was not significantly different from that of the hand-picked controls (Table 2). Ficoll-sucrose and sucrose gradients gave

Table 1. Mean buoyant density limits (g/ml) of rat islets, lymph nodes, vessels, and ducts in continuous density gradients

Gradient	Number of experiments	Islets	Lymph nodes	Vessels and ducts
Ficoll	8	1.072–1.102	1.070–1.077	1.085–1.109
Sucrose	5	1.205–1.237	1.195–1.219	1.216–1.243
Ficoll-sucrose	8	1.100–1.136	1.086–1.099	1.117–1.147
Percoll	8	1.055–1.077	1.055–1.061	1.053–1.062
Albumin	8	1.064–1.089	1.065–1.070	1.078–1.095
Dextran-40	8	1.071–1.098	1.068–1.073	1.084–1.109
Metrizamide	8	1.097–1.121	1.100–1.108	1.115–1.141

Table 2. Islet yield of the different continuous density gradients

	Number of experiments	Islet yield[a]	Number of islets in exocrine band (% of total)[a]
Hand-picked	8	694 ± 75	
Ficoll	8	628 ± 63	71 ± 14 (10 ± 2)
Sucrose	5	137 ± 49[b, c]	105 ± 36 (43 ± 15)
Ficoll-sucrose	8	400 ± 70[b, c]	160 ± 44 (29 ± 8)
Percoll	8	663 ± 58	27 ± 12 (4 ± 2)
Albumin	8	718 ± 51	32 ± 12 (4 ± 2)
Dextran-40	8	739 ± 66	39 ± 16 (5 ± 2)
Metrizamide	8	800 ± 73[c]	37 ± 17 (4 ± 2)

[a] Only islets larger than 100 μm were counted; expressed as mean ± SEM

[b] Significantly different from hand-picked control ($P < 0.05$)

[c] Significantly different from Ficoll ($P < 0.05$)

Table 3. Insulin release of rat islets after density gradient purification tested immediately after density gradient purification

Gradient	Number of experiments	Insulin release[a] at successive incubations with		
		2.75 m*M* glucose	16.5 m*M* glucose	2.75 m*M* glucose
Control	8	0.35 ± 0.05	2.70 ± 0.35	0.58 ± 0.09
Ficoll	8	0.36 ± 0.06	2.46 ± 0.34	0.55 ± 0.05
Sucrose	5	5.42 ± 2.03[b]	7.78 ± 3.17[b]	4.07 ± 2.30[b]
Ficoll-sucrose	8	0.39 ± 0.05	2.09 ± 0.31	0.56 ± 0.06
Percoll	8	0.36 ± 0.04	2.62 ± 0.25	0.51 ± 0.05
Albumin	8	0.60 ± 0.15	2.51 ± 0.36	1.01 ± 0.41
Dextran-40	8	0.42 ± 0.04	2.63 ± 0.30	0.57 ± 0.05
Metrizamide	8	0.44 ± 0.09	2.19 ± 0.27	0.56 ± 0.06

[a] (mU insulin/μg DNA/45 min, mean ± SEM)
[b] Significantly different from control ($P < 0.05$) at given glucose concentration

Table 4. In vitro function of rat islets density after gradient purification tested after 16-h tissue culture period

Gradient	Number of experiments	Insulin release[a] at successive incubations with		
		2.75 m*M* glucose	16.5 m*M* glucose	2.75 m*M* glucose
Control	8	0.46 ± 0.10	2.22 ± 0.31	0.53 ± 0.10
Ficoll	8	0.52 ± 0.10	2.04 ± 0.30	0.49 ± 0.09
Sucrose	3[b]	10.45 ± 6.86[c]	11.02 ± 4.65	4.34 ± 2.51
Ficoll-sucrose	8	0.57 ± 0.12	1.83 ± 0.33	0.54 ± 0.12
Percoll	8	0.76 ± 0.28	2.11 ± 0.35	0.56 ± 0.14
Albumin	8	0.72 ± 0.18[c]	2.55 ± 0.37[c]	0.66 ± 0.13
Dextran-40	8	0.45 ± 0.08	1.82 ± 0.25	0.50 ± 0.08
Metrizamide	8	0.62 ± 0.10	1.81 ± 0.30	0.59 ± 0.16

[a] (mU insulin/μg DNA/45 min)
[b] In two out of five experiments, no islets could be recovered after 16 h of culture
[c] Significantly different from control ($P < 0.05$) at given glucose concentration

significantly lower yields than all other gradients. Significantly higher yields were obtained with metrizamide when compared to the yield of the Ficoll gradients (32% ± 12% in paired experiments, not shown).

A very large proportion of the total number of islets in the gradient was found in the exocrine bands of the sucrose and the Ficoll-sucrose gradients (43% ± 15% and 29% ± 8%, respectively). In the Ficoll gradients, 10% ± 2% of the total number of islets was found in the exocrine bands, whereas in Percoll, albumin, dextran-40, and metrizamide this was only 5% or less (Table 2).

Islet viability was tested in vitro. Hand-picked control islets incubated successively at 2.75 m*M*, 16.5 m*M*, and 2.75 m*M* glucose immediately after the isolation and purification procedure secreted 0.35 ± 0.05, 2.70 ± 0.35, and 0.58 ± 0.09 mU insulin/μg DNA/45 min, respectively. Insulin secretion by the islets from the Ficoll, Ficoll-sucrose, Percoll, albumin, dextran-40, and metrizamide gradients was not significantly different from that by the hand-picked controls (Table 3). Insulin secretion by islets purified with sucrose gradients was very high, even during the incubations at low glucose levels. Under the microscope these islets were seen to disintegrate during the in vitro test procedure, and usually less than the initial number of ten islets per aliquot could be retrieved at the end of the test. As a result of both findings, the calculated insulin release related to the DNA content cannot be considered a true measure of the insulin secretion of the islets purified with sucrose gradients.

The in vitro function of islets purified with sucrose gradients had not improved after 16 h of culture in RPMI (Table 4). Further deterioration was illustrated by the fact that in two out of five experiments no islets could be recovered after the culture period, and the mean insulin levels had increased to 5–20 times that of controls. The secretion of islets retrieved from albumin gradients was also significantly different from the secretion of control islets in the first two incubation periods, but their mean secretion was in the same order of magnitude as that with control islets. The islets derived from all other gradients secreted similar amounts of insulin as control islets, and all could be stimulated adequately.

Discussion

This study of rat pancreases addresses the question of which density gradient is most suitable for the purification of islets for transplantation purposes. Continuous density gradients were used in order to determine the buoyant density of each of the different pancreatic tissue components obtained after collagenase digestion. Moreover, by applying continuous density gradients, the potential of each gradient to separate islets from exocrine tissue, lymph nodes, vessels, and ducts could be determined.

An effective separation of the islets from the exocrine tissue was usually obtained with each of the gradient-forming substances. In each gradient and within each experiment, a band containing islets and another band containing exocrine tissue could be identified. A small overlap of the two bands was occasionally seen in the Ficoll gradients. In the other gradients, however, these two bands were either adjacent or even separated by an area in the gradient containing neither islets nor exocrine tissue. The finding that pancreatic tissue components were retrieved from different densities in each type of gradient indicates that the density of these tissue components is subject to changes during centrifugation within the gradient, and that the magnitude of these changes varies with each type of gradient. This observation can be explained by differences in osmolarity of the gradients, since an increase in osmolarity is associated with dehydration of the pancreatic tissue components and, therefore, with an increase in their density [8].

Large numbers of islets could be recovered from the islet band when Ficoll, Percoll, albumin, dextran, and metrizamide were used. The islet yield of these gradients was comparable to the number of islets obtained from the control aliquot by hand-picking, indicating that our modified technique of chopped tissue collagenase digestion adequately dissociates the pancreatic tissue for further purification by means of a density gradient. Mutual comparison of the gradients showed that metrizamide gave significantly higher islet yields than the widely used Ficoll gradients. With sucrose and with the combination of Ficoll and 0.25 m*M* sucrose, low islet yields were obtained, and a

high percentage of the islets was found within the exocrine tissue band. The total number of islets regained from all of the tissue bands with these two gradients was lower than the hand-picked control, indicating that not only an insufficient separation but also an absolute loss of islet tissue had occurred during the centrifugation. This cannot be readily explained by viscosity-induced shear forces on the islets during centrifugation and migration causing the islets to disintegrate into islet fragments or single islet cells, since higher yields were obtained from gradients with viscosity profiles similar to those of sucrose and Ficoll-sucrose.

It could be argued that different results would have been obtained if ductal injection of collagenase, rather than chopped tissue digestion, had been applied. Indeed, larger islet yields have been reported when using ductal injection [14]; however, these authors have not restricted the islet count to islets with a diameter of 100 μm or more, as we have done. Moreover, there is no principal consideration to suggest that ductal injection, as compared to chopped tissue digestion, is associated with a different effect on the respective densities of the different components of the pancreatic tissue. Therefore, it is unlikely that a comparison of the efficacy of the density gradients in terms of islet yield would have been different if islet isolation by ductal collagenase injection had been applied.

Not only an adequate yield of purified islets but also their viability is an obvious prerequisite for successful transplantation. The viability of islets obtained from the density gradients and of hand-picked control islets was tested by static incubation, and the insulin secretion was related to the DNA content of the islets in order to exclude possible effects of differences in islet size. Both before and after the culture period, the insulin secretion per unit DNA of islets retrieved from all gradients except sucrose and albumin was similar to the secretion of hand-picked control islets, indicating that the islets had not been damaged by the density gradient. Islets recovered from the sucrose gradients were seen to disintegrate during in vitro testing, and the very high levels of insulin found with static incubation of these islets should, therefore, be interpreted as insulin release from damaged beta cells rather than as glucose-stimulated insulin secretion. The very high molar concentration of sucrose in the gradients is the obvious causative factor, since such high concentrations are associated with a high osmolarity. High osmolarity can cause loss of water from cells, resulting in a higher density of the pancreatic tissue components [8] and the release of hormones by other endocrine cells [3]. With the islets derived from albumin gradients, the levels of insulin secretion were statistically significantly higher than with control islets after the culture period. However, the mean insulin secretion was of similar magnitude to that of control islets. Batch-to-batch variability of BSA has been reported, something which may influence metabolic functions of tissue due to differing amounts of contaminants [16]. The slight alteration of the in vitro function of the islets from the albumin gradients could be due to the contaminants of the BSA batch used in our experiments.

For practical reasons, one would prefer to use discontinuous rather than continuous density gradients, as the different solutions of the gradient-forming material can be made in advance and are easily layered prior to centrifugation. Discontinuous density gradients with an interface intermediate to both bands, capable of separating exocrine tissue and islets without predetermining the density limits of both tissues [14], can only be constructed if the means of the density limits of both tissues differ significantly from each other. We found that the density limits of the islet band and the exocrine tissue band in each gradient in the different experiments showed a considerable variation, and that such a significant difference in the means of density limits is restricted to Ficoll-sucrose, albumin, dextran, and metrizamide. On the basis of this consideration, it should be possible to form an effective discontinuous gradient with each of these four materials. On the basis of other considerations discussed above, however, Ficoll-sucrose and albumin are less suitable, leaving dextran and metrizamide as the most appropriate alternatives.

The islet band in the gradients was always seen to contain either lymph nodes, vessels and ducts, or both. The mean density limits of the tissue bands containing lymph nodes or vessels and ducts showed a considerable overlap with the density limits of the islet band. It is, therefore, unfortunate that continuous or discontinuous density gradients are incapable of providing us with pure islets without some contamination by lymph node, vessel, and duct tissue.

We conclude that density gradients are only effective in separating exocrine tissue from the islets. The application of an additional technique, which either separates lymph nodes, vessels, and ducts from the islets or eliminates the immunological consequences of impurities of the islet preparation with these tissues, is essential. Of the seven gradient-forming materials tested, metrizamide appeared to be the most suitable choice, as the highest islet yield and the largest difference in densities between islets and exocrine tissue was seen with metrizamide gradients. For the routine isolation of rat islets we prefer the use of dextran gradients, followed by hand-picking to remove the nonseparated lymph nodes, vessels, and ducts, as dextran gradients, compared to metrizamide, are less expensive and can be sterilized by autoclaving.

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