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Cytology of β-Cells in Rabbit Pancreas Pieces Incubated *in vitro*; Effects of Glucose and Tolbutamide*

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Summary. The electron microscopic appearances of rabbit pancreas β-cells taken *in vivo* and after *in vitro* incubation have been compared and the effects of high glucose and tolbutamide stimulation of insulin release *in vitro* on β-cell cytomorphology investigated. Normal structure was maintained on *in vitro* incubation in a proportion of β-cells; this varied in different samples from 35—100 per cent. The cause of this variation was not apparent but it was not influenced by the time of incubation, medium glucose concentration or tolbutamide. Quantitative measurements have been made of the proportion of β-cell profiles showing marginal distribution of granules, of the number of contacts between granule sac membranes and the plasma membrane, of the number of granule sacs showing perforations, of the population density of specific secretion granules, lysosomes and autophagic bodies, and estimates have been made of the extent of the Golgi apparatus in cross section. No consistent change in these parameters was observed following stimulation of insulin release with glucose or tolbutamide. There was no evidence in any of the specimens examined of continuity between the interior of a granule sac and the outside of a β-cell. Calculations based on the rate of release of insulin and the insulin content of the pancreas indicate that the chance of visualising granule extrusion by electron microscopy is small. The significance of these results in relation to the emiocytosis theory of insulin release (Lacy and Hartroft) is discussed.

Cytologie des cellules bêta de fragments de pancréas de lapin incubés in vitro; effets du glucose et du tolbutamide

Résumé. On a comparé l'aspect au microscope électronique des cellules bêta de pancréas de lapin prélevés *in vivo* et après incubation *in vitro*, et on a étudié les effets, sur la cytomorphologie de la cellule bêta, de la stimulation de la libération d'insuline *in vitro* par un taux élevé de glucose et par le tolbutamide. Une partie des cellules bêta conservait sa structure normale au cours de l'incubation *in vitro*; ceci variait dans les différents échantillons de 35 à 100 pour cent. La cause de cette variation n'était pas apparente, mais elle n'était pas influencée par la durée de l'incubation, ni par la concentration du milieu en glucose, ni par le tolbutamide. On a fait des mesures quantitatives de la proportion de profils de cellules bêta, présentant une distribution marginale des granules, du nombre de contacts entre les membranes des sacs granulaires et la membrane plasmatique, du nombre de sacs granulaires présentant des perforations, de la densité de population des granules spécifiques de sécrétion, des lysosomes et des corps autophagiques, et on a fait des estimations de l'étendue de l'appareil de Golgi dans les coupes transversales. On n'a pas observé de modifications constantes dans ces paramètres après stimulation de la libération d'insuline par le glucose ou le tolbutamide. Dans aucun des spécimens examinés il n'y avait de signe de continuité entre l'intérieur d'un sac granulaire et l'extérieur d'une cellule bêta. Des calculs basés sur le taux de libération d'insuline et sur le contenu en insuline du pancréas, indiquent qu'il y a peu de chance de visualiser l'extrusion d'un granule au microscope électronique. L'importance de ces résultats en relation avec la théorie d'émiocytose de la sécrétion d'insuline (Lacy and Hartroft) est discutée.

Zytologie der β-Zellen aus in vitro inkubierten Pankreasteilchen von Kaninchen; Einwirkungen von Glucose und Tolbutamid

Zusammenfassung. Das elektronenoptische Bild von β-Zellen aus *in vivo* entnommenem Kaninchenpankreas wurde vor und nach *in vitro* Inkubation verglichen und die Auswirkungen der in *vitro* durch Glucose und Tolbutamid herbeigeführten Insulinfreisetzung auf die β-Zell-Zytomorphologie untersucht. Ein Teil der β-Zellen behielt auch nach *in vitro* Inkubation seine normale Struktur; dieser Anteil schwankte in den verschiedenen Ansätzen zwischen 35 und 100%. Die Ursache dieser Schwankungen ließ sich nicht nachweisen. Die Inkubationsdauer und die Glucose- oder Tolbutamidkonzentration im Medium hatten keinen Einfluß darauf. Quantitativ bestimmt wurden: Der Anteil der β-Zellprofile, die eine randständige Granulaverteilung aufwiesen; die Anzahl der Kontakte zwischen Granula- und Plasmamembran; die Anzahl der Granula, die Perforationen aufwiesen; die Bevölkerungsdichte spezifischer Sekretionsgranula und die Zahl der Lysosomen und Autophagen. Ferner wurde die Ausprägung des Golgi-Apparates in den einzelnen Schnitten geschätzt. Nach Anregung der Insulinfreisetzung durch Glucose oder Tolbutamid wurden keine konstanten Änderungen dieser Parameter beobachtet. In keinem der untersuchten Präparate ließ sich eine Verbindung zwischen dem Inneren eines Granula-Sackes und der Umgebung der Zelle nachweisen. Berechnungen, die sich auf die Insulinfreisetzungsrate und auf den Insulingehalt des Pankreas stützen, ergeben allerdings nur eine geringe Wahrscheinlichkeit dafür, daß sich ein Granulaaustritt elektronenmikroskopisch darstellen läßt. Die Bedeutung dieser Ergebnisse in Bezug auf die Emiozytose-Theorie der Insulinfreisetzung (Lacy und Hartroft) wird besprochen.

Key-words: Quantitative electron microscopy, rabbit pancreas, β-cells, *In vitro*, stimulation glucose tolbutamide

Rabbit pancreas pieces incubated *in vitro* in bicarbonate-buffered saline media supplemented with glutamate, fumarate and pyruvate (Krebs, 1950) release insulin in response to agents such as glucose and tolbutamide which effect insulin release *in vivo* (Coore and Randle, 1964). Such a system appears to offer advantages for quantitative studies of possible fine structural changes which might occur during insulin release. In particular the composition of the external medium may be more easily controlled *in vitro* than

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in vivo and pieces of pancreatic tissue containing islets may be rapidly removed and fixed for electron microscopy. Qualitative studies of the electron microscopic appearance of β-cells during stimulation of insulin release *in vivo* and *in vitro* by glucose and tolbutamide have led to the conclusion that insulin release may involve margination of β-cell granules and extrusion through the plasma membrane (WILLIAMSON, LACY and GRISHAM, 1961; WILLIAMSON, LACY and TAYLOR, 1967). In the present study the electron microscopic appearances of pancreatic β-cells obtained *in vivo* and after *in vitro* incubation have been compared. Quantitative measurements have also been made of the distribution of granules in β-cells, of the number of contacts between granules and plasma membrane and of the fine structure of β granules *in vivo* and after *in vitro* incubation with or without stimulation of insulin release by glucose or tolbutamide.

A preliminary account of some of these findings has been published (LEVER, FINDLAY, RANDLE, and GILL, 1965; FINDLAY, GILL, IRVINE, LEVER and RANDLE, 1966). The apparent retention of *in vivo* fine-structure of β-cells of rabbit pancreas after *in vitro* incubation has also recently been described by GOMEZ-ACEBO, LOPEZ-QUIJADA and R-CANDELA, 1966 and WILLIAMSON et al; 1967.

Materials and Methods

Investigations on the incubated pancreas

Male rabbits were anaesthetised with nembutal and the pancreas exposed by laparotomy. Specimens of pancreas were taken *in vivo* and immediately fixed and processed for electron microscopy. The pancreas was then removed, transferred to a petri dish containing buffer (see below) trimmed of obvious fat and divided into pieces of about 150 mg. These were then transferred into bicarbonate-buffered saline medium supplemented with glutamate, fumarate and pyruvate which contained glucose (0.6 mg/ml), and was gassed with $O_2 + CO_2$ (95:5) as described by COORE and RANDLE (1964). After an initial 30 min (pre-incubation) period in the medium, representative specimens were removed for electron microscopy. The remaining specimens were separated into three groups, and incubated (1) in the basal medium, (2) in a high glucose (3 mg/ml) medium and (3) in the basal medium to which tolbutamide (0.2 mg/ml) had been added. Pancreatic samples were removed for electron microscopy from each group after 10, 20, 30, 60 and 120 min.

Processing of material for electron microscopy

Pancreatic pieces were cut into 1 mm blocks with scissors and fixed for 1 h in 1% veronal acetate-buffered osmic acid (pH 7.6) prior to ethanol-dehydration and passage through xylol into araldite. Fine sections of islet tissue were stained on the grid with lead citrate (REYNOLDS; 1963) and viewed in a Siemens Elmiskop 1 electron microscope.

Quantitative Assessments

Electron micrographs of all biopsies were taken at screen magnifications of × 4000, and either × 8000 or × 10000.

1. Beta granule counts. Using the low magnification electron micrographs (× 12000 printed) the population density of β-granules was determined for each biopsy by counting the granules within measured areas of cytoplasm (LEVER and FINDLAY, 1964).

2. Lysosome and autophagic body counts. Using the same low magnification micrographs, the population density of both of these inclusions was determined for each biopsy as described for the β-granule counts.

3. Area estimates of the Golgi apparatus. Using low magnification micrographs, the percentage area occupied by Golgi membranes in β-cell cytoplasm was estimated by a line sampling method (LOUD, 1962). For this purpose each group of Golgi membranes was first circumscribed in ink.

4. Marginal Contacts. Using the higher magnification micrographs (× 24000 and × 30000 printed) the number of β-granule sacs in direct contact with plasma membranes was counted, and the length of these plasma membranes was measured with an opisometer. From these data the number of marginal contacts per 10 μ of β-cell plasma membrane was calculated for each biopsy. The observations were made only upon membranes present in cross section as clearly defined lines.

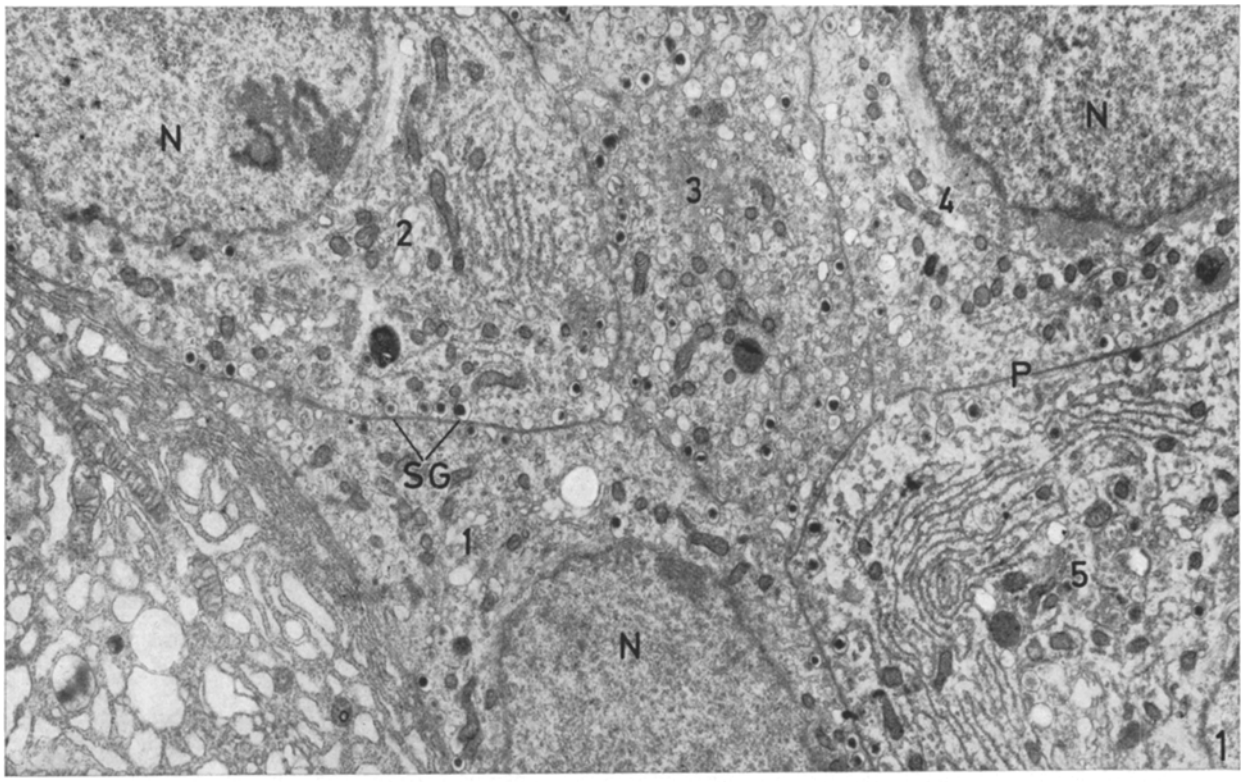
5. Percentage perforated β-granule sacs. Again using the higher magnification micrographs, counts were made of (a) the total number of β-granules and (b) the number of granules with perforated sacs (LEVER and FINDLAY, 1966), so as to obtain the percentage of perforated β-granule sacs per biopsy.

Results

Cytomorphology of rabbit islet β-cells in vivo

Our observations on the fine structure of the normal rabbit islet cells in general confirm those of LACY (1957), VOLK, LAZARUS and WELLMAN (1965), and MUNGER, CARAMIA and LACY (1965). The arrangement and appearance of the mitochondria, granular endoplasmic reticulum and Golgi membranes is essentially similar to that described in the cat (LEVER, JEACOCK and YOUNG, 1961) and guinea-pig (FINDLAY and LEVER, 1964) islets while the intercellular and cellular-vascular relationships conform to the usual endocrine pattern (LEVER, 1962).

Typically each β-granule consists of a smooth membrane sac from which the contents are separated by an electron lucid halo (Fig. 2). There is considerable individual variation in the electron density of the material contained within β-granules. In some, this consists of a well-defined dense core, whereas in others it may be diffuse and of low density (Fig. 2). The mean diameter of the β-granules in the rabbit is 0.23 μ (standard deviation 0.0299 μ). In the majority of β-cell cross-sections examined by electron microscopy secre-



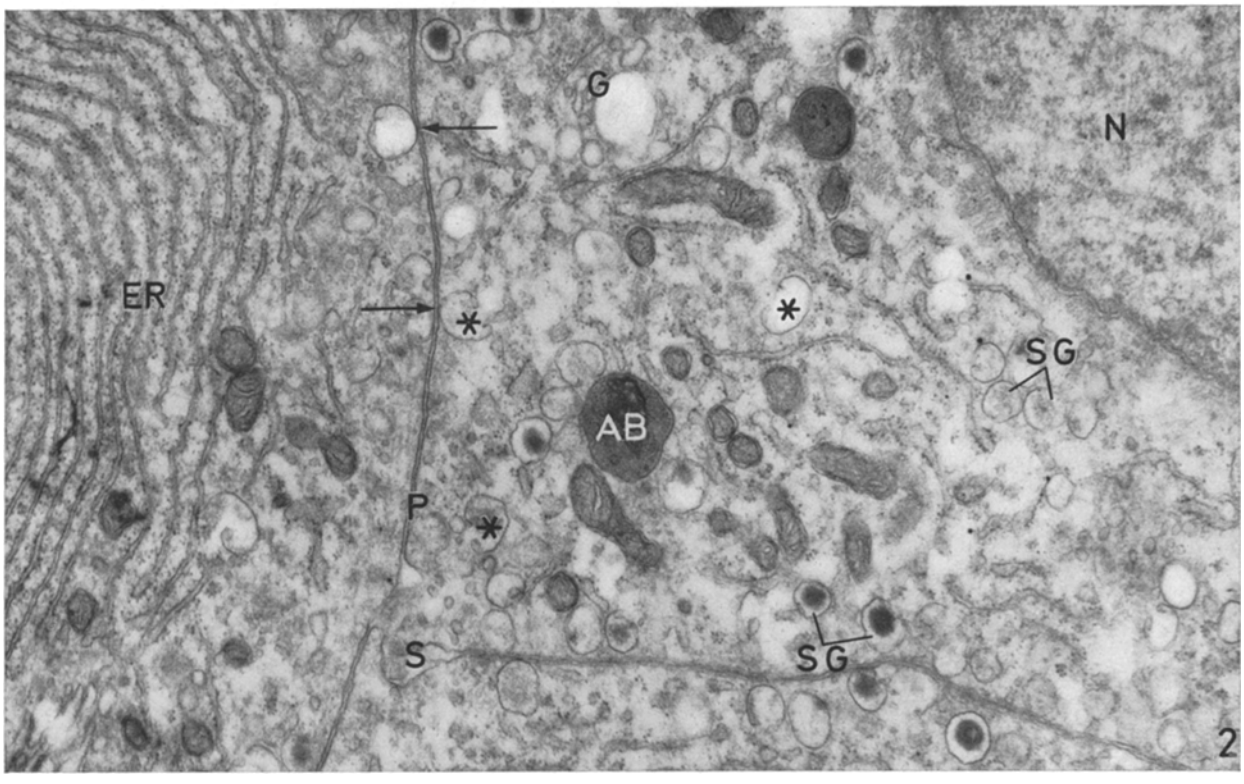


Fig. 1. Parts of five rabbit beta-cells at the edge of an islet taken directly from the animal are shown; nuclei (N); plasma membranes (P). In cell outlines 1, 2 and 4 the majority of secretion granules (SG) are peripherally disposed, while in outlines 3 and 5 they appear randomly distributed. × 10000

Fig. 2. Parts of three rabbit beta-cells — *in vivo* specimen: nucleus (N); plasma membranes (P); intercellular spaces (S). The specific secretory granular material (SG) exhibits considerable individual variation in electron density, but this is typically surrounded by a less dense halo and the whole is enclosed by a smooth membranous sac. The sacs of some peripherally disposed granules appear to be fused with the plasma membrane (arrows). About 20% of granule sacs are perforated (asterisks). Other organelles include Golgi membranes (G); endoplasmic reticulum (ER); autophagic bodies (AB). × 25000

tory granules were distributed randomly thoughout the cytoplasm, but in approximately 16% of cross-sections peripheral disposition of the secretory granules was observed (Fig. 1). Cell profiles in which this peripheral orientation of granules was an obvious feature also exhibited a low overall granule population. Contacts between the plasma membrane and some β-granule sacs were observed not only in the 16% of cell cross-sections in which the majority of granules were disposed peripherally, but also in the other β-cell profiles in which the (much higher) granule population was randomly distributed. Sacs in contact with the cell surface show fusion with the plasma membrane (Fig. 2) but do not open onto it (LEVER and FINDLAY, 1966). In none of 142 sac contacts with 454.4 μ of β-cell surface was there evidence of continuity between the outside of a cell and the inside of one of its granules. Approximately 20% of the sac profiles around β-granules in freshly-excised rabbit pancreas exhibited a single membrane perforation (Fig. 2). At the site of perforation the inner and outer laminae of the trilaminar enclosing membrane were continuous. A fuller account of these hiatuses was given in an earlier communication (LEVER and FINDLAY, 1966). The distribution of perforated sacs was random.

In all substantial cross-sectional profiles of β-cells and often in the vicinity of the Golgi region, lysosomal sacs with a speckled granular content could be identified, and in almost all these cell profiles one or more larger (up to 2 μ) dense membrane-bound bodies were present. Besides a granular lysosome-like content these larger bodies often contained myelinic figures (Fig. 2) and resembled the autophagic vacuoles reported in rabbit β-cells by LAZARUS, VOLK and BARDEN (1966).

The examination of β-cells in vitro

When tissues are divided and subjected to the mechanical interference involved in incubation some cell damage may be expected. In an earlier light microscopic study of pancreatic pieces *in vitro* (LEVER et al., 1965) it was found that some β-cells showed nuclear pyknosis, cytoplasmic vacuolation and eosinophilia during a period of 3½ h incubation in COORE and RANDLE's (1964) medium. In a survey of electron micrographs of all the islets in the present series (5 *in vivo*: 34 *in vitro*) estimates were made of the β-cell nuclei surrounded by cytoplasm which was contained by a continuous plasma membrane. In all the *in vivo* specimens (i.e. those taken direct from the animal) this was 100%. In the *in vitro* specimens this varied from 100% of intact cells (as judged from single cross-sections) to only 36%. There was no evidence that cell survival was reduced with longer times of incubation. In studying the effects of a high concentration of glucose and of tolbutamide on insulin release, measurements and deductions were made only on unstigmatised β-cells (i.e. those with intact plasma membrane and with organelles of normal appearance).

Release of insulin by pancreas in vitro

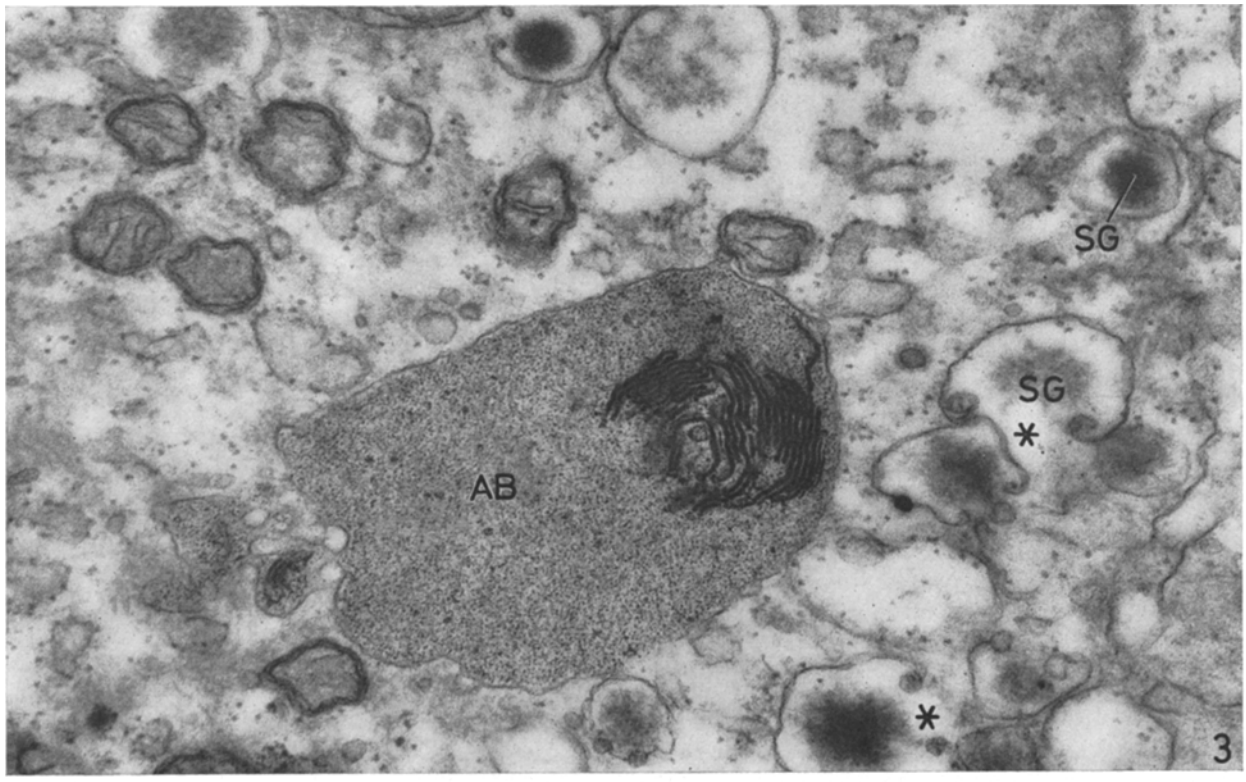
When rabbit pancreas pieces are incubated *in vitro* in a bicarbonate-buffered saline medium there is a small release of insulin, which is not changed by addition of glucose at concentrations up to 0.6 mg/ml or by addition of glutamate, fumarate or pyruvate which assists in the maintenance of tissue respiration (KREBS, 1950). Insulin release is enhanced by glucose at higher concentrations (e.g. 3 mg/ml) and by tolbutamide (0.2 mg/ml), and stimulation by these agents is improved by supplementing the medium with glutamate, fumarate and pyruvate. Table 1 records the results of typical experiments in which the outputs of insulin were measured under basal conditions (glucose concentration 0.6 mg/ml) and after stimulation by glucose (3 mg/ml) or tolbutamide (0.2 mg/ml). These data are representative of those obtained under the same conditions by COORE and RANDLE (1964) and in parallel experiments in the present study by one of us (J.R.G.). In what follows the cytology of β-cells has been examined in pancreas pieces incubated in basal medium or stimulated to release insulin with either tolbutamide or a high concentration of glucose (3 mg/ml).

Table 1. *Insulin release by rabbit pancreas in vitro, stimulation by glucose and tolbutamide. Representative data from experiments by Coore and Randle (1964) and Gill (unpublished observations). Conditions of incubation and assay of insulin in incubation media and pancreas pieces as described by Coore and Randle (1964). Insulin release was measured for the time shown after 30 min of preincubation in medium containing glucose (0.6 mg/ml)*

	Period of incubation (min)	Insulin output unit × 10^{-3}	Insulin output as per cent of pancreatic insulin
Glucose (0.6 mg/ml)	0–30	0.98 (6)	
Glucose (3 mg/ml)	0–30	29.7 (6)	
Glucose (0.6 mg/ml)	90–120	2.0 (6)	
Glucose (3 mg/ml)	120–150	15.0 (6)	
Glucose (3 mg/ml)	0–60	46 (6)	2.7
Glucose (0.6 mg/ml)	0–30	5.9 (6)	
Glucose (0.6 mg/ml) + tolbutamide (0.2 mg/ml)	0–30	36.1 (6)	

β-cell cytomorphology of rabbit islets in vitro

General features and disposition of granules. The cytology of unstigmatised β-cells in pieces of pancreas removed at 30, 40, 60, 90 and 150 min. after commencement of incubation in basal medium was ostensibly similar to that of β-cells *in vivo* (Fig. 4). This contention is supported by the fact that as between *in vivo* and all unstimulated *in vitro* specimens (1) counts of lysosomes and autophagic bodies/unit area of β-cytoplasm were not significantly different (P lysosomes > 0.2 : P autophagic bodies > 0.2), and further (2) the cross-sectional area of the Golgi membranes in the β-cells was



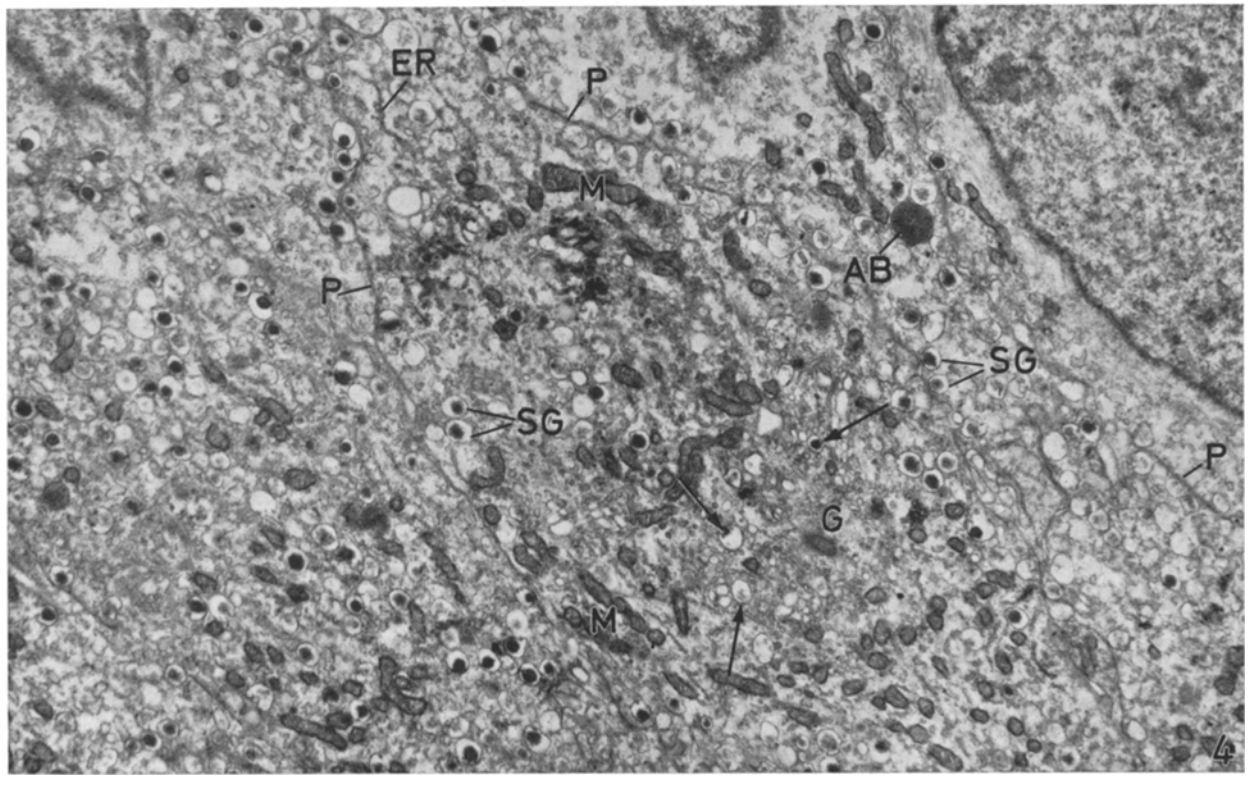


Fig. 3. Part of a rabbit beta-cell — *in vitro* specimen — 90 min in the basal medium. Notice the large autophagic body (AB) containing myelinic figures and a fine speckled granular content characteristic of lysosomes. Specific secretion granules (SG) are also seen and some of these (asterisks) show sac perforations. × 50000

Fig. 4. Parts of several rabbit beta-cells are shown after 90 min in the basal medium. Notice that the plasma membranes (P) are intact and that the cytoplasmic organelles appear normal: secretion granules (SG); mitochondria (M); autophagic bodies (AB); endoplasmic reticulum (ER). Secretory granular material is shown within some of the sacs (arrowed) of the Golgi apparatus (G). × 10000

not significantly changed ($P > 0.1$). In all the *in vitro* specimens it was possible to identify granular material within some of the Golgi sacs, a finding which may be construed as evidence of the synthetic potential of the incubated β-cells. In the unstimulated *in vitro* pancreas not change significantly with increasing time of incubation in low glucose medium. In the larger second experiment shown in Table 4 there was similarly no change in population density with time of incubation in the presence of high glucose or tolbutamide. Al-

Table 2. Margination of β-Granules
(Expressed as the percentage of β-cells showing an obvious marginal distribution of granules)

Samples were taken direct from the animal (*in vivo*) after 30 min pre-incubation in the basal medium (pre-inc.), and after various times of incubation in basal medium (LG 10′—120′) and of stimulation with high glucose (HG 10′—120′) or tolbutamide (T 10′—60′). Figures in bold face indicate percentage marginal distribution: figures in brackets indicate total number of cells examined in each biopsy.

	In vivo (90) **16**	
	Pre-inc. 30′ (57) **32**	
HG 10′ (34) **35**	LG 10′ (39) **44**	T 10′ (35) **31**
HG 20′ (53) **21**	LG 20′ (44) **32**	T 20′ (58) **21**
HG 30′ (43) **35**	LG 30′ (47) **34**	T 30′ (30) **37**
HG 60′ (52) **37**	LG 60′ (78) **17**	T 60′ (43) **16**
HG 120′ (64) **25**	LG 120′ (38) **26**	

Tables 3—8

For connotation of biopsies see legend to Table 2. Values for granule population density (Tables 3 and 4), marginal contacts (Tables 5 and 6), percentage perforated β-granule sacs (Tables 7 and 8) are given in bold face. Two tables for each parameter indicate results obtained from two experiments. The effect on these parameters of duration of incubation and of stimulation by high glucose or tolbutamide was assessed statistically by means of the "*t*" test: *t*-values appear in italics between the results which were compared: probability values are indicated by asterisks: — $p > 0.05$, NS; $0.05 > p > 0.01$*; $0.01 > p > 0.001$**; $p < 0.001$***.

Table 3. β-granule population density — experiment 1
(Number of granules/unit area cytoplasm)

			In vivo (8) **1.60**					
			1.594, NS					
			Pre-inc 30′ (3) **1.21**					
	*3.985***		*1.693, NS*			*3.008**		
HG 10′	(5) **2.40**	*3.898***	LG 10′	(5) **1.52**	*0.623, NS*	T 10′	(5)	**1.42**
	*3.976***		*1.202, NS*			*2.474**		
HG 30′	(3) **1.25**	*0.334, NS*	LG 30′	(6) **1.17**	*0.591, NS*	T 30′	(9)	**0.80**

Table 4. β-Granule population density — experiment 2
(Number of granules/unit area cytoplasm)

			In vivo (8) **1.01**					
			0.086, NS					
			Pre-inc 30′ (24) **1.04**					
	0.205, NS		*0.554, NS*			*0.318, NS*		
HG 10′	(26) **1.08**	*0.385, NS*	LG 10′	(17) **1.18**	*0.277, NS*	T 10′	(16)	**1.11**
	0.352, NS		*0.184, NS*			*0.668, NS*		
HG 20′	(41) **1.01**	*0.935, NS*	LG 20′	(31) **1.25**	*0.113, NS*	T 20′	(49)	**1.23**
	0.195, NS		*0.720, NS*			*1.000, NS*		
HG 30′	(41) **0.98**	*0.045, NS*	LG 30′	(18) **0.99**	*1.689, NS*	T 30′	(53)	**1.37**
	0.403, NS		*0.999, NS*			*1.033, NS*		
HG 60′	(19) **1.05**	*1.759, NS*	LG 60′	(33) **0.80**	*3.215***	T 60′	(42)	**1.22**
	1.388, NS		*0.255, NS*					
HG 120′	(31) **1.34**	*2.620**	LG 120′	(22) **0.84**		—		

tic specimens as in the *in vivo* specimens the majority (54—84%) of the β-cell profiles showed β-granules randomly distributed throughout the cytoplasm (Table 2). In the remainder (16—46%) of β-cell profiles the majority of β-granules were peripherally disposed (Fig. 1)

Secretory Granule Population. As shown in Tables 3 and 4 the population density of secretion granules did though significant differences in the population density of β-cell granules were seen in isolated samples (e.g. high glucose 120 min sample and tolbutamide 60 min sample in Table 4) there is in general no consistent change in the population density of granules either after stimulation of insulin release with high glucose or tolbutamide or with increasing incubation time (up to 120 min).

Marginal contacts between secretory granules and the β-cell plasma membrane. Tables 5 and 6 indicate considerable and in some instances significant variation in the number of marginal contacts both in stimulated (high glucose; tolbutamide) and unstimulated (low glucose) β-cells sampled at time intervals up to 150 min; and this rather irregular variation is also reflected by a number of significant differences between (both glucose- and tolbutamide-) stimulated and unstimulated β-cells sampled at the same time. Neither experiment, however, indicates any consistent effect of either stimulation by high glucose or tolbutamide, or the duration of incubation, on the number of marginal contacts.

Percentage of β-granule sacs exhibiting perforations. Tables 7 and 8 show a number of significant differences in percentage sac perforation both with time in stimulated and unstimulated β-cells and when stimulated and unstimulated cells are compared with each other at the same time, but no meaningful trends can be observed in either experiment.

Table 5. Marginal contacts — experiment 1
(Number of β-granule sacs/10μ plasma membrane)

					In vivo	(27)	**3.4**				
						0.492, NS					
					Pre-inc	30′ (17)	**3.7**				
			1.579, NS			*2.251**				*6.130****	
HG	10′	(23)	**3.0**	*1.329, NS*	LG	10′ (25)	**2.5**	*7.763****	T	10′ (51)	**6.2**
		0.053, NS				*5.972****				*5.332****	
HG	30′	(26)	**3.1**	*3.782****	LG	30′ (54)	**5.3**	*4.148****	T	30′ (35)	**2.4**

Table 6. Marginal contacts — experiment 2
(Number of β-granule sacs/10μ plasma membrane)

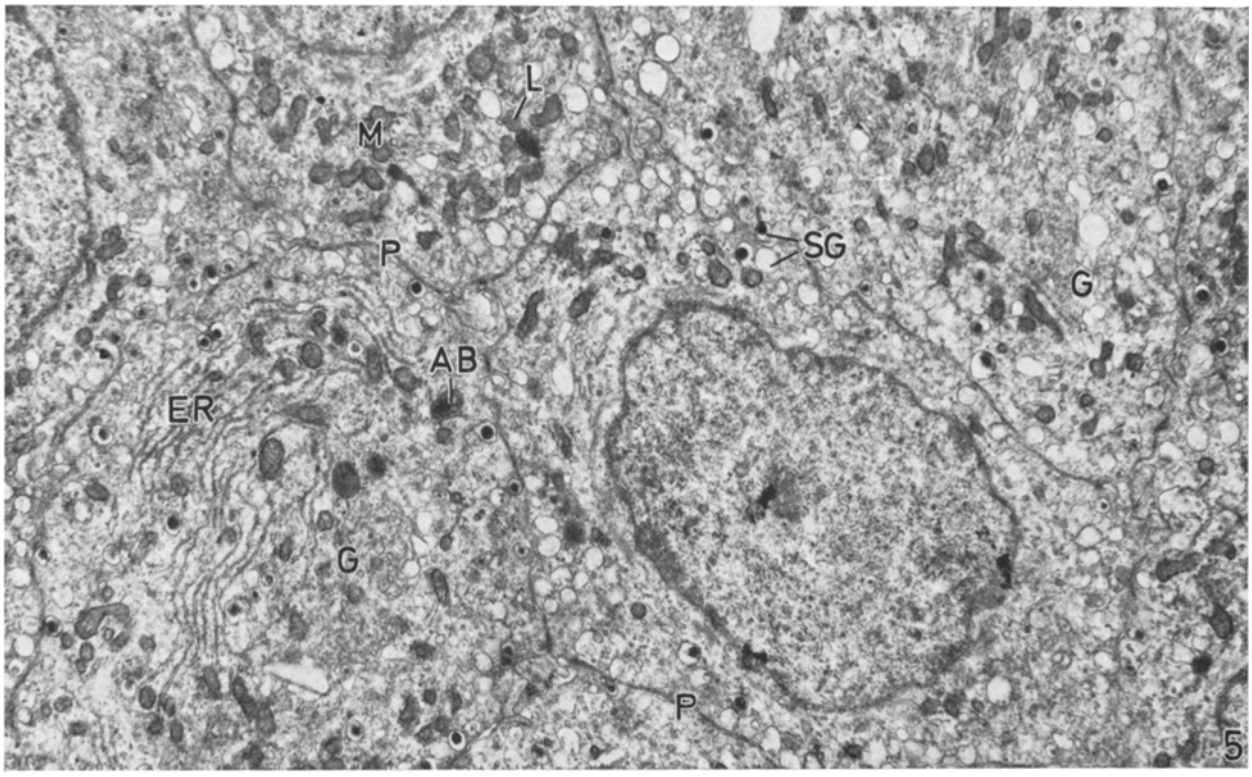
					In vivo	(15)	**3.48**				
						0.432, NS					
					Pre-inc	30′ (21)	**3.79**				
			1.204, NS			*0.235, NS*				*0.441, NS*	
HG	10′	(13)	**4.80**	*1.284, NS*	LG	10′ (18)	**3.61**	*0.579, NS*	T	10′ (24)	**4.21**
		0.161, NS				*2.149**				*0.614, NS*	
HG	20′	(32)	**4.97**	*1.160, NS*	LG	20′ (28)	**6.20**	*2.650**	T	20′ (44)	**3.65**
		*2.429**				*2.757***				*0.739, NS*	
HG	30′	(15)	**2.48**	*0.526, NS*	LG	30′ (16)	**2.88**	*1.989, NS*	T	30′ (32)	**4.18**
		*3.319***				*1.607, NS*				*1.295, NS*	
HG	60′	(33)	**4.84**	*1.919, NS*	LG	60′ (32)	**3.84**	*0.831, NS*	T	60′ (22)	**3.32**
		*3.179***				*2.345**					
HG	120′	(16)	**2.93**	*3.553***	LG	120′ (16)	**5.37**			—	

Table 7. Perforated sacs — experiment 1
(Expressed as % of total β-granule sacs)

					In vivo	(6)	**18.3**				
						0.447, NS					
					Pre-inc.	30′ (6)	**17.9**				
			*2.907**			*2.215, NS*				*3.008**	
HG	10′	(6)	**27.9**	*5.072****	LG	10′ (6)	**12.5**	*1.305, NS*	T	10′ (6)	**11.8**
		*2.385**				*1.956, NS*				*5.675****	
HG	30′	(6)	**18.3**	*3.722***	LG	30′ (7)	**10.0**	*6.538****	T	30′ (8)	**30.3**

Table 8. Perforated sacs — experiment 2
(Expressed as % of total β-granule sacs)

					In vivo	(17)	**23.16**				
						*5.946****					
					Pre-inc	30′ (24)	**9.93**				
			*5.171****			*5.826****				*4.350****	
HG	10′	(13)	**30.05**	*0.385, NS*	LG	10′ (20)	**28.10**	*1.426, NS*	T	10′ (27)	**22.76**
		*3.417***				*0.810, NS*				*1.701, NS*	
HG	20′	(33)	**18.05**	*2.706***	LG	20′ (29)	**25.12**	*0.876, NS*	T	20′ (46)	**27.52**
		*4.480****				*0.151, NS*				*5.920****	
HG	30′	(16)	**31.93**	*1.559, NS*	LG	30′ (16)	**24.52**	*3.397***	T	30′ (35)	**14.61**
		1.369, NS				*2.698**				*0.914, NS*	
HG	60′	(38)	**39.09**	*0.374, NS*	LG	60′ (32)	**37.48**	*5.076****	T	60′ (24)	**16.96**
		1.369, NS				*3.058***					
HG	120′	(16)	**32.23**	*2.313**	LG	120′ (16)	**22.52**			—	



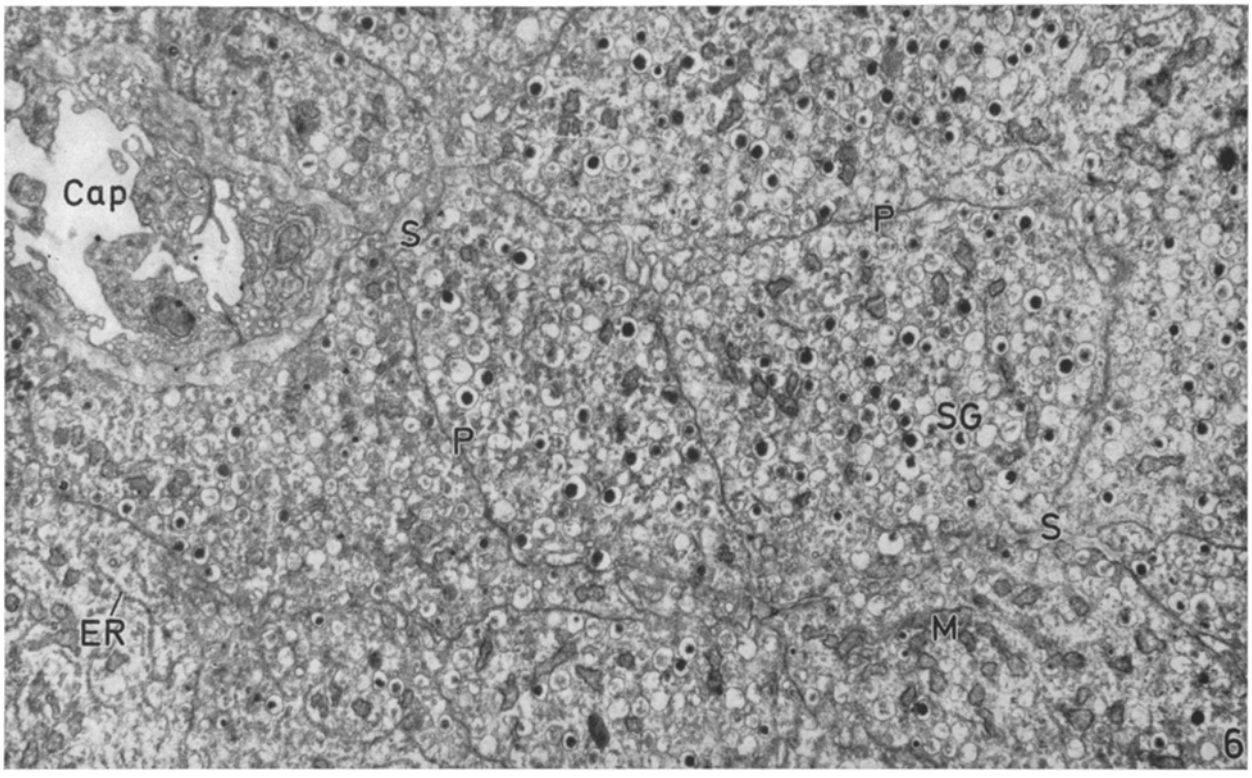


Fig. 5. Parts of several rabbit beta-cells are shown after 60 min in a high glucose incubation medium. The plasma membranes (P) are intact and the cytoplasmic organelles appear normal: secretion granules (SG); mitochondria (M); lysosomes (L); autophagic bodies (AB); Golgi membranes (G); endoplasmic reticulum (ER). × 10000

Fig. 6. Parts of several rabbit beta-cells are shown after 20 min stimulation by tolbutamide in the incubation medium. The plasma membranes (P) are intact and the cytoplasmic organelles appear normal: secretion granules (SG); mitochondria (M); endoplasmic reticulum (ER). A capillary (Cap) and several intercellular spaces (S) are shown. × 10000

Discussion

COORE and RANDLE (1964) concluded from a study of the influence of various factors on the release of insulin by rabbit pancreas pieces *in vitro* that the behaviour of the preparation appeared to be physiologically meaningful. Thus insulin release was stimulated by tolbutamide and by glucose and mannose, but not by a number of other sugars. The response to glucose was suppressed by adrenaline and by mannoheptulose. The influence of these agents on pancreatic pieces *in vitro* is qualitatively similar to those which were known or have since been shown to occur *in vivo*. From the quantitative point of view glucose caused the release of approximately 2 to 5 per cent of pancreatic insulin in 2 h of incubation and it was calculated that the rate of release may be comparable with that seen *in vivo*.

These biochemical results which indicated β-cell survival *in vitro* have been endorsed in the present study by the concurrent morphological findings. It is clear that a proportion of β-cells remain intact *in vitro* under maintenance conditions (low glucose) and during tolbutamide and high glucose stimulation for periods of at least 2 h (the maximum period of incubation in the present study). These β-cells did not differ cytologically from β-cells *in vivo*. There was no increase in the lysosome and autophagic body counts in these surviving cells nor did they show any changes in Golgi size. Evidence of secretory granule formation within Golgi sacs was found in all *in vitro* specimens. Although secretory granule formation was not apparently enhanced with high glucose or tolbutamide, increased numbers of cytoplasmic ribosomes were found in some long-term tolbutamide specimens (Fig. 8).

Although the present study has provided conclusive morphological evidence for *in vitro* survival of β-cells the proportion of morphologically intact β-cells was not constant from specimen to specimen the variation being from 36 to 100 per cent. This variation was not apparently related either to the total incubation time or to stimulation by high glucose or tolbutamide, and it cannot at present be satisfactorily explained. Some cell damage is to be expected as a consequence of the manipulations involved in removal of the pancreas and division into pieces. Moreover the effects of detaching islet tissue from its blood and nerve supply cannot be predicted. *In vitro* and *in vivo* studies have indicated a functional involvement of the autonomic nervous system in insulin release (COORE and RANDLE, 1964; PORTE and WILLIAMS, 1966; KANETO, KOSAKA and NAKAO, 1967) and synaptic connections of cholinergic and adrenergic nerves with α and β-cells have been demonstrated in cat islets by ESTERHUIZEN, SPRIGGS and LEVER (1967).

It has to be concluded that if an exact relationship is sought between insulin output and β-cell cytomorphology then the use of pancreatic pieces is open to objection. It has been shown in the present study that some cell damage occurs and the extent of this varies from sample to sample. In seeking endocrine tissue within a piece of pancreas the microscopist is necessarily selective because of the labour involved in locating islets and his selection may not reflect the condition of all the islets in the various pieces which make up the "specimen" and which in sum are responsible for the biochemical results. Morphological studies are therefore subject to sampling errors which must be taken into account in assessing the relationship between insulin output and β-cell cytomorphology. Quantitative measurements are therefore essential with *in vitro* studies and these arguments are presumably applicable to *in vivo* studies.

The population density of β-granules showed no significant alteration during incubation in the low glucose medium for periods of time up to 150 min, and no consistent changes following stimulation of insulin release either by a high glucose concentration or by tolbutamide; the population density under these conditions showed little variation. A stable β-granule population in these experiments is also suggested by the absence of any significant difference in the percentage of β-cell outlines showing a peripheral distribution of granules (as opposed to a random granule distribution) in stimulated and unstimulated pancreatic pieces. The finding of random and peripheral granule distributions in different β-cell outlines in the same plane of section may be construed as evidence of a polarity or concentration of β-granules at one end of the β-cell, and of their more sparse but peripheral distribution elsewhere.

In view of the suggestion that insulin is released from the β-granules at the surface of the cell and that a migration of granules towards the plasma membrane is a feature of tolbutamide-stimulation (WILLIAMSON et al., 1961), the marginal distribution of granule sacs was precisely quantitated from high magnification electron micrographs by counting the number of these sacs exhibiting fusion with known lengths of the plasma membrane. In the experiments listed in Tables 5 and 6 significant variations in the number of marginal contacts per unit length of plasma membrane were seen between pancreatic samples taken at intervals from the low glucose medium. Comparable variation was observed in islets stimulated by high glucose and tolbutamide, but there was no evidence that stimulation of β-cells (sufficient to increase their insulin output as shown in Table 1) consistently increased the number of contacts between marginally-disposed granules and the plasma membrane within the time limits of these experiments. Moreover, in a close scrutiny of 2284 granule sacs exhibiting fusion with 6027.4 μ plasma membrane from both stimulated and unstimulated β-cells *in vitro*, there was no evidence to indicate actual continuity between the interior of a granule and the outside of a cell — a finding that would presumably be required to support the theory of insulin release by emiocytosis as proposed by LACY and HARTROFT (1959). Since samples for electron microscopy are taken at an

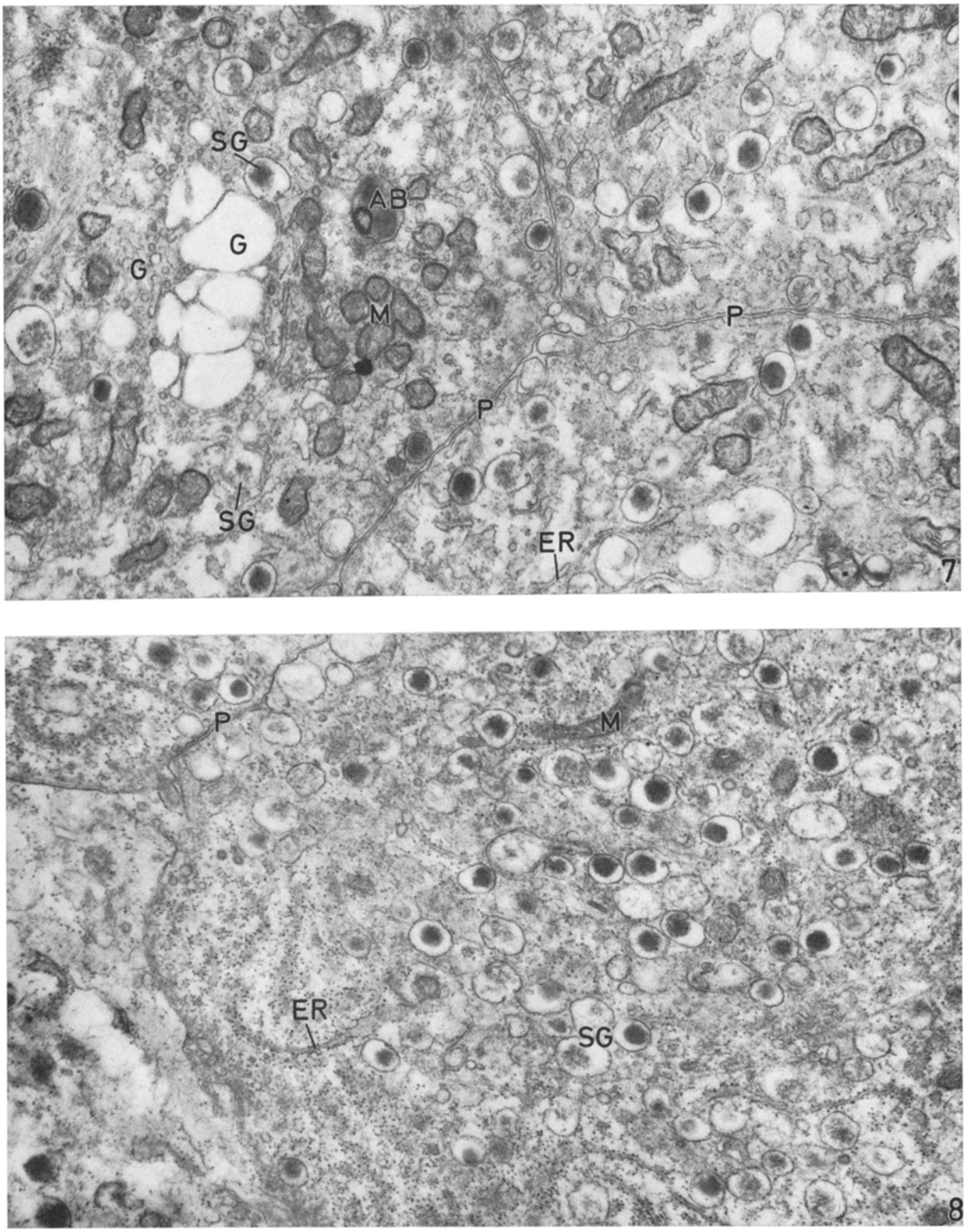


Fig. 7. Parts of three rabbit beta-cells after 20 min stimulation by tolbutamide in the incubation medium. Notice the collection of large and small Golgi sacs (G) in one of the cells and the presence of several secretion granules (SG) closely associated with these membranes. The plasma membranes (P) and cytoplasmic organelles — mitochondria (M); endoplasmic reticulum (ER); autophagic bodies (AB) — all appear normal. × 25000

Fig. 8. *In vitro* specimen of rabbit beta-cells after 60 min stimulation by tolbutamide. An increased cytoplasmic ribosome content is found in some cells (compare with appearances in figure 7). In other respects these "stimulated" cells appear normal — mitochondria (M); secretion granules (SG); endoplasmic reticulum (ER); plasma membranes (P). × 25000

instant in time the chance of visualising granule extrusion may however be slender. The proportion of insulin released from the pancreatic islets in these *in vitro* experiments after stimulation of release with glucose or tolbutamide was no more than 0.01 per cent per 0.5 min. Thus for example, if a β-cell profile contains 100 granules, no more than 1 per cent of profiles would be expected to show granule discharge. Such calculations may emphasise the importance of quantitative aspects of electron microscopy in *in vivo* as well as in *in vitro* experiments.

As an alternative to the theory of insulin release by emiocytosis we have considered the possibility of release from granules within β-cells. For this reason the number of secretion granules with perforated sacs as a percentage of all granules present was estimated from high magnification electron micrographs. No meaningful changes in the per cent of perforated sacs was detected following stimulation with tolbutamide or high glucose.

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