Is It Insulin That is Stained by Aldehyde Fuchsin and Pseudoisocyanin in the Sections of the Pancreas?*

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The B cells of the pancreatic islet are characterized by their specific granules, the beta granules. Best, Haist and Ridout (1939) stained these granules with Bowie's method and found that they reduced conspicuously in rats after subjecting these animals to fasting, after keeping them on a fat diet and after the administration of insulin and that, in concurrence with this, the insulin content of the pancreas measured by bioassay decreased markedly in these animals. Barron (1948) confirmed this result by staining the granules with Gomori's chrome-hematoxylin. A parallelism between the amount of beta granules as examined by aldehyde fuchsin (abbreviated as AF in this paper), the newer stain of Gomori, and the insulin content of the pancreas in normal and diabetic human and dog pancreases was later shown beautifully by researchers in Toronto (Wrenshall, Hartroft and Best, 1954; Hartroft and Wrenshall, 1955). The so-to-say insulin hypothesis of AF staining, i.e., the hypothesis that the substance stained by this method should correspond to insulin or at least to its close precursor, came thus to be generally accepted.

There have been, however, a few authors who doubted the validity of this hypothesis. Bangle (1956) attempted in vain to stain insulin film spread on a slide glass. Moreover, he pointed out discrepancies between the solubility of the insulin film to various solvents and that of the stainable granules of the B cell in histological sections and declared that these granules are not associated with insulin. Williamson and Lacy (1959) recognized under the electron microscope that the beta granules of the rabbit injected with alloxan lost their central core which is generally believed to be stored insulin, whereas their membrane sac together with its amorphous content remained. As, thereby, the stainability of the granules to AF was not affected, the authors suggested that the dye did not stain the core or insulin but the amorphous remnant in the granule. Lazarus and Barden (1961) confirmed this finding and postulated, in their turn, that the membrane sac of the granule was responsible for the staining. The same assumption was made by Lazarus and Volk (1962a) as they

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found the loss of granule cores and the simultaneous retention of stainability of the granules in the B cells of the rabbit stimulated by tolbutamide. These postulations seem, as pointed out by Lazarow (a discussion to Lazarus and Barden, 1961), not convincing enough as the authors did not show, by hormone assay, that insulin had been really lost from the pancreas at the very time when the stainability of the granules remained unaffected.

H. Fujita and Matsuno (1967) who recently confirmed the dissociated light and electron microscopic figures in the alloxan treated rabbit, remarked on the possibility that the granule core, i.e., insulin mass had become, in the alloxanized cells, amorphous and permeated into the cytoplasm around the granules to be still stained there by AF. These authors thus were inclined to retain the insulin hypothesis of the AF staining.

The balance of both hypotheses, insulin and non-insulin hypotheses, of this staining seems to have inclined again in favor of the former, as Kvistberg, Lester and Lazarow (1966) reported that they succeeded in staining insulin by keeping it in a fixed position on a polyacrylamide by disc electrophoresis.

Even if, however, we accept the stainability to AF of insulin in the test tube, the question remains unsettled: Is it insulin that is stained by AF in the histological sections of the pancrees? Is it only insulin, or insulin plus some other thing, or only some other thing? To gain an answer to this question, it was considered necessary to combine cytological observation with hormone assay as Hartroft and Wrenshall did. The present work, however, differs from that of the last mentioned authors in that it was designed to include two different series of animal experiments in which a correlation and a discrepancy between the granule amount and the insulin content were respectively expected.

In the present study, the significance of the metachromatic reaction of the B cell granules to pseudoisocyanin (abbreviated as PIC in this paper) was also analysed. In spite of the variety of tissue elements reacting to this dye, there have appeared few papers which questioned whether it is really insulin that is responsible for this reaction in the histological sections of the pancreatic islet as postulated by Schiebler and Schiessler (1959).

Materials and Methods

1. Glucose administration in the guinea pig

Adult guinea pigs of either sex were, after being kept unfed for 24 hrs, intraperitoneally injected with 2 g/kg of glucose (50% aqueous solution) and sacrificed every hour for 6 hrs after the injection. Small pieces of the pancreas for cytological examinations (vide infra) were taken out to be fixed in Bouin's fluid. In two of the control cases and in two animals 2 hrs after the glucose administration, the pancreas, after small samples for cytological observation had been removed, was taken out, weighed and frozen in dry-ice powder. Insulin was extracted by treating this pancreas with acidified alcohol (Best, Jephcott and Scott, 1932) and assayed by using the blood glucose level of the rabbit.

2. Alloxan administration in the rabbit

Adult albino rabbits of either sex, starved for 24 hrs, were intravenously injected
with 200 mg/kg of alloxan. At one or two minute intervals in the first 10 minutes and then at longer intervals thereafter, 0.1 cc blood was taken from the ear capillaries. The blood glucose level was measured by a glucose oxidase method. The animals were sacrificed mainly at 30 min (7 cases of which one was discarded) and at 24 hrs (7 cases of which two were discarded) after alloxan injection; some others (5 cases of which two were discarded) either were killed or died in between. The rabbits seized with hypoglycemic cramp, mostly from 6 to 15 hrs after the alloxan treatment, were rescued by glucose injection in order that they would survive till the destined time.

Tiny pieces of the pancreas were taken and dipped in ordinary Bouin's fluid, in Bouin's fluid containing lead acetate (TARAYA, 1966) and in absolute ethanol for the purpose of cytological examinations; the whole organs remaining in situ was then carefully taken out, weighed and thrown into dry-ice powder. The bioassay of extracted insulin was performed with the same method as in the guinea pig.

3. Cytological examinations

Paraffin sections were cut at 5 μ. Staining with AF was performed either with or without prior permanganate oxidation. Besides the original method of Gomori, a condensed type of AF solution (3 g basic fuchsin, 3 cc paradehyde and 3 cc HCl in 100 cc 60 % ethanol) was used with good results. Counter staining was made with ponceau-fuchsin, orange G and light green.

The metachromatic reaction of the B cells to PIC (N,N'-diethylpseuodoscyanin chloride, Leverkusen) was carried out as described by SCHIEBLER and SCHIESSLER (1960). The stained specimens were covered in water and observed either through a monochromatic filter (580 μm) or by fluorescence microscopy (STERBA, 1961).

The ethanol fixed tissues were treated with the dithizon method of OKAMOTO (modification by MAEDA, FUJIWARA and SUKENARI, 1954) for the detection of zinc contained in the islets.

Results

1. Glucose administration in the guinea pig

In the control animals the B cells were filled with granules stained intensely by AF. A substance of a corresponding granular nature and distribution in the cell was shown with its strong metachromatic reaction when treated in PIC after permanganate oxidation. This reaction was especially beautifully demonstrated by using the monochromatic filter (580 μm) or by fluorescence microscopy. As a preliminary study by the dithizon method for zinc had given only a slight coloration to the islet cells, presumably owing to the fact that the zinc content of the pancreas of this animal is very low (SHEVCHUK, 1965), this method was given up in this series of experiments.

The same dose of glucose (2 g/kg) was injected by the same method (intraperitoneal administration) as in the study of GOMORI, FRIEDMAN and CALDWELL (1939). In correspondence with the finding of these authors who stained the pancreas with Gomori's chrome-hematoxylin, a remarkable decrease in stained granules was recognized both with AF and with PIC methods. This decrease was very distinct in the case of animals killed 1 hr and 2 hrs after the glucose injection (Fig. 1). A small
amount of the stained substance remained in the apical end of some B cells, while a considerable number of other cells seemed essentially free of it. The substance then gradually increased with the lapse of time and in the animals killed 5 hrs after

Fig. 1. Islet of the guinea pig, fixed in Bouin, stained with pseudoisocyanin and observed through a monochromatic filter (580 μm).

a: From a control animal. b: From a guinea pig 2 hrs after glucose administration. Note the conspicuous decrease of the PIC metachromatic substance.
glucose administration it resumed almost the same level as in the control animals. In all the cases examined, the AF positive substance seemed completely to correspond to the PIC metachromatic element with regard to its amount and distribution. In Table 1, the amount of the stained granules in AF and PIC stainings is graded from one plus to five plus. Two persons made judgement every time. Discrepancy in the judgements made by both observers remained within one grade throughout the study. In most cases they coincided exactly but when they did differ from each other the average was given.

The result of bioassay of the insulin content of the pancreas was shown in Table 1. In spite of the small number of the cases examined, it may be clearly said that as much as 50% of insulin has been lost from the pancreas 2 hrs after the glucose injection.

Table 1. Cytological and hormone assay results in the guinea pigs given an intraperitoneal injection of glucose (2 g/kg)

<table>
<thead>
<tr>
<th>Time after glucose administration</th>
<th>Animal No.</th>
<th>Stained substance in B cells</th>
<th>Insulin content of the pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AF</td>
<td>PIC</td>
</tr>
<tr>
<td>Control</td>
<td>No. 3</td>
<td>4 +</td>
<td>4 +</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>1 hr</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

2. Alloxan administration in the rabbit

In the control animals, the B cells contained a considerably variable amount of granules which were accumulated more or less conspicuously toward the blood capillary (Fig. 2a, 3a). The feature of the granules was identical regardless of the staining used, AF or PIC. The staining of zinc by dithizon revealed a substance tinged pink which corresponded in distribution to those granules (Fig. 3b). The A cells colored more or less deeply by dithizon were often darker than the B cells. The D cells were also tinged pale pink.

The cytological changes caused by alloxan will be depicted only briefly in this report as they essentially correspond to the observations of previous authors (see Lazarus and Volk, 1962b) and as a mere description of the changes is not the purpose of this paper. In the islets of the rabbits 30 min after the administration of
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It is insulin that is stained? The title of the section.

**Table 2. Cytological and hormone assay results in the rabbits given an intravenous injection of alloxan (200 mg/kg)**

<table>
<thead>
<tr>
<th>Time after alloxan treatment</th>
<th>Animal No.</th>
<th>Damages in B cells</th>
<th>Surviving B cells</th>
<th>Stained substances in B cells</th>
<th>Insulin content of the pancreas unit/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No. 3</td>
<td>-</td>
<td>3 +</td>
<td>4 +</td>
<td>2.45</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>-</td>
<td>3</td>
<td>4</td>
<td>2.29</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>2.18</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>2.31</td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>-</td>
<td>3.5</td>
<td>4</td>
<td>2.14</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>-</td>
<td>2</td>
<td>2.5</td>
<td>2.19</td>
</tr>
<tr>
<td>Control</td>
<td>26</td>
<td>-</td>
<td>3</td>
<td>2.5</td>
<td>2.28</td>
</tr>
<tr>
<td>1/2 hr</td>
<td>8</td>
<td>+</td>
<td>2</td>
<td>2.5</td>
<td>1.91</td>
</tr>
<tr>
<td>1/2</td>
<td>10</td>
<td>+</td>
<td>3</td>
<td>2.5</td>
<td>1.98</td>
</tr>
<tr>
<td>1/2</td>
<td>20</td>
<td>+</td>
<td>4 (2)</td>
<td>4 (2.5)</td>
<td>3</td>
</tr>
<tr>
<td>1/2</td>
<td>21</td>
<td>+</td>
<td>3 (2)</td>
<td>3 (2)</td>
<td>2</td>
</tr>
<tr>
<td>1/2</td>
<td>22</td>
<td>+</td>
<td>3 (2.5)</td>
<td>3 (2.5)</td>
<td>3</td>
</tr>
<tr>
<td>1/2</td>
<td>23</td>
<td>+</td>
<td>3 (2)</td>
<td>3 (2)</td>
<td>2</td>
</tr>
<tr>
<td>1 1/2</td>
<td>11</td>
<td>+</td>
<td>2 (2)</td>
<td>2.5 (2)</td>
<td>1.97</td>
</tr>
<tr>
<td>2 1/2</td>
<td>5</td>
<td>++</td>
<td>3.5</td>
<td>3</td>
<td>1.85</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>+++</td>
<td>3</td>
<td>4</td>
<td>1.61</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>Only surviving cells encountered</td>
<td>(3) (2.5) (3)</td>
<td>1.40 Mean : 1.36 ± 0.049</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>16</td>
<td>+++</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>17</td>
<td>+++</td>
<td>4 (2.5)</td>
<td>3 (2.5)</td>
<td>1.28</td>
</tr>
<tr>
<td>24</td>
<td>18</td>
<td>+++</td>
<td>3</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>24</td>
<td>19</td>
<td>+++</td>
<td>4 (2)</td>
<td>4.5 (2)</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the reactions in surviving B cells.

Alloxan (Fig. 2b), the B cells already show slight but distinct pycnosis of nuclei; the cell cords have become slender and the pericapillary spaces enlarged. The polarity of the B cells toward the blood capillary has been more or less lost. With the lapse of time, the B cells become shrunken (1 1/2 hr) and dissociated from each other completely loosing their arrangement in cell cords (6 hrs). The nuclei, after suffering from severe pycnosis, disappear for the most part within 24 hrs after alloxan administration (Fig. 2c, 3c).

The two stainings, AF and PIC, gave again the identical results as to the amount

**Fig. 2.** Islet of the rabbit, fixed in Bouin and stained with aldehyde fuchsin (counterstained by Goldner’s trichrome method).

- **a:** Control animal (No. 13, insulin content: 2.44 unit/g)
- **b:** 30 min after alloxan treatment (No. 20, insulin content: 1.80 unit/g)
- **c:** 24 hrs after alloxan treatment (No. 19, insulin content: 1.34 unit/g)
Fig. 3. Islets of the normal and the alloxan treated rabbit in pseudoisocyanin staining (a and c) and in dithizon reaction (b and d).

a and b: Control animal (No. 13, insulin content: 2.44 unit/g)

b and d: 24 hrs after alloxan treatment (No. 19, insulin content: 1.34 unit/g)

Note that zinc can hardly be detectable in the necrotized B cells in Figure d (the peripheral cells shown dark are A cells) whereas the PIC metachromatic substance shows no decrease in Figure c.
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and distribution of the stained substance of the B cells. The substance was repre-
sented, even in the completely necrotized cells of the 24 hr cases, not by a homo-
geneous element but by distinct granules which were round and a little larger than
the beta granules in the control animals. The granular substance stained by either
of both dyes showed no tendency to decrease with the lapse of time after alloxan
administration (Fig. 2, 3). The amount of the substance in the B cells was graded
from one + to five + for each pancreas examined (Table 2). The grading was made
by the same method as described in the case of the guinea pig. This table clearly
indicates that 1) the AF positive substance always occurred in proportion to the
PIC metachromatic one and 2) neither of them decreased until at least 24 hrs after
the alloxan treatment.

In dissociation from this result on the stained substance, the insulin content of
the pancreas showed a 26% decrease in 30 min and 41% decrease in 24 hrs after the
alloxan administration (Table 2). If one takes into account that there remain a
certain number of surviving B cells (Fig. 4) which probably contain insulin, the
decrease in insulin within the necrotized cells may be by far more conspicuous than
indicated by these figures. In favor of this assumption, the zinc content as observed
by the dithizon method decreased considerably in the 30 min cases and very much
in the damaged B cells of the 24 hr cases. The last mentioned cells showed granular
substance filling the cytoplasm tinged only with a pale yellowish color, whereas the
A cells in the periphery of the islet were intensely colored pink (Fig. 3 d). The surviv-
ing B cells in the 24 hr cases, on the other hand, were stained pink indicating the

Fig. 4. Two islets, one necrotized (right) and another surviving (left), in a single section of the
pancreas from a rabbit killed 24 hrs after alloxan treatment. Insertions show each of them in a
higher magnification. Fixation in Bouin, staining in aldehyde fuchsin plus trichrome.
presence of a considerable amount of zinc (see Table 2). It may be worthy of note that these cells contained the AF and PIC stained substance rather in less amount than the necrotized B cells in the same single section (Fig. 4).

The changes in blood glucose level after alloxan treatment will be mentioned only in Discussion, as a detailed report on this subject will appear elsewhere (HASEGAWA et al., in preparation).

**Discussion**

The present study is characterized by the combination of examinations on the B cell stainability and on the insulin content in one and the same pancreas. The first experiments with glucose given guinea pigs confirm the result of HARTROFT and WRENSHALL (1955) that the amount of the substance stained by aldehyde fuchsin (AF) reflects the insulin content of the cell. This holds true also in the substance reacting metachromatically in pseudoisocyanin (PIC). Not necessarily, however, does this mean that the stained substance is nothing but insulin itself as was postulated by those and many other authors. On the contrary, the second experiments of the present study demonstrated a clear discrepancy between the amount of stained substance in the B cells and the insulin content in the pancreas. As far as the authors know, there is no previous report that showed this discrepancy in the alloxanized pancreas by combining the cytological observation with the hormone assay.

In the present study, a distinct decrease in insulin content of the pancreas was revealed in the rabbits killed 30 min after alloxan administration. As seen from Table 2, 26% of insulin was lost from the pancreas within this time. This finding of an early release of insulin from the alloxanized islets, though never recorded hitherto (see HOWELL and TAYLOR, 1966), is supported by the significant decrease in zinc content in those islets and by an early drop in blood glucose recognized in the majority of the alloxanized animals in the present study. A detailed description and interpretation on this initial hypoglycemia which occurs generally within 5 min after alloxan administration and continues only a few min will be published elsewhere (HASEGAWA et al., in preparation). A preliminary study on the changes in the serum insulin level in short time lapses after alloxan injection also seems to support an early release of insulin into the blood.

From 30 min after alloxan injection on, the insulin content did not show a marked decrease with the lapse of time, and it reached the value corresponding to 59% of control value in the animals killed 24 hr after alloxan treatment. This value should be regarded to allow an estimation at most: The insulin content in each necrotized islet may be by far less than indicated by this figure because a considerable number of islets may have escaped the damage by alloxan which presumably contains most of the insulin of the alloxanized pancreas. In accordance with this assumption, zinc reaction was considerably intense in those surviving islets whereas essentially negative in the necrotized ones. It seems thus very probable that the discrepancy between the amount of the stained substance and the insulin content in the necrotized islets is much greater than indicated by the figures shown in Table 2.

Since SCHIEBLER and SCHIESLER (1959) the metachromatic reaction of the pancreatic B cells to PIC is ascribed to the presence of insulin. As far as is known, few authors doubted this relation in spite of the fact that a considerable variety of tissue
and cell elements other than the pancreatic B cells react metachromatically in this dye. Now that the insulin theory for AF staining has been contradicted, one may have a right to analyse the corresponding theory for PIC. It may be quite possible that the pancreatic B cell would contain a PIC metachromatic substance other than or besides insulin. In this connection, it seems worthy to add that the B cell granules react metachromatically also in routine basic dyes such as toluidin blue (Fujita and Takaya, in preparation) in spite of the description of Schieessler and Schiessler that PIC is highly specific for this reaction of B cells.

There now occurs the question, what is then stained in AF and PIC in the pancreatic B cells. The stained matter should be sought among the elements which are associated with insulin within the cell. It must increase, move and diminish together with insulin according to the different functional phases of the cell as shown in guinea pigs after glucose administration. It becomes, however, dissociated from insulin in the acute intoxication of the cell as in the case of alloxan treated rabbits. The membrane sac of the beta granules which has been recognized in some electron microscopic works (Williamson and Lacy, 1959; Lazarus and Barden, 1961; H. Fujita and Matsuno, 1967) to be left on the spot when the core of the granule disappears after the administration of alloxan, seems to be competent for this substance in search. The possibility proposed in the electron microscopic study by H. Fujita and Matsuno (1967) that the stained substance in the alloxanized B cells is insulin which has permeated into the cytoplasm was neglected by the present study.

As for the chemical nature of the AF positive and PIC metachromatic substance, it seems worthy of attention that both stainings fail after fixation of the tissue either in absolute ethanol or in aceton as already described by Schiessler and Schiessler (1959). This fact may imply that the substance is soluble in these lipolytic solvents and thus may correspond to lipid containing matter such as lipoprotein.

**Summary**

Rabbits were intravenously given alloxan (200 mg/kg) and the changes in the cytology of the pancreatic B cells as well as in the insulin content of the pancreas were examined. Although 26% of insulin was shown to have been lost from the pancreas in 30 min and 41% in 24 hrs after the administration of alloxan, no decrease was found in the amount of the substance stained by aldehyde fuchsin and reacting metachromatically to pseudoisocyanin. The content of zinc in the B cells examined by the dithizon method diminished considerably in 30 min and almost disappeared in the 24 hr case. These results indicate that it is not, or at least not only, insulin that is responsible for the aldehyde fuchsin and pseudoisocyanin stainings.

On the other hand, it was confirmed, that the stained substance in the B cells decreased in amount in clear concurrence with the insulin content of the pancreas when the guinea pig was given an intraperitoneal injection of glucose (2 g/kg). This implies that the substance reacting in both stainings corresponds to a certain structure which is associated with insulin as far as the cell physiologically functions. A hypothesis that the substance is in the membrane sac surrounding the beta granule seems to fit in with this condition.
アルデヒド フクシン と プソイド イソチアニン で腸臟の切片に
染められるものはインシュリンか？（内容自抄）

ウサギにアロキサン（200mg/kg）を静注し、腸臓のB細胞の細胞学的変化と腸内インシュリン量の変化をしぼらべた。アロキサン注射後30分で腸内インシュリンの26%が、24時間で41%が失われるのでに対して、アルデヒド フクシン可染性およびプソイド イソチアニン異染性の物質は減少する気配を示さなかった。腸臓内の亜鉛の量をディチゾン法でしぼらべると、30分でかなりの減少、24時間ではほぼ完全な消失が見られた。これらの所見は、アルデヒド フクシンとプソイド イソチアニンに染まる物質がインシュリンではない（少なくともインシュリンだけではない）ことを示している。

他方 モルモットにぶどう糖（2g/kg腹腔内）を与えると、B細胞の可染物質は腸内インシュリン量の減少とよく一致して減少することが認められた。この所見は両染色に関与する物質が、細胞が正常な機能サイクルにあるときには、インシュリンに伴なって存在することを暗示する。この物質がβ顆粒の限界膜にある可能性を論じた。

References

Bangle, R. jr.: Factors influencing the staining of beta cell granules in pancreatic islets with various basic dyes including paraldehyde fuchsin. Amer. J. Pathol. 32: 349-362 (1956).


Schiebler, T. H. und S. Schiessler: Über den Nachweis von Insulin mit den metachromatisch
Is It Insulin That is Stained?


