FACTORS INFLUENCING THE STAINING OF BETA-CELL GRANULES IN PANCREATIC ISLETS WITH VARIOUS BASIC DYES, INCLUDING PARALDEHYDE-FUCHSIN *

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Gomori,[1] in 1950, reported that basic fuchsin, in the presence of strong mineral acids, forms an intensely purplish dye with certain aldehydes. The dye was referred to as *aldehyde-fuchsin* and was found to impart a deep purple color to elastic fibers and to a few other tissue structures, among which are the beta-cell granules in pancreatic islets. Gomori[2] considered the staining properties of paraldehyde-fuchsin as being unique in the sense that no other dye is known to stain both intensely and selectively the beta-cell granules without the influence of prior oxidation.

The effect of prior oxidation upon the paraldehyde-fuchsin staining of various tissue structures has been described by Scott and Clayton[3] and by Scott.[4] They observed that previous oxidation of sections in acidified permanganate for 2 minutes resulted in more rapid and intense staining of pancreatic beta-cell granules than if the sections were treated with Lugol's solution for 30 minutes, as recommended by Gomori.[1] The effect of oxidation with periodic acid was intermediate.

In the present investigation it was found that pancreatic beta-cell granules, after oxidation or bromination, could be stained rapidly and selectively not only with paraldehyde-fuchsin but also with other basic dyes. This article deals principally with such staining reactions, with particular reference to factors that influence them. The staining methods also were applied to purified insulin crystals to determine if any similarity exists between the staining of beta-cell granules and of purified insulin. The principal objective of these studies is to shed some light upon the complex subject of the mechanism of staining pancreatic beta-cell granules with basic dyes.

STAINING OF PANCREATIC BETA-CELL GRANULES WITH BASIC DYES

Material. The material consisted of pancreases obtained from 3 pancreatectomized dogs, from 2 necropsies upon non-diabetic adults, and from one surgical specimen of an insulin-producing islet-cell adenoma. The fixations employed included (1) Bouin's picroformalin

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acetic acid solution (pH, 1.43), (2) formol sublimate buffered with sodium acetate (pH, 5.8), and (3) 10 per cent formalin buffered with calcium acetate (pH, 6.93). Thin (2 to 3 mm.) blocks of pancreatic tissue were fixed for 20 hours. The material was embedded in paraffin and sections cut serially at 5 to 6 μ. Prior to staining, the slides were carried through xylene and graded alcohols to water. In the case of the material fixed in formol sublimate, the mercurial precipitates were removed with Lugol's solution (2 minutes), the iodine then being removed by a rinse in 5 per cent aqueous sodium thiosulfate.

Stock Dye Solutions. The dye solutions employed possessed the following features common to all: (1) constant concentration of the dye in solution (0.50 per cent), except for spirit blue (0.25 per cent); (2) constant use of 60 per cent ethanol for the dye solvent; and (3) preparation and use of the dye solutions at room temperature (about 25° C.) and in closed Coplin jars.

For all experiments the following basic triphenylmethane dyes, excepting acid fuchsin which is an acid dye, were employed:

1. Basic fuchsin (C. I. no. 677)
2. Pararosaniline (C. I. no. 676)
3. Crystal violet (C. I. no. 681)
4. Acid fuchsin (C. I. no. 692)
   In selective instances the following basic dyes were used:
5. Methyl violet 2-B (C. I. no. 680)
6. Thionine (C. I. no. 920)
7. Malachite green (C. I. no. 657)
8. Methyl green (C. I. no. 684)
9. Spirit blue (C. I. no. 689)
10. Standard "cold Schiff" reagent, manufactured according to Lillie's specifications.[5]

Direct Staining with Stock Dye Solutions

With basic fuchsin (pH, 6.9) or pararosaniline, the islet-cell granules were stained pink to red with no differentiation between the alpha- and beta-cell types. With crystal violet (pH, 5.6) or methyl violet 2-B, the islet-cell granules, in general, were colored light to moderate purple with no distinct separation into the granular types. Acid fuchsin (pH, 3) stained the beta-cell granules pink, the alpha-cell granules possessing a more intense shade. Thionine (pH, 3.9) stained the islet-cell granules faint blue with no differentiation between the granular types. In all cases the cytoplasm of the acinar cells was stained similarly to, or more intensely than, the islet-cell granules. Nuclei were

colored orthochromatically, being dull and indistinct only with the Bouin's fixed material. Elastic fibers could not be distinguished readily from the general background staining of connective tissue.

The Effect of Prior Oxidation upon Staining with Stock Dye Solutions. The various oxidizing agents and other pretreatments employed are listed as follows:

1. Periodic acid (H_5IO_6), 1% aqueous solution
2. Potassium permanganate ($KMnO_4$), 0.5% aqueous solution, with 0.5% H_2SO_4 (freshly prepared)
3. Performic acid (HCO_3H), prepared according to Greenspan's[6] specifications; aged 1 hour before use; stable for 1 day
4. Peracetic acid (CH_3CO_3H), prepared according to Greenspan's[6] specifications; aged overnight before use; stable for 2 to 3 weeks
5. Acetic acid control: same concentration of acid as specified for the preparation of peracetic acid; distilled water used in place of hydrogen peroxide
6. Bromine:carbon tetrachloride (Br_2:CCl_4), 1:39 volume dilution.

Deparaffinized sections of pancreatic tissue were treated with one of the above reagents at 25° C. for specified intervals. Following the use of any of the first five reagents, the sections were washed in running tap water for 5 minutes. The sections oxidized with permanganate were treated with 1 per cent oxalic acid to remove the manganese deposits. Following the use of bromine:carbon tetrachloride, the sections were rinsed in 3 changes of carbon tetrachloride and then taken through graded alcohols to water. The sections were stained separately in each of the stock dye solutions for 5 to 20 seconds unless otherwise stated. Following this, they were rinsed quickly in water, mounted in water, and examined immediately.

The results are presented in Table I.

The Effect of pH upon Staining. The stock dye solutions employed in this experiment consisted of basic fuchsin (pH, 6.9), crystal violet (pH, 5.6), and acid fuchsin (pH, 3.0). The pH of each of these solutions was adjusted by adding drops of concentrated HCl. One set of dye solutions possessed a pH of 1.3, and another set a pH between 2.13 and 2.46. Staining was performed both directly and after oxidizing the sections with peracetic acid for 2 to 3 minutes. After staining, the sections were rinsed quickly with running tap water, mounted in water, and examined immediately.

The results are presented in Table II.

The Effect of Paraldehyde-Dye Mixtures upon Direct Staining. The stock dye solutions employed in this experiment consisted of basic fuchsin, crystal violet, thionine, and acid fuchsin. Three sets of these dye solutions were prepared: one set in which the pH was not adjusted; another set with pH adjusted to 1.3 (this corresponded to 1 per cent HCl in the basic fuchsin solution); and a third set with pH adjusted to 2.1 to 2.5. To 50 ml. aliquots of each dye solution from

TABLE I

The Effect of Prior Oxidation upon Staining Pancreatic Islet-Cell Granules with Stock Dye Solutions

Oxidant or pretreatment	Oxidation interval	Stain (stock dye solution)	Staining interval	Coloration of beta-cell granules*	Coloration of alpha-cell granules*
Peracetic acid or performic acid	2 to 3 min.	Crystal violet or methyl violet 2B	5 to 10 sec.	Brilliant purple-red	Murky blue
	1, 2, 6, and 24 hrs.	Crystal violet	5 to 10 sec.	Dark purple	Murky blue
	2 to 3 min.	Basic fuchsin	5 to 10 sec.	Moderate to dark red	Moderate to dark red
	2 to 3 min.	Malachite green or methyl green	1 to 5 min.	Dull green	Pale green
	2 to 3 min.	Spirit blue	1 to 5 min.	Dull blue	Pale blue
	2 to 3 min.	Thionine or acid fuchsin	1 to 30 min.	Faint color or colorless	Faint color or colorless
	2 to 3 min.	Schiff's reagent	15 min.	Colorless	Colorless
Potassium permanganate, acidified (0.5% aqueous)	5 to 10 min.	Crystal violet or methyl violet 2B	5 to 15 sec.	Brilliant purple-red	Dull blue
	5 to 10 min.	Basic fuchsin	10 to 15 sec.	Dark red	Dark red
	5 to 10 min.	Schiff's reagent	15 min.	Colorless	Colorless
Periodic acid (1% aqueous)	2 hrs.	Crystal violet	5 to 10 sec.	Dark pinkish purple	Pale purple
	6 and 24 hrs.	Crystal violet	5 to 10 sec.	Brilliant purple-red	Murky blue
	30 min.	Schiff's reagent	15 min.	Colorless	Colorless
Bromine:carbon tetrachloride (1:39 volume dilution)	15 to 60 min.	Crystal violet or methyl violet 2B	5 to 10 sec.	Dark purple	Pale purple
	45 min.	Schiff's reagent	15 min.	Colorless	Colorless
Acetic acid control	2, 10 min.; 1, 6, and 24 hrs.	Crystal violet	5 to 15 sec.	Colorless	Colorless
No pretreatment		Crystal violet, methyl violet 2B, or basic fuchsin	5 to 15 sec.	Colorless	Colorless

* Sections mounted in water and examined immediately.

each set there was added 0.5 ml. of paraldehyde (aldehyde-free). Thus, the basic fuchsin solution containing 1 per cent HCl and 1 per cent paraldehyde corresponded to Gomori's specifications for the preparation of paraldehyde-fuchsin. The solutions were allowed to age at 25° C. for 15 hours and for 48 hours before use. The added paraldehyde did not influence significantly the pH of the solutions. Deparaffinized sections of pancreatic tissue were immersed separately in each dye

TABLE II

The Effect of pH upon Staining Pancreatic Islet-Cell Granules

Stain	pH of dye solution	Staining interval	Oxidation prior to staining	Coloration of beta-cell granules*	Coloration of alpha-cell granules*
Basic fuchsin	1.3	6, 10, and 30 min.	None	Pale pink	Pale pink
	2.3	6, 10, and 30 min.	None	Pink	Pink
	6.9	1 to 5 min.	None	Pink to red	Pink to red
	1.3 and 2.4	3 to 10 min.	Peracetic acid (2 to 3 min.)	Bright red	Pale pink
	6.9	5 to 10 sec.	Peracetic acid (2 to 3 min.)	Moderate to dark red	Moderate to dark red
Crystal violet	1.3	6, 10, and 30 min.	None	Faint purple	Faint purple
	2.3	6, 10, and 30 min.	None	Pale purple	Pale purple
	5.6	1 to 5 min.	None	Light to moderate purple	Light to moderate purple
	1.3 and 2.3	1 to 2 min.	Peracetic acid (2 to 3 min.)	Dark purple	Faint purple
	5.6	5 to 10 sec.	Peracetic acid (2 to 3 min.)	Brilliant purple-red	Murky blue
Acid fuchsin	1.3, 2.2, and 3.0	5 to 6 min.	None	Pink	Pale red
	1.3	5 to 10 sec.	Peracetic acid (2 to 3 min.)	Pink	Red
	3.0	1 min.	Peracetic acid	Faint pink	Faint pink

* Sections mounted in water and examined immediately.

solution for 1 to 10 minutes. The sections were examined through a water mount as before.

The results are summarized as follows: Direct staining with the various paraldehyde-dye mixtures, excepting paraldehyde-basic fuchsin, led to results similar to those with direct staining in the same dye solution of corresponding pH but without added paraldehyde. Direct staining with paraldehyde-basic fuchsin at pH 1.3 or 2.3 and

aged for 15 hours resulted in bluish red beta granules and faint pink alpha granules. Elastic fibers were colored a deep bluish red. After the dye solution had aged 48 hours, the beta granules and elastic fibers were stained a deep purple or purple-red. The paraldehyde basic fuchsin solution at pH 6.9 did not give rise to differential islet-cell staining.

The Effect of Prior Oxidation upon Staining with Paraldehyde-Fuchsin. The reagents and the procedures employed in this experiment were identical with those described under the heading "The effect of prior oxidation upon staining with stock dye solutions." The only difference in the present experiment was that staining was performed with Gomori's paraldehyde-fuchsin solution, aged 48 hours before use. The staining interval was 10 to 15 seconds. Non-oxidized controls were carried along for each fixative.

The results are presented in Table III.

Destaining Procedures. The stained sections in the experiments just described were examined microscopically through a water mount.

TABLE III

The Effect of Prior Oxidation upon Staining Pancreatic Beta-Cell Granules with Paraldehyde-Fuchsin, Aged 48 Hours

Oxidant or pretreatment	Oxidation or pretreatment interval	Coloration of beta-cell granules*
Peracetic acid	2 to 3 min.	Deep purple
	2, 6, and 24 hrs.	Deep purple
Performic acid	2 to 3 min.	Deep purple
Acetic and formic acid controls	2 to 10 min.; 1, 6, and 24 hrs.	Colorless
Potassium permanganate, acidified (0.5% aqueous)	5 to 10 min.	Deep purple
Periodic acid (1% aqueous)	2 to 15 min.	Pale purple
	1.5 hrs.	Purple
	2 hrs.	Deep purple
	24 hrs.	Deep purple
Bromine:carbon tetrachloride (1:39 vol. dil.)	5 to 10 min.	Light purple
	30 min.	Deep purple
No pretreatment		Faintly colored or colorless

* Staining interval: 10 to 15 seconds. Direct staining for 3 to 10 minutes, depending upon the type of fixation used, was necessary to give results equivalent to those with staining for 10 to 15 seconds after oxidation or bromination.

These sections then were treated with a variety of organic solutions, listed as follows:

1. 70% ethanol containing 1% HCl, referred to as "acid-alcohol" (pH, 1.34)
2. 1% aqueous solution of borax ($Na_2B_4O_7 \cdot 10H_2O$), referred to as "borax solution" (pH, 9.16)
3. Sequence of 95% ethanol, 100% ethanol, ethanol-xylene (equal parts), xylene, and permount
4. Sequence of acetone, acetone-xylene (equal parts), xylene, and permount

The *results* are summarized as follows: The sections of pancreas stained by any of the basic dye solutions with or without prior oxidation were decolorized completely by acid-alcohol within 2 minutes but largely resisted decolorization by the borax solution for 30 minutes. This applied to all the pancreatic structures, including beta-cell granules, that were stained by a given method. Decolorization with acid-alcohol was extremely rapid (few seconds) in beta granules stained by crystal violet and least rapid (1 to 2 minutes) in those stained by paraldehyde-fuchsin. The pancreatic structures, including the alpha-cell granules, that were stained by acid fuchsin were decolorized by the borax solution within 2 minutes but largely resisted decolorization by acid-alcohol for 30 minutes. The coloration of beta granules by crystal violet was extracted completely in 95 to 100 per cent ethanol within 1 minute and in acetone within 3 minutes. The metachromatic staining of beta granules by crystal violet after peracetic acid oxidation was transformed into an orthochromatic coloration when the sections were dehydrated rapidly in the acetone sequence. By this rapid dehydration method the beta granule staining was extracted minimally, whereas acinar-cell staining was decolorized to a variable extent within the same section. The usual non-rapid dehydration sequence in ethanol resulted in a minimal loss of dye from sections stained with paraldehyde-fuchsin. Actually, the intensity of beta granule staining did not appear to be affected. Beta granules stained by crystal violet or by paraldehyde-fuchsin not only could be decolorized completely with acid-alcohol but also could be restained in undiminished intensity. In other words, under the conditions employed, the use of decolorizing agents did not result in dissolution of the beta granules.

*The Effects of Various Fixations upon Direct Staining with Paraldehyde-Fuchsin.** The effects of varied fixations upon direct staining of

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beta-cell granules with paraldehyde-fuchsin are summarized as follows: With tissue fixed in buffered formol sublimate, the optimal interval for staining beta granules varied between 1.5 and 3 minutes. With tissue fixed in Bouin's fluid or in 10 per cent formalin, the optimal interval was 10 minutes. With staining for intervals longer than these, there was no appreciable change in the intensity of beta granule coloration, though the general background staining was accentuated. Under these conditions of staining, the beta granules were colored brilliantly in tissue fixed in formol sublimate or in Bouin's fluid, and slightly less brilliantly in tissue fixed in 10 per cent formalin. Under these conditions of staining, the general background coloration was nil with tissue fixed in Bouin's fluid or in 10 per cent formalin, and was slight to moderate in tissue fixed in formol sublimate. The short (1 to 2 minute) treatment of sections with Lugol's solution, necessary to remove mercury precipitates from tissue fixed in formol sublimate, did not affect the results of staining when applied to tissues fixed in Bouin's fluid or in 10 per cent formalin. Thus, under the conditions employed, the use of Lugol's solution does not appear to be a factor in accelerating the staining of beta granules in tissue fixed in formol sublimate. Fixation of tissue in absolute alcohol or in Zenker's fluid was found to be unsuitable.

Factors That Affect the Staining Properties of Paraldehyde-Fuchsin Dye. The following factors influenced the staining properties of the paraldehyde-fuchsin dye: (1) The use of paraldehyde solutions from different sources seemed to alter somewhat the ability of paraldehyde-fuchsin to stain the beta granules. The paraldehyde which had been most satisfactory in our hands was a preparation sold at a local pharmacy for medicinal purposes. (2) The use of commercial basic fuchsin dyes of low maximal spectral absorption (435 to 440 mμ) failed to produce paraldehyde-fuchsin dyes which were satisfactory for staining beta granules, though grossly a purple dye was formed. This confirms the observations of Gomori.[2] (3) Paraldehyde-fuchsin dyes which had aged over a prolonged period (e.g., 2 months at 25° C.) failed to stain the beta granules directly. However, after selective oxidation, the granules could be colored about as quickly and intensely as with dye preparations aged for short intervals.

Summary of the Results of Staining Pancreatic Beta-Cell Granules with Basic Dyes

Basic triphenylmethane dyes, such as basic fuchsin, methyl violet 2-B, or crystal violet, were capable, in the absence of HCl and of paraldehyde, of staining elastic fibers and beta-cell granules in

pancreatic islets. However, this direct staining resulted in such a generalized and intense coloration of pancreatic structures that differentiation between the connective tissue fibers or the granular types in the islets was difficult or impossible. Lowering the pH of these dye solutions led to a depression of their affinity for various pancreatic structures, but the islet-cell granules, though stained less intensely, still could not be distinguished readily as two types. Only paraldehyde-fuchsin was capable of staining, both directly and selectively, the beta-cell granules.

Oxidation of sections in performic or peracetic acid for 2 to 3 minutes had a pronounced effect upon the subsequent staining of beta-cell granules by basic dyes. The dyes found to be most suitable for such staining after oxidation included basic fuchsin, paraldehyde-fuchsin, methyl violet 2-B, and crystal violet. Malachite green, methyl green, and spirit blue were less satisfactory. The optimal interval of staining with these dyes was 5 to 20 seconds, whereas in the case of paraldehyde-fuchsin without prior oxidation, the staining interval, though dependent upon the fixation employed, had to be considerably longer to give equivalent results. The basic fuchsin solution was effective only if used at a pH below 3; if the pH was 6.9, a differential staining of the islet-cell granules did not occur. Methyl violet 2-B and crystal violet worked effectively without adjusting the pH of their solutions. Both resulted in an intense metachromatic staining of the beta granules. This staining could be transformed into an orthochromatic coloration upon rapid dehydration of the sections in acetone. A slow dehydration of sections in acetone or ethanol resulted in complete extraction of the dye from the beta granules. Lowering the pH of the basic fuchsin, methyl violet 2-B, or crystal violet solutions did not affect the intensity of beta granule coloration, though the general background staining was suppressed.

Oxidation of sections in 1 per cent aqueous periodic acid for 2 or more hours at 25° C. or in 0.5 per cent aqueous acidified potassium permanganate for 5 to 10 minutes at 25° C., or bromination in bromine: carbon tetrachloride for 30 minutes at 25° C. produced results similar to those when peracetic acid was used for 2 to 3 minutes. Prolonging the oxidation or bromination interval, in general, accentuated the background staining.

Paraldehyde-fuchsin which had aged 2 months at 25° C. failed to stain the beta granules directly, though the elastic fibers were colored intensely. However, if the sections were first oxidized with peracetic acid, the beta granules were stained just about as quickly and intensely with old paraldehyde-fuchsin as with the relatively fresh dye.

No matter by which method the beta granules were stained, the granules were decolorized within 3 minutes with acid-alcohol (pH, 1.3), whereas the dye resisted extraction in a borax solution (pH, 9.2) for 30 minutes. The reverse occurred upon decolorizing the alpha-cell granules which had been stained by acid dyes, such as acid fuchsin. After decolorization, the granules were restainable in undiminished intensity, indicating that they did not dissolve in the solvent of the dye.

Staining Properties of Purified Insulin Crystals

Material. The insulin crystals used had been specially purified (The Lilly Research Laboratories, lot no. 466368).

Solubility of the Insulin Crystals. Approximately 20 mg. of the insulin crystals were found to be soluble at 25° C. in 20 ml. of each of the following solvents: N/10 NaOH in distilled water, N/10 HCl in distilled water or in 70 per cent ethanol, 1 per cent aqueous periodic acid, and peracetic acid. The dried insulin was found, under the same conditions, to be largely insoluble in distilled water (pH, 5.5), acetone, 95 per cent ethanol, and absolute ether.

Preparation of Insulin "Films." The "films" were fixed separately in the following fluids for 4 to 6 hours at 25° C.: (1) Bouin's picroformalin acetic acid solution (pH, 1.4); (2) formol sublimate buffered with sodium acetate (pH, 5.8); and (3) 10 per cent formalin buffered with calcium acetate (pH, 6.9). The insulin "films" did not dissolve appreciably in these fixatives.

Staining of Fixed Insulin "Films." The fixed "films," after rinsing in distilled water, were stained separately in the following dye solutions for 45 seconds and then rinsed quickly in water: (1) 0.5 per cent basic fuchsin in 60 per cent ethanol (at pH 6.9, 2.5, and 1.3); (2) 0.5 per cent crystal violet in 60 per cent ethanol (at pH 5.5, 2.3, and 1.3); (3) 0.5 per cent acid fuchsin in 60 per cent ethanol (at pH 3.0, 2.2, and 1.3); (4) Gomori's paraldehyde-fuchsin, aged 48 hours (pH, 1.3).

The *results* are summarized as follows: Each dye solution, except paraldehyde-fuchsin, stained the insulin "films" orthochromatically and intensely regardless of the fixation employed or of the pH of the dye solution. The dyes obscured, without alteration of their natural color, the yellow coloration of the insulin-picrate (Bouin's fixed) "films." Paraldehyde-fuchsin failed to color the fixed "films" in 3 minutes, though at 5 minutes a dull pinkish tinge existed, and after 5 minutes the "films" dissolved in the dye solvent.

Oxidation with peracetic acid for 1 or more minutes led to dissolution of the fixed insulin "films."

Discussion

From the experimental data it appears that by peracetic acid or potassium permanganate oxidation and by bromination there is produced, in pancreatic beta-cell granules, an acid substance capable of *rapidly* taking up various basic dyes even from pH 1.3 solution. At this pH level, the staining of alpha-cell granules and acinar-cell cytoplasm is almost nil. Those basic dyes of strong affinity for the oxidized beta granules include basic fuchsin, paraldehyde-fuchsin, crystal violet, and methyl violet 2-B. The paraldehyde-fuchsin dye is unique in that it is capable of staining the beta granules selectively without the influence of prior oxidation, though the latter greatly accelerates the subsequent uptake of dye.

There is evidence, though indirect, that the above staining reactions depend upon the protein constituent of the beta-cell granules. So far as can be determined from various histochemical methods applied to frozen and paraffin sections, such granules do not possess lipid or carbohydrate components.[7]

The findings from the present investigation support the theory that the mechanism of staining pancreatic beta-cell granules is based upon those physicochemical factors affecting the interaction of basic dyes with proteins. Of importance in this regard is the reversible nature of the staining reaction when the solution environment of the tissue sections is changed Thus, beta-cell granules stained by a given basic dye can be decolorized readily with a solution free of dye if the pH of such solution is adjusted downward—a pH region which favors dissociation of a basic dye-protein combination. As observed in the present study, the extent and rate of destaining depends upon the particular dye and its affinity for the beta granules and the conditions of washing. These features appear to follow the general principles of staining tissue proteins as reviewed by Singer.[8]

It seems improbable that the staining reactions employed in the present investigation depend upon the formation of azomethines (Schiff's bases) between tissue aldehydes and basic dyes possessing open amino groups because of the following: (1) The beta granules fail to react with Schiff reagent either directly or after oxidation, indicating the absence of reactive aldehyde groups; (2) acid fuchsin, which possesses open amino groups, fails to stain the beta granules, whereas crystal violet, in which the amino groups are methylated, is capable of staining the beta granules after oxidation; (3) the formation of colored Schiff's bases in tissue sections is a slow process, whereas the staining of beta granules after oxidation is rapid; and (4) the use of

aldehyde blocking reagents (phenylhydrazine-HCl or aniline-HCl) fails to prevent the staining of the beta granules. A previous report[9] dealt with the physicochemical properties of basic fuchsin and of paraldehyde-fuchsin and with the conditions under which these dyes are capable of forming azomethines in tissue sections.

The problem now arises as to the nature of the protein substance characterizing the pancreatic beta-cell granules. Is it insulin or insulin precursor? Hartroft and Wrenshall[10] reported their finding of a high degree of correlation between bioassays of extractable insulin and numbers of beta-cell granules stained by paraldehyde-fuchsin. The material consisted of pancreases obtained at necropsy from diabetic and non-diabetic subjects. A loss in extractable insulin content, such as occurs in cases of diabetes mellitus, was found to parallel a loss in granulation of beta cells. An abnormal metabolic state in animals similar to diabetes mellitus in man may be produced experimentally in a variety of ways.[11] In such cases of experimental diabetes there also is an associated loss in beta-cell granulation.[11]

Thus, as is so often stated in recent literature, there is evidence that the beta-cell granules represent stored insulin or, at least, a precursor of insulin. If this is true, paraldehyde-fuchsin would be expected to react with a protein similar or identical to insulin. This hypothesis is controverted by the findings in the present investigation. Of importance are those observations dealing with the staining properties of purified insulin crystals in the form of insulin "films" subjected to the same fixations as applied to the pancreatic tissue. It was found that not only did paraldehyde-fuchsin fail to stain the fixed insulin "films" within 3 minutes, but also that after 5 minutes the insulin dissolved in the dye solvent. Furthermore, the fixed insulin "films" were stained rapidly by various basic dyes and by acid fuchsin regardless of the fixation employed or the pH of the dye solution, though at low pH levels the "films" eventually dissolved in the dye solvent. Such staining properties do not correspond with those of pancreatic beta-cell granules, though the dye solutions employed were identical.

Also of importance are those findings related to the solubility of fixed insulin "films" as compared to the solubility of pancreatic beta-cell granules fixed in an identical manner. As mentioned previously, the fixed insulin "films" dissolved in the dye solvent within a specified interval depending upon the pH of the solution, there being rapid dissolution in alcoholic solutions of low pH. The use of corresponding dye solutions did not result in dissolution of the fixed beta-cell granules even at prolonged intervals (24 to 48 hours). Furthermore, performic and peracetic acids are known to increase the solubility of

insulin by cleaving the cystine-disulfide bonds.[12] This results in the formation of two distinct polypeptide chains, each with characteristic solubility properties. In the present investigation purified insulin, whether subjected to fixation or not, dissolved rapidly in peracetic acid. In contrast, fixed pancreatic beta-cell granules did not appear altered in number or structure after tissue sections had been oxidized with peracetic acid for 24 hours, followed by washing in acid or alkaline solutions and then staining with paraldehyde-fuchsin or crystal violet. Thus, the solubility properties of purified insulin did not correspond with those of pancreatic beta-cell granules under the conditions employed.

It is evident, then, that pancreatic beta-cell granules do not represent stored insulin, assuming that the latter is in a form similar to extractable insulin. Yet, there remains to be explained the high degree of correlation between extractable insulin and numbers of beta-cell granules stained by paraldehyde-fuchsin in a particular pancreas. Since the nature of the protein characterizing the beta-cell granules is not known, it is impossible to determine the true significance of this granulation. At present, the degree of beta-cell granulation may be regarded as reflecting the state of biochemical activity for synthesizing insulin.

Summary

After specific oxidation or bromination, beta-cell granules in pancreatic islets were stained quickly and intensely by certain basic dyes, including paraldehyde-fuchsin. Factors that influenced such staining reactions are the type of fixation employed, the nature of the oxidant and interval of oxidation or bromination, the pH of the dye solution, the staining interval, and the use or omission of varied dehydrating agents after staining. Those basic dyes of strong affinity for the oxidized beta-cell granules included basic fuchsin, paraldehyde-fuchsin, crystal violet, and methyl violet 2-B. Paraldehyde-fuchsin was unique in that it stained the granules without the influence of prior oxidation, though the staining interval had to be considerably longer than if sections first were oxidized.

It appears that by oxidation with peracetic acid or potassium permanganate and by bromination with bromine:carbon tetrachloride there was produced, in beta-cell granules, an acid substance of protein nature capable of taking up rapidly and selectively certain basic dyes from pH 1.3 solution. At this pH level the staining of alpha-cell granules and acinar-cell cytoplasm was almost nil. The beta granules were decolorized readily and completely by acid-alcohol at pH 1.3, a pH region which favors dissociation of basic dye-protein combinations.

From the studies dealing with the staining and solubility properties of purified insulin crystals as compared to those properties of pancreatic beta-cell granules, it is concluded that the granules do not represent stored insulin.

ADDENDUM

George Gomori, at the University of Chicago, reviewed this article shortly after it was accepted for publication. He made the comment that removal of paraldehyde-fuchsin from beta-cell granules by acid-alcohol does not occur unless the dye is freshly prepared. Since, in the present investigations, the paraldehyde-fuchsin dye was aged for only 48 hours, it was decided to try acid-alcohol destaining procedures on beta granules colored by paraldehyde-fuchsin aged for 8 days. In such instances, little or no dye was removed from the granules, thus confirming Gomori's findings. This indicates that the staining of beta-cell granules by paraldehyde-fuchsin does not depend upon ionic forces but is based upon a genuine chemical reaction between the dye and the protein in the granules.

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