Immunological Methods in Endocrinology

Symposium in Ulm, February 25, 1970 in Connection with the 16th Symposium of the German Endocrine Society

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Contents

Introduction

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This symposium, planned as a "workshop" on immunological methods in endocrinology, was originally scheduled to be held in Ulm some time earlier. However, the rather turbulent developments connected with starting a new University caused a delay in the fulfilment of this plan. Thus, when the German Endocrinological Society chose Ulm as the place of their Annual Meeting 1970 and Prof. Tonutti and Prof. Kracht proposed a "pre-symposium", we gratefully accepted the opportunity.

The first draft of our program had a much wider purview than the final one. It contained other main topics such as immunopathology in endocrinology, comparisons of biological, chemical and immunological structures of hormones, practical use of the new radioimmunological methods for demonstration of hormones in the organism, not to mention a special differentiation in the techniques of labeling, antibody-production, standardization, clinical application including the development of solid phase systems as well as the problem of automatization. However, we had to realize that such an extensive program — comparable to the programs for the symposia on similar subjects in Milan and Liège in 1968, and for the symposium in preparation in Edinburgh for September 1970 — would alone have occupied the full time scheduled for the entire Annual Meeting of the Society for Endocrinology.

It was for these reasons that we decided to consider the practical needs of the clinical endocrinologist. General aspects were left to the introductory papers for the various sessions, while it was left to the other speakers to deal with the specific methodological aspects of the single hormones.

Undoubtedly, the techniques for measuring the tiny amounts of protein hormones in blood provided the most important impetus to the broad field of endocrinology. However, the basic facts related to those hormones had already been established on the grounds of biological assays and clinical observations in patients as well as in healthy persons. The new situation can be characterized by the fact that at present any laboratory interested has the chance to examine the secretory capacity of the different glands, i. e. pituitary, pancreatic or parathyroid glands, in the *individual* patient.

On the other hand, the rather simple principles of the radioimmunologic assay should not conceal two main problems not yet satisfactorily solved: 1) the unspecific cross-reactions between antisera and glycoproteins and 2) the difficulties of raising antibodies to protein and peptide hormones of low molecular weight. Add to this the unsatisfactory standardization of antisera over longer periods of time as well as the lack of generalized standardization.

Therefore, a closer collaboration between the various laboratories should be encouraged and promoted. In this symposium we invited as many colleagues from foreign countries as possible — mainly from European countries (financial considerations prevented us from extending our efforts to countries overseas) — guided by the conviction that medical science in one country alone cannot solve all the existing general and common problems. It has been the intention of this symposium to facilitate scientific contacts and cooperation in the European area. Hopefully, other symposia, which will be arranged in the not too distant future, will encompass a wider spectrum of participants and speakers.

PART I

The Demonstration of Hormones in Tissues by Means of Antibodies

Methodological Problems in the Use of Immunohistological Techniques for the Localization of Hormones

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Hormones are characterized by a specific biological effect. The qualitative and quantitative determination of these effects requires extraction of the hormones from tissues or body fluids. For this reason the biological effect cannot serve in the exact localization of hormones in tissue structures.

In order to demonstrate hormonally active substances in organs, cells or cell organelles it is necessary to make use of a further property of the hormone molecules. This property is antigenicity, i. e. the property of inducing the formation of antibodies reacting specifically with the hormone used. This property is found in hormones having a protein or polypeptide character. Insofar as steroid hormones are concerned it has only been possible to obtain specific antibodies against 17-β-estradiol.

The principle of immunohistological localization of hormones in tissue consists of incubating specific labeled antibodies with histological tissue sections. After the section has been washed, antibodies will adhere only to those tissue sites where the hormone being investigated is concentrated. Due to the labeled antibodies these sites of antigen-antibody reaction become visible under the microscope (Fig. 1). This technique requires the use of specific antibodies which will only react with the hormone to be investigated. This aim can only be reached if absolutely pure hormones are used for immunization. Optimally, this would mean synthetic pure hormones. Preparations obtained by extraction involve the risk of impurities. These impurities are also to be expected if only a solitary specific hormonal effect can be demonstrated. Even denaturated inactive hormone impurities have an antigenic effect. This reservation should always be kept in mind in related studies.

Antigenicity of the various hormones differs greatly. Broadly speaking, it may be said that high molecular hormones can induce a stronger antibody production than low molecular hormones. However, the development of special immunization techniques has permitted the production of antisera against even extremely short-chain polypeptides. The decisive factor of more effective immunization is to be found in the fact that short-chain hormones, by being coupled to larger molecules, are completed similar to haptens. Suitable coupling vehicles are serum albumins, polyaminó acids, particular substances such as charcoal (Dietrich and Rittel 1969), plastics (Kracht, Hachmeister, Breustedt and Zimmermann 1967) or the hormone itself by polymerizing it (Heding 1969). Antibodies against the vehicles can be absorbed. In systems in which the vehicles themselves are not present, they are of no importance. The fact that emulgation of the antigen preparations with adjuvant based on mineral oil with or without addition of mycobacteria of the

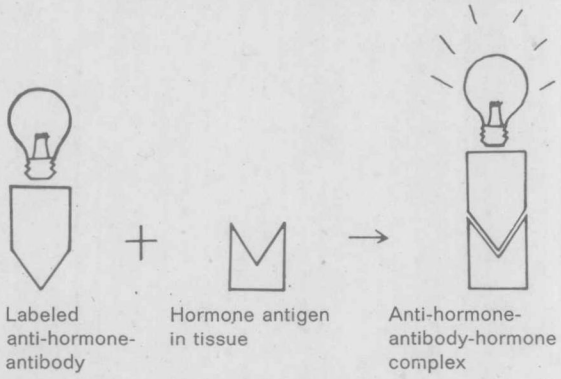


Fig. 1. Direct immunohistological technique.

type of Freund's adjuvant has decidedly improved results is generally known.

Standard animals for immunization are rabbits and guinea pigs. The use of monkeys, sheep, dogs and chickens has been repeatedly described. Some hormones only induce good antibody production in certain animal species. This selectivity is partly due to the fact that there are varying differences in the amino acid sequence between the endogenously produced hormone of the immunized animal and the sequence of the hormone used as an antigen. The higher this difference, the more readily will the injected hormone be recognized as a foreign antigen. Example: since insulin of the guinea pig greatly differs from the insulin molecule of other mammalian species it is particularly suitable for obtaining insulin antibodies.

The more or less marked accordance of the amino acid sequences of homologous hormones in various species and the similarity of tertiary structure form the basis for the phenomenon of cross-reactions transcending species barriers. This is to say that antibodies against the hormone of one species may react with hormones of the same biological activity of other species. This cross-reaction also depends on another fact, namely against which sequence portion of a hormone the antibodies are directed. If the sequences bearing the species differences prove determinant antigenically, the cross-reaction range will be minimal. If common sequences are determinant the cross-reaction will show a broad spectrum. It is significant that the chain portions necessary for biological activity are mostly identical among the species.

Difficulties arise if identical chain portions are present in several hormones of different biological characteristics. This is the case, for example, in gastrin and pancreozymin-cholecystokinin and cerulein, a substance from the chromophores of Hyla caerulea. In these three substances a sequence of five amino acids is identical. Antibodies against these sequences cannot discriminate between the two hormone molecules and the frog substance. The same situation prevails with ACTH and MSH (Kracht et al. 1967) and TSH and LH of bovine origin (Ta-Hsin Liao, Hennen, Howard, Shome and Pierce 1969).

The most widely used labeling substance for immunohistological studies is fluorescein isothiocyanate (FITC). This dye is easily coupled to antibody proteins via thiocarbamide binding with the epsilon-amino groups of lysine. In like manner tetramethyl rhodamine isothiocyanate binds itself to the proteins. Under stimulation with highly energetic light fluorescein gives an intense apple-green fluorescence in the 300–400 nm range, whereas rhodamine, depending on the wave length of stimulation, yields a yellow to orange fluorescence. Numerous studies on the optimal dye/protein binding rate are available for FITC. It was repeatedly observed that an excessive dye load of the antibody molecules leads to pseudospecific tissue reactions (Goldstein, Slicys and Chase 1961) which – due to the selectivity of reaction with certain cell groups – may feign specific results (Kracht et al. 1967). The only remedy is consequent fractionation of the coupling product according to dye binding rates by anion exchangers (Dietrich and Rittel 1969, Goldstein, Slicys and Chase 1961). Determination of the fraction neither overcoupled nor undercoupled must be individually made.

In newer studies antibodies are often coupled with enzymes which yield a visible reaction product after corresponding reaction (Nakane 1968/69). The most suitable substance at present is horseradish peroxidase since this enzyme shows very little interference with endogenous enzyme activities. However, glucose oxidase and alkaline phosphatase may also be used as labeling substances. Compared with preparations giving a fluorescent microscopic evidence of antibody binding sites, enzyme immunological methods have the advantage of yielding durable preparations. Fluorescent dyes quickly lose their luminosity. Moreover, the enzyme method seems to be more sensitive if highly active enzymes are used. Another advantage of horseradish peridoxidase labeling is that the electron density of the resulting reaction product is also highly osmiophilic. Thus the essential prerequisites for extending immunohistological hormone localization into electron microscopy are given. The first results with this method are already available (Hachmeister 1970, Nakane and Kawarai 1970). Our own studies show, however, that unspecific tissue reaction with the detection reagent for peroxidase diaminobenzidine in the spontaneously oxidized form is a source of misinterpretation, especially with regard to the anterior pituitary and the pancreatic islets. Ferritin, the classical labeling substance in electron microscope immunohistology (Singer and Schick 1961), has proved unsatisfactory in the localization of hormones. This is probably due to the fact that the resulting antibody-ferritin coupling product with an MW more than 600,000 is hardly suitable for tissue penetration. Its use on thin sections is restricted due to the unavoidable unspecific adsorption of almost all embedding substances used in electron microscopy. Studies published so far on the localization of growth hormone in the adenohypophysis (Tani, Kawamura, Ametani, Handa, Imura and Kato 1969) have shown that reaction can only be obtained if the tissue is destroyed to a great extent. An interesting labeling substance, 3-carboxy-4-ferrocenylphenyl isothiocyanate has been developed by Franz (Franz 1968). This substance will have to undergo more detailed investigation. The iron content and possibly the special structure of the ferrocen ring make this substance visible under the electron microscope. The isothiocyanate group permits careful coupling to the antibody proteins. Since the molecular weight is low the antibodies should be able to penetrate tissues

Table 1. Labeling of Antibodies for Immunohistological Studies

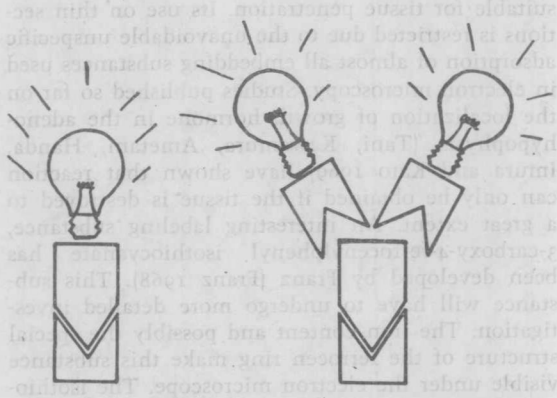
1. Fluorescein isothiocyanate	green fluorescence
2. Rhodamine-B-isothiocyanate	yellow-red-fluorescence
3. Peroxidase 4. Glucoseoxidase 5. Phosphatase	after reaction the enzyme substrate becomes visible light optically and also electron microscopically due to the electron density
6. Ferritin	single molecule visible
7. Uranyl acetate 8. Mercury (after thiolation)	increased contrast of antibody linkage sites
9. Diazothioester-osmic acid 10. Carboxy-ferrocenylphenyl-isothiocyanate	accumulation of iron = electron density

sufficiently. The presently known and used antibody labelings for immunohistological methods are shown in Table 1. The principle of autoradiographic demonstration of antigen-antibody complexes has not been considered.

Since immunohistological methods aim at the association of the hormone being investigated to the appropriate tissue structures, fixation of the hormone-containing tissue is usually required. This fixation in most cases must differ from the fixations generally used in histology.

Optimally, preliminary model studies should be made to find a method of fixing the hormone in the tissue, preserving the antigenicity of the hormone and its accessibility for the antibody as well as the fine structure of the tissue.

Optimal conditions for each individual case cannot be generalized. However, it has frequently been shown that fixations in the cold are of advantage. At these temperatures the proteins seem to be cross linked in the tissue without considerable denaturation. Under these conditions neutral buffered formalin and ethanol have found wide application. The time of fixation is short. The safest preservation methods are freeze-drying and cryostat sections, although misplacing of the antigens must be borne in mind.



a) Direct technique b) Indirect technique (sandwich) Multiplier effect

Fig. 2.

Reaction of the antibodies with hormone antigens in the tissue may take place in two ways:

1) In the *direct method* the hormone antibody is coupled with the labeling substance and reaction is effected in one step. 2) The *indirect sandwich method* in which the binding of the unlabeled hormone antibody in the tissue is made visible by a second reaction step with a labeled antibody against the gamma-globulin class of the species used in the first step. This method is the most widely used. It has the advantage of requiring only one labeled antibody for various reactions. Moreover, sensitivity is greater due to a simultaneously occurring multiplication effect (Fig. 2).

Reaction between tissue and antibody is often carried out at 37° C. Our own studies have shown that prolonged incubation at 4° C lessens the unspecific adsorption of labeled proteins without impairing the binding of antibodies.

Before reaction with antibodies in tissue can be recognized as localization of the antigen under investigation, immunological specificity must be tested under stringent categories. Validity of an immunohistological reaction requires inhibition of the reaction if the corresponding antigen is added to the antibody before tissue incubation. The antibody must be neutralized by the antigen, not by another substance. In the direct method reaction must be blocked by pre-incubation with an unlabeled hormone antibody. In the sandwich method reaction of the second step must be prevented by pre-incubation with an unlabeled anti-species gamma-globulin. If enzyme labeled antibodies are used, it must be safeguarded that endogenously-present enyzme activities do not feign hormone localization and that the reaction product is not unspecifically fixed by the tissue. The validity of all published methods for hormone localization should be scrutinized under the following aspects:

1) Whether the hormones used for immunization are sufficiently pure or whether measures for the absorption of antibodies against impurities have been taken or both. The side fractions of extraction methods are suitable for such absorptions.

2) Whether FITC-coupled antibodies have been sufficiently processed after coupling by the ion exchange method. Our experience has been that commercially available sera are always overcoupled.

3) Whether specificity tests have been sufficiently applied.

Summary

A brief review of methodological problems in the localization of hormones in tissue by antibody techniques is outlined.

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The Immunohistological Demonstration of Insulin in Pancreatic Tissue

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Lacy and Davies (1957, 1959) described for the first time the demonstration of insulin in beta cells on the basis of a specific immunological reaction with a fluorochrome labeled antibody. This antibody was produced in the guinea pig and directed against bovine crystalline insulin. It reacted also with the insulin of some other species such as rat, rabbit, dog, cat and mouse. In further studies the cross reactivity of anti-insulin serum with different mammalian species was examined (Costanzi, Mancini and Zampa 1961). Pancreatic insulin could also be demonstrated immunohistologically in human beings (Mancini, Zampa, Vecchi and Costanzi 1965).

Guinea pigs are preferred as the source of antibodies against insulin. This species is especially suitable because of the great differences in the amino acid sequence of its insulin compared with insulins of other species. Nevertheless, it is possible to demonstrate pancreatic insulin in this species with guinea pig antibodies even in the same animal (own observation). For the production of insulin antibodies in guinea pigs, different procedures are recommended. In an approved system, 300 to 400 gm animals are injected with 400 μg crystalline bovine insulin emulsified with complete Freund's adjuvant into the foot pads. The booster injections are given monthly. Animals are bled four to six days after the last immunization. Even after two to three antigen injections antibody titer reaches values which permit the demonstration of insulin in tissue. Gamma-globulin preparations of the antisera can be performed by precipitation with ammonium sulfate, but the loss of the antibody activity will be less by using DEAE-Sephadex columns according to Bruchhausen and v. Mayersbach (1967). After labeling with fluoresceinisothiocyanate (FITC), using 10 μg dye per mg protein, the removal of free dye and the fractionation of antibodies with different F/P-ratio's has been proven to be done most conveniently by DEAE-Sephadex chromatography. The optimally labeled insulin antibodies were obtained using a 0.06 M phosphate buffer, pH 7.4 and antibodies with a F/P ratio ranging from 2.5—3.75 x 10^{-3} (or 1.0—1.5 expressed on molar basis) which, in our experience led to the best immunohistological results. According to the general rules of immunofluorescence (IF) of Coons and Kaplan (1950) the tissue is incubated for 30 minutes in an incubator (37° C) or 45 minutes at room temperature. However, in this respect several changes of the schedule are possible or may be necessary due to the preparation of the tissue. For technical details which cannot be given here, see Hemm (1971).

The pancreatic tissue can be used for the reaction with the antibody in different states: 1) unfixed, cryostat sections, 2) formalin fixed, cryostat sections, 3) formalin fixed and paraffin embedded, 4) freeze dried with subsequent paraffin embedding. Unfixed tissue gives nonsatisfactory results because an equal thickness of the slides can not be kept as in fixed tissue. Better results are obtained with formalin treated pancreas. Lazarus, Brancato, Shapiro and Volk (1967) washed the pancreatic tissue for 24 hours (after fixation in formalin) using phosphate buffer and 0.25 M sucrose solution and then froze the tissue with liquid nitrogen cooled isopentane. Sections were then cut in a cryostat. However, it is also possible to use a regular but shortened paraffin embedding procedure after fixation of the tissue in formalin. In both instances the beta cells are recognizable mainly with a fine granular fluorescence of the cytoplasma and a dark nucleus (Fig. 1). The fluorescence is more dense and sometimes homogeneous at the vascular pole of the cells but in some islets the majority of the beta cells show a homogeneous pattern. This was observed more frequently in embedded material. One could assume that the homogeneous fluorescence is caused by the process of shrinkage during the embedding procedure. But this disadvantage is more than counterbalanced by the possibility of obtaining thin slides of the paraffin material. The fourth method mentioned above (freeze drying and paraffin embedding) can be considered as ideal because it avoids nearly all influences of a chemical nature on insulin and on insulin's location within the cell. Using this technique, a granular form of fluorescence was found in the stained islet cells; homogeneous staining was also observed but it was not as common as in formalin-fixed embedded material. An additional advantage of the method is the negligible nonspecific staining of the surrounding tissue by labeled antibodies (Fig. 2).

Finally, it must be mentioned that immunohistological demonstration of insulin is also possible with the indirect immunofluorescence technique, i. e. incubation of tissue with unlabeled guinea pig anti-insulin antibody followed by a labeled antibody

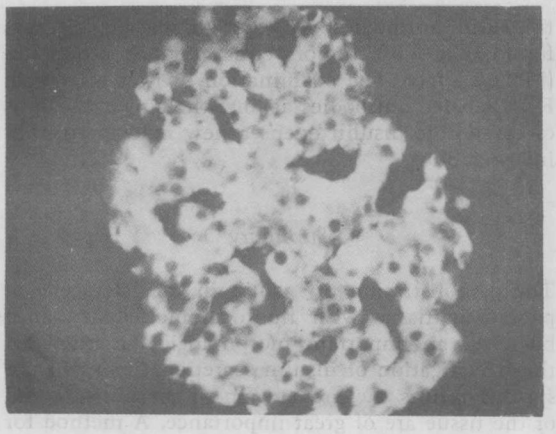

Fig. 1. Normal islet of the rat. Formalin fixation and paraffin embedding. Stained with FITC-labeled antibodies of guinea pig anti-bovine insulinserum (F/P ratio 2.6 x 10^{-3} or 1.1 expressed on molar basis).

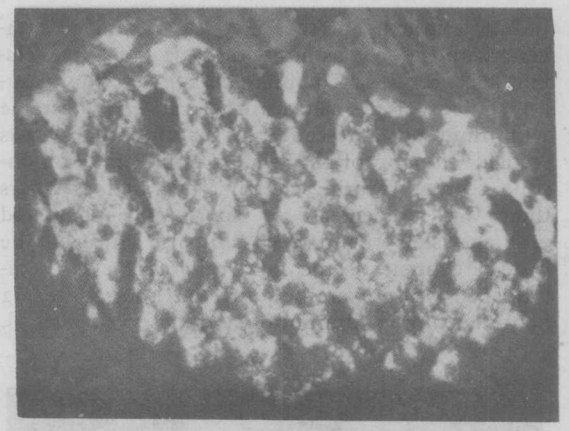

Fig. 2. Freeze-dried islet (paraffin embedding). Same staining as in Fig. 1.

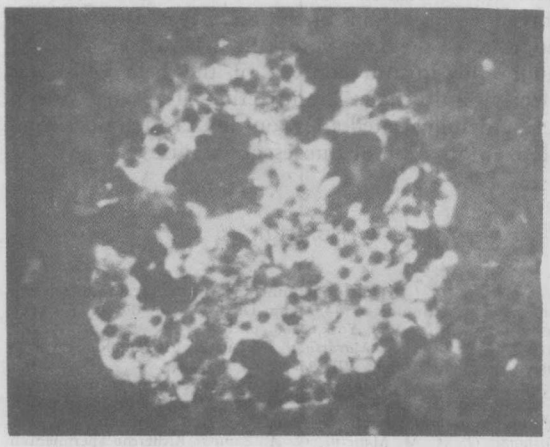

Fig. 3. Islet of a guinea pig stained with autologous anti-bovine insulin antibody.

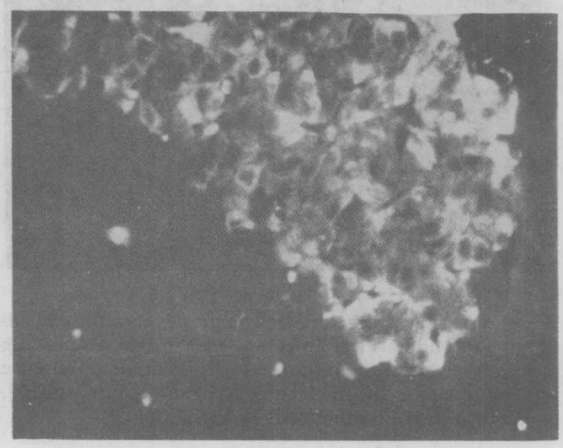

Fig. 4. Rat islet 24 hours after a single injection of glibenclamid (1 mg/kg). Marked reduction of fluorescence of the beta cells.

against guinea pig globulin. However, the results are less satisfactory compared with the direct immunofluorescence technique, as proven by our experience. Also, from the literature it can be seen that the direct immunofluorescence is used mainly for demonstrating insulin in tissue.

The immunohistological staining of beta cells has found different applications.

The distribution of beta cells within the islet can be compared with the discovery of other staining methods. Thus the typical localization of beta cells in the center of the islet in rats has been found while the A and D cells form a dark margin of unstained elements. In guinea pigs, according to other staining methods, strands of A and B cells show a more irregular pattern (Fig. 3). Furthermore FITC-labeled insulin antibodies have been used in the microscopic examination of islet cell tumors in patients with severe hypoglycemia (Lacy and Williamson 1960, Pfeiffer 1967, Breustedt and Kracht 1968, Federlin, Raptis, Beyer and Pfeiffer 1969). The results were contradictory (for possible explanation see below.)

Finally, the effect of the agents stimulating insulin secretion on the insulin content of beta cells has been studied with immunofluorescence (Lazarus et al. 1967). The authors found granular staining of the beta cells of normal rabbits and heavier staining at the vascular poles of the cell. In the pancreas, from cortisone or sulfonylurea treated rabbits in which the beta cells were degranulated, fluorescent staining was markedly reduced. In moderately degranulated beta cells heavy staining was still present at vascular poles of some cells, while in heavily degranulated cells only sparse individual granules along the cell borders were observed.

In our experiments the effect of a single intravenous injection of sulfonylureas (1 mg Glibenclamid/kg, 400 mg Tolbutamide/kg) on the insulin content of the beta cells of rats as detectable with immunofluorescence (at varying times) was studied. In spite of the known rapid effect on insulin secretion during the first two to four hours there was no decrease of fluorescence compared with the islets of untreated animals. However, the insulin content of the beta cells was clearly diminished when examined after 24 hours; after 12 hours beginning diminution was observed (Fig. 4).

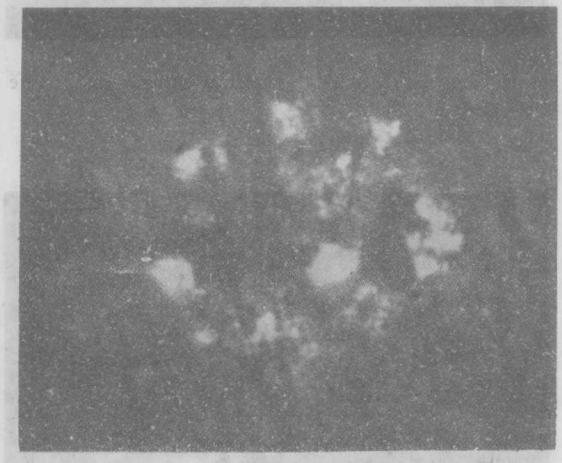

Fig. 3. Rat islet (freeze drying technique, paraffin embedding) four hours after injection of 2 ml of guinea pig anti-bovine insulin serum. Nearly complete absence of fluorescence in the center ("washing out effect"), some insulin containing cells in the periphery.

In contrast, the single injection of 2 ml of an anti-insulin serum from guinea pigs led to almost completely "washed out" islets even after four hours (Fig. 5). It is not the purpose of this paper to go deeper into the discussion of insulin secretion. These experiments were only mentioned to emphasize that – apart from studies with other methods including the electron microscope – insulin secretion investigations can be performed with the method of immunofluorescence.

The immunohistological demonstration of "insulin" must be considered with the stipulation that it is not specific in the sense of pure insulin (monocomponent insulin according to Schlichtkrull et al. 1969). It includes a still unknown extent proinsulin and other related proteins. In this respect it is an important fact that the species differences in proinsulin are much greater than in insulin (Steiner, Hallund, Rubenstein, Cho and Bayliss 1968). Possibly the contradictory immunohistological results obtained in islet cell tumors by different authors are caused by the variable content of anti-proinsulin antibodies among the population of "anti-insulin antibodies" of the guinea pig. It was found that those tumors contain much proinsulin (Melani 1970). The examination of islet cells with FITC-labeled antibodies against the single fractions of crystalline insulin opens a new field for further investigations.

Summary

The demonstration of insulin in the islet cells of pancreatic tissue with fluorochrome labeled antibodies is an important morphological method for the investigation of insulin secretion. To avoid unspecific results the preparation of the antisera and of the tissue are of great importance. A method for fluorochrome labeling of antisera and the preparation of the conjugate by column chromatography are mentioned and the different types of tissue processing are discussed. Using these techniques no decrease of fluorescence was found after the intravenous injection of sulfonylureas two to four hours after the rats were killed. However, after 24 hours the insulin content of the beta cells was clearly diminished. The injection of an anti-insulin serum demonstrated after four hours an almost complete elimination of fluorescent staining.

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Discussion

P. C. Scriba: In your studies in vivo injection of insulin antibody results in decreased islet fluorescence upon subsequent incubation with fluorescent insulin antibody. This was interpreted as indication for stimulation of insulin secretion and decreased insulin content of islets after in vivo injection of unlabeled insulin antibody. An alternative explanation of your observation could be, that insulin contents of islets are not changed, and that the insulin is not reached by the fluorescent antibody, since it is blocked by the non-fluorescent antibody injected previously in vivo.

K. Federlin: The possibility of a fixation of heterologous anti-insulin antibodies within islets was studied in previous experiments. After I.V. injection of guinea pig anti-bovine insulin antisera with high titers into rats the islets were examined immunohistologically for the presence of guinea pig globulins, but no fluorescence could be observed. Therefore it is rather improbable that the reduced demonstration of insulin by the immunofluorescent method is mainly originated by masking the insulin due to the previously injected antibodies.
Some antibodies might reach the islet tissue but their amounts seem to be too low for immunohistological detection in our system.

H. J. Breustedt: I would like to ask Dr. Federlin if he has plans to do more experiments which will support his ideas in relation to other immunological reactions with tumor insulin and insular insulin?

K. Federlin: Further immunological and immunohistological studies on islet tumors and tumor extracts are projected especially in regard of their proinsulin content, but this depends on new tissue specimen which were not yet available.

U. Hachmeister: How much of the demonstrated material is proinsulin? How can you explain the brown color of the pancreatic islets in the control incubations in contrast to the greenish fluorescence of the excretoric pancreatic parts?

K. Federlin: We can not yet answer the question regarding pro-insulin from the point of practical results. Perhaps it will be possible using specific antisera to the various compounds of extracted insulin.
To your second question: The typical color of islets in control incubations is a dark green. The contrast to the brighter surrounding is caused by the nonspecific staining of the excretoric tissue with fluorescent dyes which is low in unfixed cryostat sections but marked in formalin fixed and paraffin embedded material.

L. Kerp: I would like to comment on *Dr. Hachmeister's* question. The antisera against insulin used by *Dr. Federlin* cross-react completely with pro-insulin, but not with the free pro-insulin C-chains. Apparently the pro-insulin impurities in bovine insulin used for the stimulation of antibodies in guinea pigs are not sufficient to elicit antibodies specific for the pro-insulin C-chain.

Another question to *Dr. Federlin:* Is there a difference in immunofluorescence when specific insulin antibodies are used which have been produced with the aid of an immuno-adsorbent, for example when immunoglobulin fractions from the anti-insulin serum are used?

K. Federlin: Up to the present we did not use specific antibodies to insulin isolated with the aid of immuno-adsorbents.