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STUDIES ON ISOLATED ISLETS OF LANGERHANS (BROCKMANN BODIES) OF TELEOST FISHES

I. METABOLIC ACTIVITY *IN VITRO*

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SUMMARY

Conditions are described for incubation of islet tissue of toadfish (*Opsanus tau*). Sterile tissue culture medium (Eagle) was used at relatively low temperature (15–20°) for prolonged incubation periods. Tissue viability up to 48 h was demonstrated by the linearity of several metabolic indices: glucose uptake, lactate production, oxidation of substrate carbon to CO_2, and incorporation of substrate carbon into both total trichloroacetic acid precipitable and acid ethanol soluble islet protein. The comparative use of different substrates showed about equal metabolism of carbon from glucose, mannose and pyruvate, whereas fructose carbon was metabolized at a much lower rate. Results obtained with differentially labeled glucose add further evidence for an operational hexose monophosphate pathway in islet tissue.

Incorporation rates of glucose carbon and leucine into total and acid ethanol extractable islet protein suggest that this preparation might be a useful tool for the study of insulin biosynthesis *in vitro*.

INTRODUCTION

The study of insulin biosynthesis, storage and secretion is rendered difficult by the distribution of the islets of Langerhans within the exocrine pancreas. As yet, isolation of normal surviving mammalian islet tissue for metabolic studies *in vitro* has not been achieved. It is well known, however, that in some teleost fishes the islets of Langerhans form a distinct organ, the BROCKMANN bodies[1] or principal islets[2]. LAZAROW and his collaborators[3–5], among others, have explored the possible usefulness of islets prepared from toadfish (*Opsanus tau*), and the present investigation has been stimulated by their work. This report deals with initial studies on the metabolic activity of isolated toadfish islets, while a subsequent paper will deal with the incorporation of glucose carbon and amino acids into islet protein. The ultimate aim

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of these studies is that of contributing to the elucidation of the metabolic processes involved in insulin biosynthesis, storage and secretion. Some of these studies have been reported in preliminary form[6,7].

MATERIALS AND METHODS

Adult toadfish (*Opsanus tau*) were used as experimental animals. They were obtained either from the Marine Biological Laboratories, Woods Hole, Mass., or from the New England Biological Laboratories, Point Judith, Rhode Island, or from a fisherman in Narragansett, Rhode Island. They were transported to Boston in cooled containers and stored in continuously circulating sea water at the American Lobster Company in Boston. On experimental days they were brought to the laboratory in aerated sea water and killed by stunning just prior to use. The weight of the fish varied between 0.5 and 3 pounds. The largest islet, situated in the mesentery near the spleen, was rapidly dissected by careful cleaning of the fibrous capsule surrounding the islet. Although this fibrous capsule frequently contains some exocrine pancreatic tissue, it was left intact, since preliminary histological observations indicated loss of viability of islet tissue during prolonged incubation, if the capsule was completely removed. The weight of each islet varied between 2 and 6 mg. After dissection the islets were kept in buffer on ice until incubation was begun. Between 5 and 15 islets were as a rule incubated in any one flask.

The incubation medium used in preliminary experiments was Krebs–Ringer bicarbonate buffer[8]. In subsequent experiments, however, EAGLE's basal medium[9] was used (Hyland Laboratories), supplemented with nonessential amino acids[10]. No serum was added. Incubation was carried out in small flat-bottomed, straight-walled flasks of 2 cm diameter and 10 ml capacity, the incubation volume per flask being either 0.75 or 1.0 ml. The flasks were capped with sleeve-type rubber stoppers and individually gassed through stainless-steel needles for 5 min with O_2-CO_2 (95:5). Incubation was carried out in an Eberbach metabolic shaking incubator equipped with a cooling coil to permit incubation below room temperature when indicated. When the incubation was prolonged (at temperatures below 25°), the islets were transferred into fresh medium every 12 or 18 h. Incubation flasks containing the same medium but no tissue were always carried along as controls for bacterial contamination, which was held to a minimum by using sterile medium and sterilized glassware.

Glucose randomly labeled with ^{14}C or specifically labeled in the 1 or 6 position was obtained from New England Nuclear Corporation. Randomly labeled fructose, mannose and L-leucine, as well as mannose labeled in C-1 were obtained from Nuclear Chicago Corporation. The radioactivity in the incubation medium was of the order of 0.5–10 μC/ml. After incubation, $^{14}CO_2$ was collected and assayed as previously described[11]; medium glucose was measured according to SOMOGYI[12] and NELSON[13], or with the glucose oxidase technique of FROESCH *et al.*[14], and medium lactate was assayed with lactic dehydrogenase (Boehringer Company) according to HORN AND BRUNS[15]. The islets were homogenized in 10 % trichloroacetic acid and the precipitate was washed 5 times with 5 % trichloroacetic acid. For the determination of radioactivity in total trichloroacetic acid precipitable protein, the precipitate was heated in 5 % trichloroacetic acid at 90° for 15 min, washed once with 5 % trichloroacetic acid and twice

with ethyl ether (the customary additional extraction with ethanol was omitted since ethanol acidified with trichloroacetic acid removes a considerable portion of insulin). The dried precipitate was then taken up in dilute ammonium hydroxide, plated and counted in a proportional flow counter. The radioactivity of insulin-enriched protein subfractions was measured as follows: The trichloroacetic acid precipitate was extracted with acid ethanol (1.56 N HCl in 65 % ethanol)[16] for 30 min at room temperature, and again for 10 min. The extracts were combined, dialyzed in Visking 23/32 tubing against 4 changes of distilled water at 4°, and lyophilized. The residue was redissolved in a small amount of acid ethanol (0.18 N HCl in 74 % ethanol)[7] and plated with carrier albumin for counting. Radioactivity was measured in a proportional flow counter and self-absorption was corrected for according to KARNOVSKY *et al.*[18].

On some occasions, the acid ethanol extractable protein was hydrolized in 6 N distilled HCl at 110° for 12 h, subjected to two-dimensional ascending chromatography on Whatman paper No. 52 in pyridine – acetone – ammonia – water (45:30:5:20, v/v) and in isopropanol – formic acid – water (80:10:10, v/v). The chromatogram was then subjected to autoradiography on Kodak no-screen film (exposure time 2 weeks), stained with 2 % ninhydrin in ethanol, and fixed with 6 % nickelous chloride.

RESULTS

Initial experiments served to establish conditions suitable for incubation of tissues from this poikilotherm marine organism. In these experiments, oxidation of glucose carbon to CO_2 was used as a convenient reference index for overall tissue viability. In subsequent experiments, glucose uptake was also measured, as well as lactate production and the incorporation of labeled carbon into trichloroacetic acid precipitable and acid ethanol extractable protein. On occasion, the appearance of incubated tissue was checked histologically. Radioautography of islets incubated with L-[^{14}C]leucine showed even distribution of the isotope over the entire thickness of the tissue, indicating satisfactory diffusion of the substrate during incubation*.

Incubation temperature

Four batches of islets were incubated at either 25° or 37° for 1 or 3 h. As can

TABLE I

EFFECT OF TEMPERATURE ON THE OXIDATION OF UNIFORMLY LABELED [^{14}C]GLUCOSE TO $^{14}CO_2$

Glucose concentration 20 mM. Krebs–Ringer bicarbonate buffer. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Incubation period (h)	*Temperature*	*Glucose-C to CO_2*	*Number of islets*
1	25°	1.95	3
3	25°	9.58	3
1	37°	4.27	3
3	37°	5.46	4

* The authors are indebted to Dr. W. MEISSNER, Department of Pathology, New England Deaconess Hospital, for his help with the interpretation of the histological observations, and to Dr. D. JOFTES of the Cancer Research Institute, New England Deaconess Hospital, for carrying out the radioautography.

be seen from Table I, the oxidation of glucose to CO_2 was higher at 37° than at 25° after 1 h, whereas after 3 h the reverse was true. This suggested failure of islet tissue to survive at 37° beyond 1 h. Prolonged survival of islet tissue at relatively low temperature was demonstrated in the next experiment which was carried out at 23° (Table II). Two batches of six islets each were incubated for 1 h, then transferred into new medium for further consecutive periods of 2, 3 and 3 h, the total incubation time thus being 9 h. The results obtained clearly indicate an almost linear rate of oxidation of glucose carbon to CO_2. With still longer incubation periods, it was thought that nutrients such as amino acids might become important, and for these experiments Eagle's basal tissue culture medium was used as described above.

TABLE II

EFFECT OF INCREASING INCUBATION PERIODS ON CUMULATIVE OXIDATION OF UNIFORMLY LABELED [^{14}C]GLUCOSE TO $^{14}CO_2$

Number of islets, 12. Glucose concentration, 20 mM. Temperature, 23°. Krebs–Ringer bicarbonate buffer. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Incubation period (h)	*Glucose-C to CO_2*
1	2.14
3	6.81
6	15.12
9	22.88

TABLE III

EFFECT OF Na^+ CONCENTRATION ON GLUCOSE UPTAKE, UNIFORMLY LABELED [^{14}C]GLUCOSE OXIDATION TO $^{14}CO_2$ AND INCORPORATION OF UNIFORMLY LABELED [^{14}C]GLUCOSE INTO TRICHLOROACETIC ACID PRECIPITABLE PROTEIN

Glucose concentration, 5 mM. Temperature, 20°. Eagle's medium. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Na^+ (mM)	*Incubation period (h)*	*Glucose uptake*	*Glucose-C to CO_2*	*Glucose-C in total protein*	*Number of islets*
141	24	206	73	5.6	9
191	24	174	82	5.8	9
141	48	563	207	22.6	9
191	48	409	205	27.4	9

Tonicity

Four batches of islets were incubated for 24 and 48 h respectively in Eagle's medium (141 mM Na) and in Eagle's medium with sodium concentration brought to 191 mM by the addition of sodium chloride. As shown in Table III, the higher (and more physiological one for marine organisms) sodium concentration did not appear to influence the rate of glucose oxidation. Accordingly Eagle's medium without supplement was used in all subsequent experiments.

Antibiotics

As shown in Table IV, penicillin and streptomycin at the concentration of 50 μg/ml were without effect upon glucose uptake, lactate production, oxidation of

glucose carbon to CO_2, or the incorporation of glucose carbon into trichloroacetic acid precipitable protein. Accordingly, these antibiotics were subsequently added routinely to the incubation medium.

TABLE IV

EFFECT OF PENICILLIN AND STREPTOMYCIN (50 μg/ml EACH) ON GLUCOSE UPTAKE, LACTATE PRODUCTION, UNIFORMLY LABELED [^{14}C]GLUCOSE OXIDATION TO $^{14}CO_2$ AND INCORPORATION OF UNIFORMLY LABELED [^{14}C]GLUCOSE INTO TRICHLOROACETIC ACID PRECIPITABLE PROTEIN

Glucose concentration, 10 mM. Temperature, 20°. Eagle's medium. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Penicillin and streptomycin	*Incubation period (h)*	*Glucose uptake*	*Lactate production*	*Glucose-C to CO_2*	*Glucose-C in total protein*	*Number of islets*
—	24	218	45.0	52.1	7.06	8
+	24	258	70.0	68.6	8.56	8
—	48	538	145.5	124.0	16.90	8
+	48	507	149.3	128.6	16.25	8

On the basis of these preliminary studies, the following conditions were chosen for the incubation studies using different substrates. Eagle's basal tissue culture medium was used throughout and supplemented with nonessential amino acids, 50 μg/ml each of penicillin and streptomycin, without the addition of serum. The incubation temperature was 20° in summer and 15° in winter, and the total duration of incubation usually varied between 36 and 48 h, with transfer to new medium every 12 or 18 h.

Metabolism of glucose

The data shown in Fig. 1 demonstrate glucose uptake, total lactate production, and oxidation of glucose to CO_2 by two batches of 15 islets each, one incubated for 24 h and one incubated for 48 h. As judged by these metabolic indices, islet metabolism appeared to continue at a fairly constant rate for as long as 48 h, although oxidation to CO_2 tended to diminish between 36 and 48 h. Glucose carbon incorporated into protein was measured after 24 and 48 h and, as can be seen in Table V, approx. twice as much trichloroacetic acid precipitable radioactivity was found at the end of 48 h, when compared to 24 h. Approx. 60 % of this trichloroacetic acid precipitable radio-

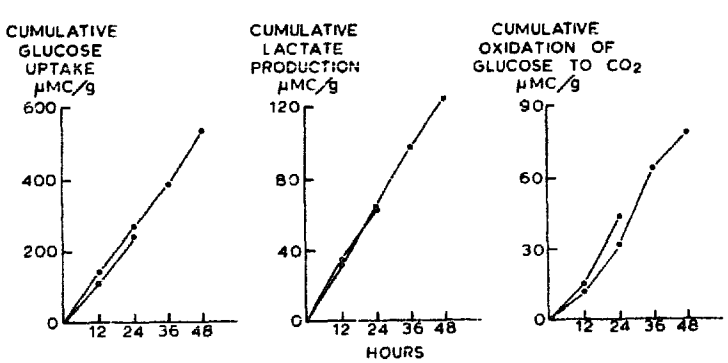

Fig. 1. Cumulative glucose uptake, lactate production, and oxidation of uniformly labeled [^{14}C]glucose to CO_2 by isolated islets of toadfish. Results expressed as μatoms glucose carbon per gram tissue (wet wt.). Glucose concentration, 8 mM; temperature, 20°; Eagle's medium.

activity could be extracted with acid ethanol in both instances. From the data shown in Fig. 1 and Table V, it may be calculated that lactate production accounted for 23% of the glucose taken up by the tissue (neglecting lactate formation from endogenous substrate), while CO_2 production accounted for 13.5%, and incorporation into protein for 3%. Thus, approx. 40% of the disappearing radioactivity could be accounted for, while the fate of 60% of the glucose carbon remained unestablished, although later studies indicated the occurrence of considerable labeling of islet tissue lipids under these conditions. As shown in Table VI, the concentration of glucose in the medium greatly influenced the metabolism of glucose by islet tissue. A 10-fold increase in glucose concentration, from 1 to 10 mM, accelerated glucose oxidation and the incorporation of glucose carbon into trichloroacetic acid precipitable and acid ethanol extractable protein approx. 5-fold.

TABLE V

INCORPORATION OF UNIFORMLY LABELED [^{14}-C]GLUCOSE INTO TRICHLOROACETIC ACID PRECIPITABLE AND ACID ETHANOL EXTRACTABLE PROTEIN

Glucose concentration, 8 mM. Temperature, 20°. Eagle's medium. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Incubation period (h)	*Glucose-C in total protein*	*Glucose-C in acid ethanol soluble protein*	*Number of islets*
24	7.7	4.5	15
48	17.8	10.6	15

TABLE VI

EFFECT OF GLUCOSE CONCENTRATION ON GLUCOSE OXIDATION TO CO_2 AND INCORPORATION OF GLUCOSE CARBON INTO TRICHLOROACETIC ACID PRECIPITABLE AND ACID ETHANOL EXTRACTABLE PROTEIN

Temperature, 20°. Incubation period, 36 h. Eagle's medium. Results expressed as μatoms glucose-C per gram tissue (wet wt.).

Glucose (mM)	*Glucose-C to CO_2*	*Glucose-C in total protein*	*Glucose-C in acid ethanol soluble protein*	*Number of islets*
1	16.8	0.89	0.38	12
10	82.1	4.96	2.00	12

When the acid ethanol extractable protein obtained after 48 h incubation of islet tissue with uniformly labeled [^{14}C]glucose was subjected to acid hydrolysis, with subsequent 2-dimensional chromatography of the hydrolysate, radioactivity was found in the amino acids shown in Fig. 2. Aspartic acid, glutamic acid, alanine, proline and serine clearly appeared on the radioautograph of the chromatogram. In addition, radioactivity was found in three ninhydrin-negative spots, of which one could be identified as glycerol, while the identity of the other two has not as yet been established.

Metabolism of L-*leucine*

The oxidation of uniformly labeled L-[^{14}C]leucine to CO_2 both in the presence and in the absence of unlabeled glucose, is shown in Fig. 3. Again the rate of leucine oxidation to CO_2 appeared to be constant for as long as 48 h, both with and without the addition of unlabeled glucose. Four batches of 12 islets each were used, two batches

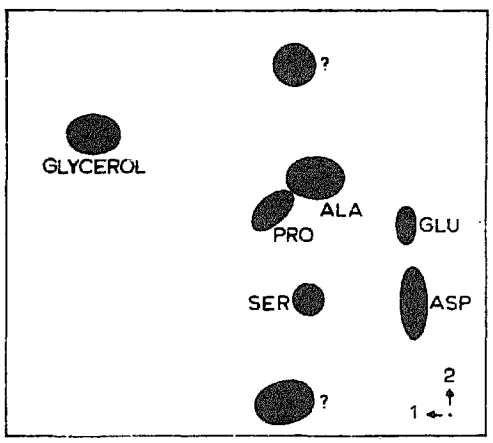

Fig. 2. Radioautograph of 2-dimensional chromatogram (pyridine–acetone–ammonia–water and isopropanol–formic acid–water) of acid hydrolysate of the acid ethanol extractable protein obtained after 48 h incubation of islet tissue with uniformly labeled [^{14}C]glucose.

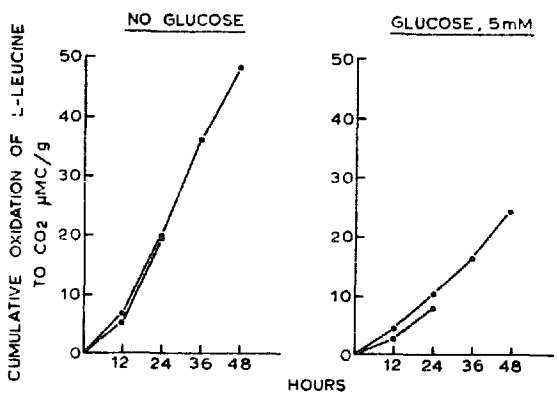

Fig. 3. Cumulative oxidation of uniformly labeled L-[^{14}C]leucine to CO_2 by isolated islets of toadfish. Results expressed as μatoms leucine carbon per gram tissue (wet wt.). Leucine concentration, 0.65 mM; temperature, 20°; Eagle's medium.

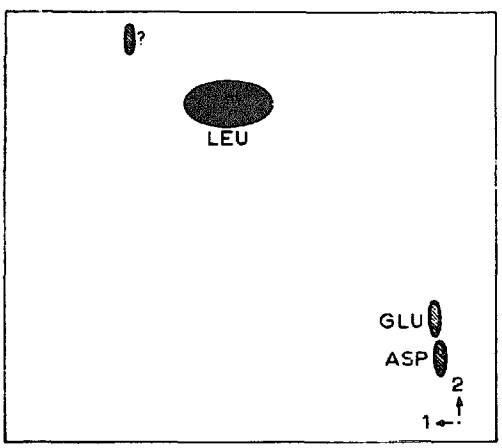

Fig. 4. Radioautograph of 2-dimensional chromatogram (pyridine–acetone–ammonia–water and isopropanol–formic acid–water) of the hydrolysate of the acid ethanol extractable protein of islet tissue incubated with uniformly labeled L-[^{14}C]leucine.

being incubated for 24 h, and 2 batches for 48 h. The incorporation of leucine carbon into trichloroacetic acid precipitable and acid ethanol extractable protein was measured after 48 h of incubation and is shown in Table VII. Whereas the addition of unlabeled glucose (5 mM) depressed the oxidation of leucine carbon to CO_2 by about 50 %, presumably as a result of dilution, the incorporation of leucine carbon into protein was not affected by the addition of glucose. Again, a portion of the acid ethanol extractable protein was hydrolyzed and the hydrolysate subjected to chromatography and radioautography as shown in Fig. 4. Radioactivity appeared almost exclusively in the leucine spot, although traces were seen in glutamic acid and aspartic acid. There was one unidentified faint ninhydrin-negative additional radioactive spot.

TABLE VII

EFFECT OF GLUCOSE CONCENTRATION ON INCORPORATION OF UNIFORMLY LABELED L-[^{14}C]LEUCINE INTO TRICHLOROACETIC ACID PRECIPITABLE AND ACID ETHANOL EXTRACTABLE PROTEIN

Leucine concentration, 0.65 mM. Temperature, 20°. Incubation period, 48 h. Eagle's medium. Results expressed as μatoms leucine-C per gram tissue (wet wt.).

Glucose (mM)	*Leucine-C in total protein*	*Leucine-C in acid ethanol soluble protein*	*Number of islets*
0	28.1	14.0	12
5	27.1	14.8	12

Comparative metabolism of glucose, mannose, fructose and pyruvate

In the mammalian organism, the administration of certain sugars results in stimulation of insulin secretion, while the administration of other substrates does not. The studies shown in Table VIII were carried out in order to explore whether metabolic peculiarities which might be correlated with the specialized function of islet tissue might be found. Glucose and mannose were oxidized to CO_2 at approximately the same rate, while fructose oxidation was much slower. Similarly, glucose carbon incorporation into protein, both trichloroacetic acid precipitable and acid ethanol extractable, again indicated that glucose and mannose were metabolized at very similar rates. The addition of equimolar amounts of unlabeled mannose to labeled glucose, or unlabeled glucose to labeled mannose, reduced the oxidation of labeled carbon to 66 and 72% respectively. On the other hand, the addition of unlabeled fructose to labeled glucose was practically without effect, while the addition of unlabeled glucose to labeled fructose somewhat suppressed the oxidation of fructose carbon.

Using either [1-^{14}C]glucose or [6-^{14}C]glucose as substrates, the C-1 to C-6 ratio was found to be 2 in CO_2, while it was approx. 0.5 in protein.

TABLE VIII

COMPARATIVE METABOLISM OF GLUCOSE, MANNOSE, FRUCTOSE, AND PYRUVATE

Each hexose is present at 5 mM, pyruvate at 10 mM. Temperature 20°. Incubation period, 36 h. Eagle's medium. Results expressed either as μmoles substrate per gram tissue (wet wt.) or as per cent of results obtained with uniformly labeled [^{14}C]glucose.

Substrate		*Substrate-C to CO_2*		*Substrate-C in protein*				
Labeled	*Unlabeled*	*μmoles*	*%*	*Total μmoles*	*%*	*Acid ethanol soluble μmoles*	*%*	*Number of islets*
Uniformly labeled [^{14}C]glucose	—	7.8	100	2.4	100	0.90	100	5
Uniformly labeled [^{14}C]glucose	Mannose	5.1	66	0.9	38	0.52	58	5
Uniformly labeled [^{14}C]glucose	Fructose	7.2	93	1.4	58	0.30	33	5
[1-^{14}C]Glucose	—	8.8	112	1.4	58	0.88	98	5
[6-^{14}C]Glucose	—	4.3	55	2.3	96	1.63	181	5
Uniformly labeled [^{14}C]mannose	—	8.4	108	2.0	83	0.71	79	5
Uniformly labeled [^{14}C]mannose	Glucose	6.1	78	1.3	54	0.59	66	5
Uniformly labeled [^{14}C]fructose	—	2.9	37	1.0	43	0.32	36	5
Uniformly labeled [^{14}C]fructose	Glucose	2.2	28	0.6	23	0.31	34	5
[2-^{14}C]Pyruvate	—	17.8	228	4.4	184	1.58	174	5

The oxidation of pyruvate (10 mM) approximated the oxidation of glucose, if the results are expressed in terms of C_6 units. Similarly, incorporation of labeled C-2 from pyruvate into trichloroacetic acid precipitable and acid ethanol extractable protein was of the same order of magnitude as for glucose carbon. Although each of these experiments was carried out with only 5 islets, it is evident that the pattern obtained was quite consistent.

DISCUSSION

The topography, histology, electron microscopy and insulin content of Brockmann bodies are known in many species of teleost fishes[2,19–24]. The occurrence of these Brockmann bodies and thus of relatively isolated fish islet tissue has played an important role in the discovery of insulin, since the hypoglycemic effect of islet extracts, but not of zymogen tissue extracts, was considered at the time the final proof of the origin of insulin in the islets of Langerhans[21]. Furthermore, extirpation of these islets leads to long-lasting hyperglycemia[25]. For many years, fish islets were used as a commercial source of insulin in Japan[26] although this has not been true in recent years because of difficulties in crystallizing fish insulin[27] (obtained for the greater part from tuna and bonito). It is of interest that, in studies carried out in 1956, MASKE based his hypothesis concerning insulin secretion from the β-cell upon investigations of islets of Langerhans of flounder[28]. The proposed mechanism emphasizes the role of zinc in altering the solubility of insulin stored in islet tissue. WATKINS, COOPERSTEIN AND LAZAROW[29] described a specific effect of alloxan on the β-cell membrane in toadfish islets.

The purpose of the experiments described in this report was that of defining suitable conditions for incubation *in vitro* of fish islet tissue, in order to study certain aspects of its carbohydrate metabolism with the subsequent aim of exploring insulin biosynthesis in this preparation. The choice of the toadfish for our studies was based on its relatively easy availability on the Atlantic seacoast, its apparent resistance to possible damage from prolonged transport and storage (provided a sudden increase in temperature is avoided) and its convenient size. The Brockmann body of a 2-lb toadfish weighs approx. 3 mg and thus, since it is generally a flattened ovoid structure. its maximum thickness rarely exceeds 1 mm. Because of the difficulties involved in obtaining a large number of fish simultaneously, and because of the slow but persistent metabolism of the tissue, long incubation periods at rather high specific activity for radioactive substrates were used. The decision to incubate the whole islets without slicing was a deliberate one, based on early evidence that the survival time of unsliced islets was considerably in excess of that of sliced ones. Furthermore, if rates of insulin secretion are to be studied in the future, the use of intact tissue is likely to prove a significant advantage.

It was found that a relatively low temperature of incubation, not much in excess of the temperature to which the fish had been exposed for several weeks prior to incubation, greatly favored the survival of isolated islet tissue over prolonged periods of time. At 37° the metabolic function of islet tissue as well as its histologic appearance grossly deteriorated by approx. 1 h. Similarly, HELLMAN AND LARSSON[30] have reported a lack of further glucose utilization when islet tissue of *Cottus quadricornis* was incubated beyond 1 h at 37°. In tissue-culture work, 26° was found to be the maximal temperature suitable for the culture *in vitro* of gonadal cells of the rainbow

trout[31] obtained from water considerably warmer than that in the circulating sea water used at the American Lobster Company, which averaged 15° in summer and anywhere between 2 and 8° in fall and winter. Although Eagle's medium was selected for prolonged incubations rather than Krebs bicarbonate buffer, with the thought that protein synthesis might be impaired by exhaustion of endogenous amino acid supplies, data objectivating this hypothesis were not obtained. Both nonessential and essential amino acids were added to the medium since the characterization of amino acids as "nonessential" is derived exclusively from studies of mammalian tissues[9].

The ionic composition of serum from aglomerular fishes is known, particularly from studies on *Lophius piscatorius*[32]. Serum sodium concentration is about 200 mM as compared with the concentration of approx. 140 mM in mammalian serum and thus in Eagle's medium[9]. However, since the addition of 50 mM sodium to Eagle's medium failed to significantly improve the oxidation of glucose to CO_2 and the incorporation of glucose carbon into trichloroacetic acid precipitable protein, the mammalian concentration of sodium was used in these studies. Indeed, glucose uptake was slightly depressed with the higher sodium concentration, although the significance of this observation is as yet unestablished. Similarly, media originally designed for mammalian tissues have been used successfully for culturing fish tissues[31]. Also, LAZAROW, in his studies on the respiration of toadfish islet tissue *in vitro*, found that the highest oxygen uptakes were seen in hypotonic media.

The linearity with time of the metabolic indices measured when either uniformly labeled [^{14}C]glucose or uniformly labeled L-[^{14}C]leucine were used as substrate, encouraged us to incubate routinely for between 24 and 48 h, a procedure which results in higher specific activities of the protein isolated at the end of the incubation. Increasing the glucose concentration increased the rate of incorporation of glucose carbon into protein. The concentration effect appears to concern an early phase of glucose metabolism, or glucose transport, since the incorporation of glucose carbon into protein was increased to approximately the same extent as the oxidation of glucose carbon to CO_2. The incorporation of leucine carbon into protein was not affected by the simultaneous presence of glucose, while leucine oxidation to CO_2 was diminished by the add glucose, probably as a result of a dilution effect at the level of 2-carbon intermediates entering the tricarboxylic acid cycle.

The radioactivity studies on hydrolysates of islet protein at the end of 24 or 48 h incubation with labeled leucine indicate that leucine was mostly incorporated as such, and not as a degradation or transamination product. In mammals the carbon skeleton of leucine cannot be synthesized from glucose[9,33] and transamination with leucine is not a particularly active process in most mammalian tissues. The data obtained in toadfish tissue suggest that leucine is similarly handled in this species. In particular, leucine found in protein hydrolysates was not labeled after prolonged incubation of the tissue with uniformly labeled [^{14}C]glucose. On the other hand, several non-essential amino acids exhibited definite radioactivity after incubation with uniformly labeled [^{14}C]glucose, an observation which is in agreement with results reported for islets from *Cottus quadricornis*[30].

When considering the comparative metabolism of glucose, mannose, fructose and pyruvate, it was of particular interest to find that glucose and mannose were utilized at approximately the same rate, both in the presence and absence of equal

amounts of the other hexose. Whereas both these sugars are utilized at approximately the same rate by several mammalian tissues[34] and are both known to be responsive to insulin[34,35], glucose induces the release of pancreatic insulin in the mammalian organism, whereas mannose does not[36,37]. If insulin secretion in fish is stimulated by the same sugars which stimulate insulin secretion in mammals, and if the stimulating property of glucose upon insulin secretion is related to glucose metabolism by the insulin-secreting cells, then a discrepant metabolic behavior of glucose and mannose might have been expected. This discrepant behavior was not found, but it is evident that this observation may be subjected to only the most limited interpretation, since the assumptions just outlined are as yet unproven.

FIELD AND LAZAROW[38] have studied the oxidation of glucose labeled specifically in C-1 or C-6 to CO_2 by isolated islets of toadfish and goosefish. The results reported here confirm their finding that CO_2 is formed more rapidly from C-1 than from C-6 of glucose and, in addition, a preferential incorporation of C-6 over C-1 into protein has been demonstrated. These data, together with the recent histochemical demonstration of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in islets of Langerhans of rabbits[39,40] and mice[41] suggest the presence of an operational hexose monophosphate shunt in islet tissue.

In agreement with HELLMAN AND LARSSON[30], who studied the islets of *Cottus quadricornis*, fructose was utilized at a much slower rate than glucose by the islets of toadfish. In the presence of equimolar amounts of glucose and fructose 3.3 times more glucose than fructose was oxidized to CO_2, a relationship very similar to that reported for rat diaphragm muscle[42].

In conclusion, therefore, it would appear that the preparation of islets of Langerhans from toadfish described in this study allows for prolonged survival of islet tissue *in vitro*, is capable of metabolizing various substrates added in the medium, and actively incorporates carbon from glucose or leucine into protein. These observations confirm the working hypothesis of this study and suggest that the preparation might be profitably used for the more detailed investigation of insulin biosynthesis *in vitro*. As yet, the observations made with a few substrates do not indicate the presence of striking differences in the metabolism of substances which are known to stimulate insulin secretion, and of substances which do not. Of course, insulin-producing β-cells account for only about one-third of the cells present in the preparation used.

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