Extended Production of Insulin by Isolated Rabbit Pancreatic Islets; Evidence for Biosynthesis of Insulin (33912)

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In recent publications methods for the long-term cultivation of adult mammalian pancreatic tissue (1, 2) and methods for the isolation and short-term incubation of individual islets of Langerhans of various mammalian species have been reported (3, 4). These studies with intact pancreas tissue have been supported by the demonstration of newly synthesized insulin in the incubation medium of both fetal and adult tissue slices (5, 6) incubated *in vitro* with radioactive precursors. Short incubations of isolated islets from the rabbit have demonstrated the incorporation of leucine-^{3}H into insulin (7). Inherent difficulties with organ cultures (8) and limitations on biochemical measurements have prompted us to attempt establishment of larger scale primary submerged islet cultures from young rabbits capable of insulin synthesis over an extended period of time.

Methods and Materials. New Zealand white female rabbits (3.5–4 lb littermates) were killed by injection of air into the marginal ear vein. Following laparotomy the pancreatic duct was injected with 15–20 ml of Hanks' balanced salt solution (HBSS) or in later experiments HBSS containing 1 mg/ml of collagenase (Worthington Biochemical Corp). The pancreata were excised and adhering fat, blood vessels, and ducts were aseptically removed with the aid of a dissecting microscope. The remaining pancreas tissue was minced with scissors and incubated at 37° for 30–35 min in HBSS containing 5.0 mg/ml collagenase. In later experiments collagenase concentration was reduced to 1.0 mg/ml. Enzyme treatment was interrupted by dilution with cold HBSS and the islets were subsequently sedimented and washed as described by Lacey (4). Aliquots (1.0 ml) of islets were placed in nutrient media and incubation was carried out at 37° in Bellco micro spinner flasks containing 10 ml of medium. Media were purchased from Grand Island Biological Co., Grand Island, N. Y. Each flask was fitted with provisions for constant gassing with 95% O_2–5% CO_2.

Media were replaced every 24 or 48 hr by sedimentation of the islets and aspiration of the spent media. Extracts of islet tissue were made by the acid–ethanol procedure used for commercial production of insulin. Insulin concentration was measured for each medium sample and for each tissue extract by a modification of the method of Heding (9).

Labeling was accomplished by supplementation of the media with a mixture of 13 amino acids uniformly labeled with carbon 14 (Reconstituted ^{14}C protein hydrolysate, Schwarz Bioresearch Inc). The ^{14}C amino acid mixture was added to the medium to obtain a final concentration of 0.2 μCi/ml. Determination of incorporation into protein was made by cold TCA precipitation of the culture medium.

Column chromatography and polyacrylamide disc-gel electrophoresis. Ten ml of media were combined with 10 ml of 2 *M* acetic acid and allowed to equilibrate 16 hr at 4°, after which this solution was chromatographed on a 2.5 × 80-cm Sephadex G-50 (fine grade) column equilibrated and eluted with 1 *M* acetic acid at 4°. The flow rate was 50 ml/hr and 5.4-ml effluent fractions were collected. In addition to continuous monitoring of the column effluent at 276 mμ, 0.1-ml aliquots from each fraction were examined for ^{14}C activity, and another set of 0.1-ml aliquots from fractions most likely to contain insulin was examined by the insulin radioimmunoassay. Based on the immunoassayable insulin detected (Fig. 3) fractions 52–59 were combined. Eighty percent of this pooled fraction was lyophilized and assayed by the double antibody assay method of Morgan and Lazarow (see below). The remaining 20% of this pool was lyophilized in the presence of 1 mg of single component porcine

insulin. This fraction was used in several preliminary studies to determine if ^{14}C activity could be isoltated out of the insulin band from a polyacrylamide disc-gel electrophoresis column. Although only small amounts of labeled materials were observed in the insulin band, there was encouraging evidence that a labeled fraction was associated with marker insulin. In view of these results, a second chromatographic run was performed as described above. In this run the entire peak of insulin activity was pooled and lyophilized. The sample was reconstituted in 150 μl of 7 *M* urea (freshly deionized) containing about 50 μg of single-component porcine insulin for an internal marker. The sample was applied directly to a 20% polyacrylamide disc-gel electrophoresis column for further evaluation. Proinsulin, insulin, and glucagon were electrophoresed simultaneously for comparison.

The polyacrylamide disc-gel columns were prepared from 20% acrylamide and 0.2% *N*,*N*^{1}-methylene-bisacrylamide according to the method of Davis (10), and the protein components were stained with the TCA–Coomassie blue system (11). The protein samples were applied directly on to the top of the gel using 7 *M* urea for the sample solvent. Electrophoresis was performed at 1 mA/gel tube for 15 min and 2 mA for 90 min at 25°, similar to that described by Chance *et al.* (12).

The gel used to analyze the radioactive sample was sliced into twenty 3-mm sections. Each section was digested in 1 ml of 30% H_2O_2 (25° for 16 hr and 60° for 4 hr), diluted with 15 ml of scintillation solution (50:50 toluene:methyl cellosolve containing 0.5% DOP and 0.01% POPOP) and counted for 10 min, five different times.

Double antibody identification of insulin. The remainder of the lyophilized material (large portion) was made up to 10 ml with phosphate buffer containing 0.1% human albumin. The double antibody insulin assay method of Morgan and Lazarow (13) was used to measure the insulin in 1.0- and 2.0-ml samples of the reconstituted lyophilized materials.

Results. Numerous preliminary experiments were completed in order to study the effects of gassing, type of medium, serum supplement, and frequency of medium change on insulin secretion into the medium and also on the longevity of viable islets. A typical result is shown (Fig. 1). Synthesis and/or release of insulin into the medium was depressed by the addition of exogenous insulin. Similar experiments in medium supplemented with bovine pancreatic RNA show a marked elevation of insulin in the medium. Various agents such as glucose and xylitol previously reported by other workers to stimulate insulin secretion had similar effects in our system.

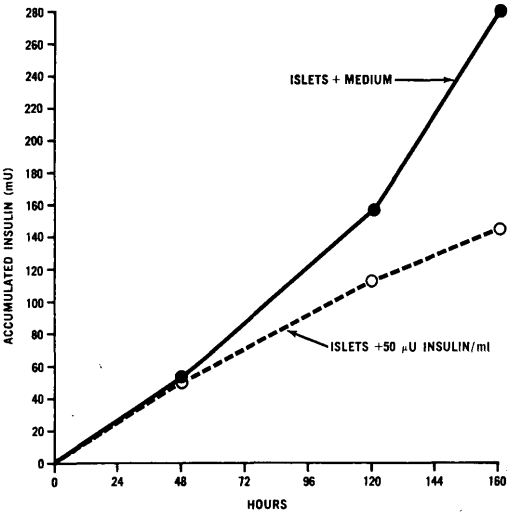


FIG. 1. Rabbit islet suspension culture: insulin released into the medium (T.C. 199 supplemented with 5% horse serum) by rabbit islets. Media were replaced at 48 and 120 hr. Each point represents the amount of insulin assayed in the medium at that time plus the amount of all previous samplings.

Preliminary experiments have shown that isolation and measurement of newly synthesized ^{14}C-labeled insulin from serum supplemented medium is quite difficult due to the interference of serum proteins with the isolation procedures. It was therefore necessary to evaluate several serum-free media for capacity to support the synthesis and release of newly formed insulin during adequate periods of islet incubation. Results of such an experiment are shown (Fig. 2). In this experiment 2–3% of 13 of the available amino acid mole-

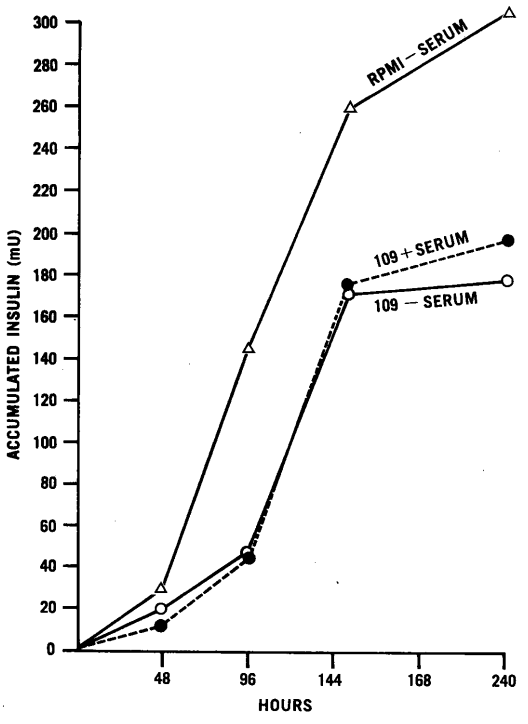


FIG. 2. Rabbit islet suspension culture: effect of incubation media on insulin release by rabbit islets. Media were replaced at 48, 96, and 144 hr. Incubation was terminated at 240 hr. Media are RPMI without serum supplement and T.C. 109 with and without serum supplement. The amount of insulin found at each sampling was added to amounts at previous samplings to establish an accumulative plot.

cules are carbon-14 labeled. Media from this experiment was collected and used in the biochemical and immunological analyses for insulin.

Column chromatography, double antibody, and polyacrylamide disc-gel electrophoresis identification of insulin. The elution profile resulting from G-50 Sephadex chromatography of the culture media is illustrated in Fig. 3. The peak of immunoassayable insulin was eluted in a position consistent with standardization runs using porcine insulin. This strongly suggests that insulin was synthesized by the tissue and released into the medium. The combined eluates under the insulin peak (fractions 52–59) were calculated to contain 72.5 mU of insulin. This lyophilized fraction was identified as insulin by the double antibody method of Morgan and Lazarow (see Figs. 4 and 5). It was calculated from these results that the specific activity of the labeled insulin was 11–14 cpm/μg.

The polyacrylamide disc-gel electrophoresis pattern of the concentrated insulin peak from the second chromatographic run with added 50 μg of single-component insulin demonstrated that other protein components were also present in the insulin peak isolated from the G-50 Sephadex column. Porcine proinsulin and mixed bovine–porcine glucagon are shown for comparison although no distinct bands matching these two proteins are observed. Immunoassayable glucagon, however, was detected in the medium (Table I).

The polyacrylamide disc-gel used to analyze the labeled insulin Sephadex peak is shown in Fig. 6 as it was divided into the twenty 3-mm sections along with the ^{14}C activity obtained from each slice. The results indicate that although there was considerably more radioactivity in other sections of the gel, a strikingly significant band of radioactivity was found in gel slice no. 8—the same

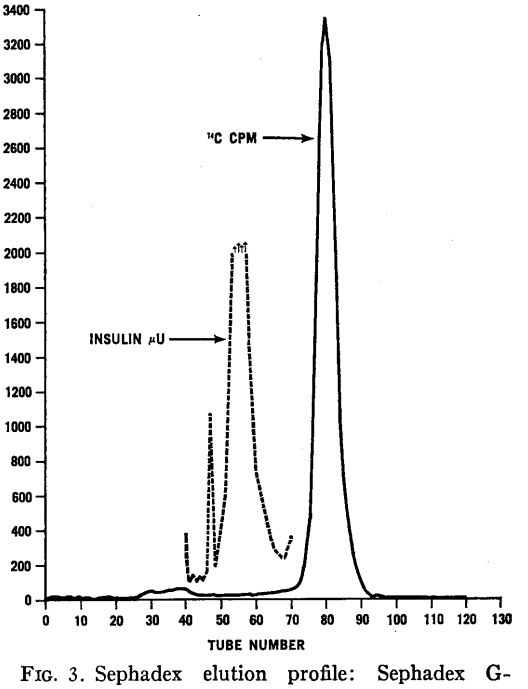
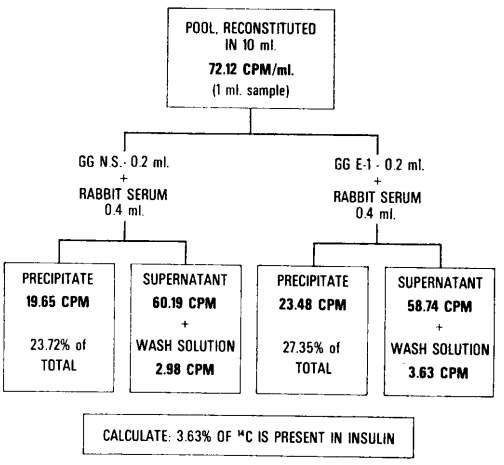
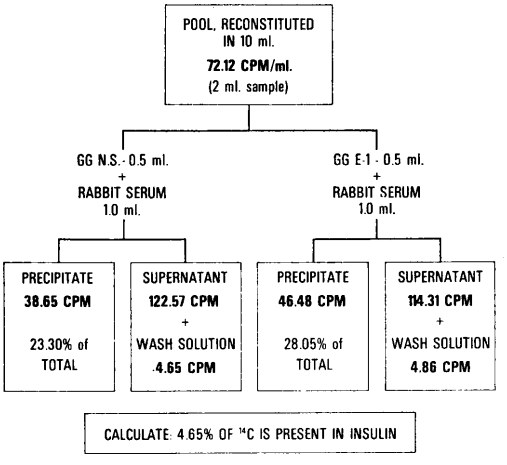


FIG. 3. Sephadex elution profile: Sephadex G-50 column chromatography of incubation medium. Medium RPMI 1640 without serum from Fig. 3 was pooled and placed on the column; ^{14}C as cpm/0.1 ml of eluate; insulin as μU/ml.





FIGS. 4 and 5. Double antibody determination of insulin in the lyophilized and pooled fractions 52–59 (Fig. 3). Abbrev.: GG N.S. = nonimmune guinea pig gamma globulin; GG E-1 = antipork insulin guinea pig gamma globulin.

one that contained the stained insulin band used as an internal marker. By assuming that the two boundary slices (nos. 7 and 9) represent background, the net counts in the insulin band range from 30 to 34 cpm. Based on the immunoassay, 2.8 μg of insulin was in the Sephadex peak of Run no. 1. Assuming an incorporation of 11–14 cpm/μg, the theoretical activity of 31–39 is remarkedly close to the amount found.

Discussion. Standardization of the islet cell preparation to completely eliminate all acinar tissue has not been accomplished in our laboratories. Histological preparations and electron micrographs have demonstrated the presence of varying amounts of acinar tissue early in the periods of incubation. The acinar tissue, however, degenerates rapidly and viable islets, essentially free of acini have been recovered from the medium after incubation periods as long as 140 hr. These incubated islets when plated in stationary cultures grow readily and produce very small amounts of immunoassayable insulin in the medium. Although not as sensitive as the immunoassay, a rat diaphragm assay detects significant amounts of insulin in our culture media, an indication that biologically active insulin is present.

TABLE I. Insulin and Glucagon Content of Islets and Media.

Incubation time (hr)	Insulin (mU/ml)	Glucagon (mμg/ml)	Total insulin release (mU)
48	3.140	23	31.4
96	11.400	15	114.0
168	11.350		113.5
240	5.230		52.3
Total released			311.2
Insulin content of islets at zero time			137.5
Synthesized during incubation			173.7

A role for exocrine tissue in supplying precursors or proteolytic enzymes for the formation of free insulin is possible. Indeed, deliberate addition of acinar cells to the islets has produced an increased level of immunoassayable insulin in one of our experiments. Steiner (14) and later Tung and Yip (6) demonstrated the formation of insulin at the expense of proinsulin in their systems. The higher specific activity of our proinsulin in slice 17 (Fig. 6) gives tentative support to their hypothesis.

Double antibody methods also indicate a low level of ^{14}C-amino acid incorporation into insulin. When it is considered that approximately 1/50 of the available amino acid molecules are ^{14}C labeled, the amount of incorporation nears 100% of theoretical value. Although small amounts of radioactivity were observed in the insulin region on the polyacrylamide disc-gel columns, most of the

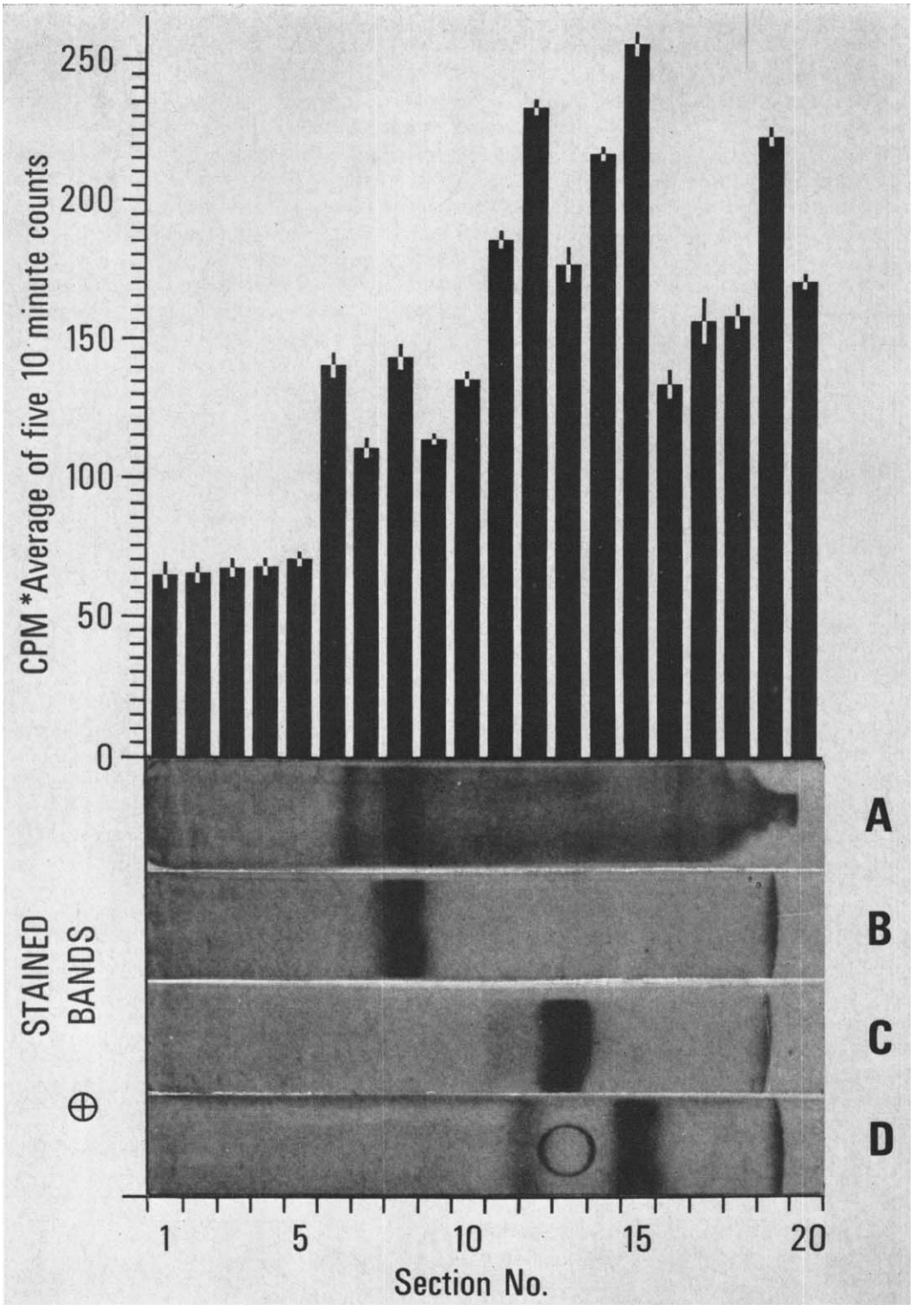


FIG. 6. Polyacrylamide disc-gel electrophoresis of lyophilized and pooled fractions 52–59 (Fig. 3), and ^{14}C distribution of gel slices. (A), porcine insulin + insulin peak pool; (B), porcine insulin; (C), porcine proinsulin; and (D) glucagon.

radioactivity from the insulin region in the G-50 Sephadex chromatograms (Fig. 3) was in other protein bands generally more basic than insulin (Fig. 6). This observation has been made repeatedly when media and islet cell preparations were electrophoresed on disc-gels. In addition, protein bands have been detected which correspond to amounts ranging from 1 to 50 μg as judged from the intensity of the stained bands. The calculated insulin could not have exceeded 0.3 μg. It appears that G-50 Sephadex Chromatography does not separate insulin and several other pancreatic proteins with similar molecular weights. Those proteins that were eluted in the insulin region are probably more basic in nature, as judged by the electrophoretic behavior, with molecular weights in the range of 3000–6000. Of particular interest is the protein band in slice No. 17 from the 500-μg sample band of (Fig. 6). The electrophoretic migration is almost identical to that of porcine proinsulin. Porcine proinsulin, however, has a molecular weight of about 9000 and would be expected to elute in fractions 35–40 (Fig. 3), assuming that rabbit proinsulin is similar in size and electrophoretic mobility. If the molecular weight of porcine and rabbit proinsulin are similar, the component in slice no. 17 might be glucagon since glucagon has the same electrophoretic migration and would be expected to overlap slightly with insulin in this chromatographic system. Indeed, there is some immunoassayable glucagon present in these samples (Table I).

The following calculations are presented to illustrate that the ^{14}C activity in the insulin band can be explained in part by ^{14}C-labeled insulin and that other labeled proteins may also be present in this band. By assuming that 1 mU of insulin activity = 0.04 μg of insulin, then 14.7 mU in the 20% fraction = 0.59 μg of newly synthesized rabbit insulin in the added 1μg of lyophilized pork insulin sample. Using the incorporation values of 11–14 cpm/μg insulin, the theoretical level of radioactivity in 1 μg of the sample should be 6–8 cpm. Since 0.5 μg was electrophoresed, 3–4 cpm would be expected in the insulin band providing only insulin was present; 5–9 cpm were found and the overall ^{14}C recovery was less than 50%. This discrepancy may have been the result of different counting machines being used on various phases of this work.

Table I demonstrates another method for establishing synthesis during culture. Calculations show a net synthesis of 173.7 mU of insulin during incubation. This probably represents a conservative figure since it disregards the amount of insulin remaining in the islets at the end of incubation. Additional experiments have demonstrated that 75–90% of the amount of insulin extractable from the islets prior to incubation can generally be recovered from the islets after extended periods of incubation.

Modification of the preparative methods to obtain more uniformly pure islets is clearly indicated. Various methods are currently being investigated in this laboratory to obtain islet preparations which are more nearly acinar free. This will allow more definitive studies as to the cellular contributions to the formation of proinsulin and insulin.

The current state of primary islet cell suspension cultures offers a convenient and reliable pharmacological tool for the evaluation of drug action on insulin synthesis and secretion. Continued development of this technique may lead to greater yields of radioactive insulin with labels at specific positions. The resolution of complex pancreatic function by specific cell types and cellular interrelationships of biosynthetic capacity is a conceivable goal.

Summary. Methods for prolonged primary suspension cultures of rabbit pancreatic islets were established, and incorporation of carbon-^{14} labeled amino acids into insulin and other proteins was demonstrated.

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Stimulation of *p*-aminohippurate Transport by Slices of Rat Renal Cortex Following *in Vivo* Administration of Triiodothyronine* (33913)

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Thyroxine administration causes an increase in oxygen consumption in kidney slices (1, 2) as well as stimulating renal protein synthesis (3–5). Furthermore, thyroidectomy is known to depress uptake of *p*-aminohippurate (PAH) by rat kidney slices (6, 7). These results suggested that thyroid hormone may exert a trophic or regulatory effect on renal function. The present study was carried out to determine if thyroid hormone would act as a direct, nonspecific stimulus of renal transport in rats. The effect of *in vivo* administration of triiodothyronine (T_3) on organic ion transport was determined by measuring PAH and NMN (*N*-methylnicotinamide) uptake in renal cortical slices. Inasmuch as the growth response of the remaining kidney after unilateral nephrectomy is influenced by age (8), a comparison of the response to T_3 in both weanling and adult rats was made. The effect of T_3 on the renal uptake of PAH and NMN, when added *in vitro* to kidney slices, was also determined.

Materials and Methods. Male Sprague-Dawley rats were used in all experiments. The weanling rats weighed 50–60 g at the beginning of each experiment, while the adult rats weighed 200-220 g. DL-Triiodothyronine was dissolved in alkaline saline and injected intraperitoneally into rats in doses of 200 or 500 μg/kg once daily for 3–7 days. Control animals received alkaline saline for the same period of time.

Twenty four or 48 hr after the last injection the animals were killed by a blow on the head. The kidneys were removed immediately, weighed, and placed in ice-cold normal saline. Renal cortical slices weighing about 100 mg were prepared freehand and kept in cold normal saline until incubated. Slices were incubated in 2.7 ml of the phosphate buffer devised by Cross and Taggart (9), which contained 7.4×10^{-5} *M* PAH and 6.0×10^{-6} *M* ^{14}C-NMN. In *in vitro* studies, a solution of 1×10^{-4} *M* T_3 was diluted with distilled water so that when 0.3 ml was added to the incubation media the final concentration of T_3 was 1×10^{-6} *M* or 1×10^{-8} *M*; the pH of the media was 7.4 Control beakers received 0.3 ml of distilled water. All incubations were carried out in a Dubnoff metabolic shaker for 90 min at 25° under a gas phase of 100% oxygen. At the end of the incubation period the slices were quickly removed from the media, blotted, and weighed. Both tissue and media were treated as outlined by Cross and Taggart (9) and the S/M ratio for PAH and NMN was determined. PAH was estimated by the method of Smith *et al.* (10) while 1.0 ml of slice and media homogenate was added to 10 ml of modified Bray's solution (2.5 g of 2,-5-diphenyloxazole and 100 g of napthalene/liter of dioxane) and the amount of ^{14}C NMN was counted in a Beckman LS-100 liquid scintillation counter.

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