Biotin-Mediated Protein Biosynthesis

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The effect of administration of biotin to biotin-deficient rats on protein biosynthesis was studied. Biotin treatment resulted in stimulation by more than twofold of amino acid incorporation into protein, both *in vivo* and *in vitro* in rat liver, pancreas, intestinal mucosa and skin. Analysis of the products of amino acid incorporation into liver proteins *in vivo* and *in vitro* indicated that the synthesis of some proteins was stimulated more than twofold, but others were not stimulated at all. This indicates a specificity in the stimulation of protein synthesis mediated by biotin.

Biotin deficiency causes a marked decrease in the activities of several glycolytic enzymes in liver, e.g. glucokinase (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2), phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) and pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40). The activities of these enzymes increased rapidly after administration of biotin (Dakshinamurti & Cheah-Tan, 1968, 1970; Dakshinamurti & Hong, 1970; Dakshinamurti *et al.*, 1970). These increases in enzyme activities could be abolished by treating the animals with inhibitors of protein or RNA synthesis (Dakshinamurti & Cheah-Tan, 1970; Dakshinamurti *et al.*, 1970). Certain other glycolytic enzymes, e.g. hexose phosphate isomerase (D-glucose 6-phosphate ketol isomerase, EC 5.3.1.9), were not affected by biotin administration (Dakshinamurti *et al.*, 1970).

We have shown previously that amino acid incorporation into proteins, both *in vivo* and *in vitro*, was significantly decreased in biotin deficiency, and that a stimulation in amino acid incorporation can be seen as early as 8h after biotin treatment (Dakshinamurti & Mistry, 1963; Dakshinamurti & Litvak, 1970). Further, the stimulation of amino acid incorporation was preceded by a stimulation in the incorporation of orotic acid into nuclear and ribosomal RNA, an effect which can be observed as early as 2–4h after biotin treatment. Nuclear RNA isolated from biotin-treated rats had a greater template activity than similar RNA isolated from biotin-deficient rats (Dakshinamurti & Litvak, 1970).

The present investigation explores in greater detail the role of biotin in protein biosynthesis by determining which specific proteins or types of proteins are affected by biotin.

Experimental

Materials

ATP, GTP, D(+)-biotin, phosphoenolpyruvic acid and pyruvate kinase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-[U-¹⁴C]-Leucine (180 μ Ci/mol) and L-[4,5-³H]leucine (50 Ci/mol) were purchased from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. Rabbit anti-(rat serum albumin) antiserum was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sephadex G-25 and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of animals

Biotin deficiency was produced in weanling (45–50g) male Holtzman rats by feeding them for 5–6 weeks on an avidin diet (Dakshinamurti & Cheah-Tan, 1968). In all experiments reported the effect of a single injection of biotin into biotin-deficient rats was examined. In addition, since the experimental (biotin-injected) and control (biotin-deficient) rats were starved for the duration of the experiment (18h), complications owing to differences in food intake between the two groups were eliminated. All animals used in an experiment were of the same age and weight. The animals received either a single intraperitoneal injection of 1ml of 0.82mM-D(+)-biotin in 0.15M-NaCl, or 1ml of 0.15M-NaCl. The animals were starved for 18h and were then killed by decapitation. Tissues were excised and immediately chilled in ice-cold 0.9% NaCl. All further procedures were performed at 4°C.

Amino acid incorporation in vivo

Biotin-treated rats were injected with 10 μ Ci (50nmol) of L-(U- 14 C)leucine/100g body wt. Control animals (biotin-deficient) received 100 μ Ci (50nmol) of L-[4,5- 3 H]leucine/100g body wt. Labelled leucine, in 0.5ml of 0.15M-NaCl, was injected intraperitoneally 30min before death.

Hair was removed from the skin of the abdomen by using a 'wax-sheet' technique. The skin was freed of connective tissues and fat, and placed on a pre-cooled (0°C) glass slab with the hair up. Paraffin, at 55°C, was poured over the tissue and allowed to solidify for 2min. The wax sheet and the skin were pulled apart and the depilated skin was homogenized by the method of Freedberg *et al.* (1967).

The intestinal mucosa was prepared and homogenized by the method of De Luca *et al.* (1969). Liver and pancreas respectively were homogenized in 2.5vol. of TKMS buffer (50mM-Tris-HCl-25mM-KCl-10mM-MgCl₂-0.25M-sucrose-5mM-2-mercaptoethanol), pH7.6, with ten strokes of a Teflon-glass Potter-Elvehjem homogenizer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.; clearance 0.2mm) rotating at 1500rev./min.

For assays of total protein synthesis, an equal volume of 10% (w/v) trichloroacetic acid was added to the homogenates, and protein was isolated by the method of Peterson & Greenberg (1952). The protein was dissolved in 1M-NaOH and counted for radioactivity. Protein assays were done by the method of Lowry *et al.* (1951).

In other experiments with liver, the homogenates were centrifuged for 10min at 12000g. The post-mitochondrial supernatants were treated with 10% (w/v) sodium deoxycholate to a final concentration of 1.3% (w/v) and centrifuged for 2h at 105000g. The supernatant was applied to a column (2.5cm $\times$ 45cm) of Sephadex G-25 and eluted with 0.05M-Tris-HCl, pH7.8. The material eluted in the void volume was collected and analysed by column chromatography as described in the Results section. In dual-label experiments, equal weights of 14 C- and 3 H-labelled livers were mixed before homogenization.

Protein biosynthesis in vitro

Liver microsomal fractions were prepared by the method of Siekevitz (1962). A pH5.0 fraction was prepared from normal (laboratory-chow-fed) male Holtzman rats by the method of von der Decken & Campbell (1962). The pH5.1 precipitate was gently suspended in 0.4M-Tris-HCl, pH7.8, and applied to a column (2.5cm $\times$ 45cm) of Sephadex G-25. The material was eluted with TKMS buffer. The material eluted in the void volume was collected, diluted to a protein concentration of 5mg/ml, and samples of this solution were stored at -80°C. The

pH5.0 fraction stored in this manner was stable for up to one month.

The amino acid-incorporating system *in vitro* contained the following, per ml final volume: 2 μ mol of ATP; 0.5 μ mol of GTP; 10 μ mol of phosphoenolpyruvate; 50 μ g of pyruvate kinase; 200 μ mol of sucrose; 15 μ mol of KCl; 9 μ mol of MgCl₂; 35 μ mol of Tris-HCl, pH7.6; 1 μ mol of 2-mercaptoethanol; 2mg of microsomal protein and 1mg of pH5.0-fraction protein. Either 1 μ Ci (5nmol) of L-[U- 14 C]leucine or 10 μ Ci (5nmol) of L-[4,5- 3 H]leucine were used, and 50nmol of each of the other 19 amino acids were added per ml of final volume. Incubations were performed at 37°C.

The incubations for the measurement of protein synthesis *in vitro* for skin and intestinal mucosa were identical with those used by Freedberg *et al.* (1967) and DeLuca *et al.* (1969) respectively, except that 14 C- and 3 H-labelled leucine were used as with liver.

For assays of total protein biosynthesis, the incorporation was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The precipitated proteins were treated as described for the experiments *in vivo*.

In experiments where the pattern of protein labelling was examined, puromycin was used to stop the reaction. Puromycin dihydrochloride and leucine were added so that the final concentrations of these compounds in the reaction mixture were 50 μ g/ml and 4mM respectively. The incubations were continued for a further 10min, after which the reaction mixture was chilled rapidly on ice and sonicated for a total of 2min with a Bronwill Biosonok sonicator. Sonication was performed at 80% maximum power in four 30s intervals with 1min cooling between each. The sonicated preparations were centrifuged at 105000g for 2h. Equal amounts of protein from the 14 C-labelled (biotin-treated) and 3 H-labelled (control) supernatants were combined and the mixture was applied to a column (2.5cm $\times$ 45cm) of Sephadex G-25. The column was eluted with Tris-HCl buffer (0.05M, pH7.8). Material eluted in the void volume was collected and analysed further by ion-exchange chromatography and sodium dodecyl sulphate-polyacrylamide-gel electrophoresis.

Isolation of serum albumin

Serum albumin was isolated from liver homogenates by using the acidified-ethanol method of Campbell *et al.* (1960). Proteins, precipitated from homogenates with 1 vol. of 10% (w/v) trichloroacetic acid, were washed three times with 5% (w/v) trichloroacetic acid. The pellets were extracted with 1% (w/v) trichloroacetic acid in 96% (v/v) ethanol by gentle homogenization. The extracts, clarified by centrifugation, were dialysed against water. The precipitate was removed from the dialysate and the

resulting solution was freeze-dried. The serum albumin isolated in this way was assayed by the single linear-immunodiffusion technique of Oudin (1952). This method also served to prove the identity of serum albumin. Samples containing 40–5000 μ g of albumin in a volume of 200 μ l were layered on a gel (0.5 cm $\times$ 4 cm) containing 0.6% agarose and 5% (w/v) rabbit anti-(rat serum albumin) antiserum. The gels were left undisturbed for 25 h at 37°C. After this time, a definite precipitin band was observed. The distance which the band had moved down into the gel was measured and it served as a specific assay for serum albumin. This method in our laboratory has enabled us to assay amounts of serum albumin as low as 40 μ g.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Samples of freeze-dried protein were dissolved to a concentration of 2 mg/ml in 10% (w/v) glycerol–1% (w/v) sodium dodecyl sulphate–1% (w/v) 2-mercaptoethanol–0.01 M-sodium phosphate, pH 7.1–0.002% (w/v) Bromophenol Blue as described by Tzagoloff (1971). Electrophoresis was done on 10% (w/v) sodium dodecyl sulphate–polyacrylamide-gels by the method of Weber & Osborn (1969).

After electrophoresis the gels were frozen at –80°C and were sliced with a device consisting of razor blades stacked 1 mm apart. Gel slices were dissolved in 6% (v/v) H₂O₂ as described by Tzagoloff (1971). Scintillator was added and the gels were counted for radioactivity as described below.

Analysis of liquid-scintillation spectrometer data

All determinations of radioactivity were done with a Beckman LS-250 liquid-scintillation system. A maximum of 0.5 ml of sample was dissolved in 10 ml of a scintillation solution consisting of 0.25% (w/v) 2,5-diphenyloxazole, 0.009% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene and 31% (v/v) ethanol in toluene. Samples were counted for radioactivity by using the external-standard channels-ratio method of Hall & Weiser (1964). Data were recorded on paper tape and analysed with a CDC 1700 computer and program SCINT (Boeckx *et al.*, 1973).

Results

The effect of parenteral administration of a single dose (200 μ g) of biotin to biotin-deficient rats on amino acid incorporation *in vitro* with microsomal and pH 5.0 fractions from the liver, intestinal mucosa and skin of biotin-injected or NaCl-injected (control) rats was examined. In all of the tissues studied, biotin administration resulted in a 1.5–2-fold stimulation of amino acid incorporation into total protein. The

smallness of the incorporation of labelled amino acids in the skin cell-free system is due to the high activity of ribonuclease in this tissue (Freedberg *et al.*, 1967).

The difference in incorporation of labelled amino acids into protein *in vitro* is not due to a difference in the amount of proteolytic activity present in the cell-free system. A ¹⁴C-labelled protein mixture was incubated with the amino acid-incorporating system (less the labelled amino acids) for up to 60 min at 37°C. No acid-soluble ¹⁴C appeared, indicating that no proteolytic activity was present in either of the liver systems (biotin-treated or control).

The liver amino acid-incorporating systems from biotin-treated and control animals *in vitro* showed the same requirements. The use of protein-synthesis inhibitors shows that there is no difference in the response of the systems to the addition of puromycin or ribonuclease. Deoxyribonuclease had no effect in either case.

The possibility that differences in the amount of rRNA in the microsomal preparations from biotin-deficient and biotin-injected rats might be responsible for the smaller amino acid incorporation in the preparation from deficient rats was examined. Table 1 shows the RNA/protein ratios of microsomal preparations from normal (Laboratory-Chow-fed), biotin-deficient and biotin-injected rats. All three groups had the same RNA/protein ratio. There was no difference in the yields of liver microsomal RNA among the three groups. In all cases, approx. 3.5–4.0 mg of microsomal RNA was recovered/g of fresh liver. Thus any difference between microsomal preparations from biotin-deficient and biotin-injected rats cannot be ascribed to the rRNA content.

This was further confirmed by experiments where the amount of microsomal RNA in the amino acid-incorporation system *in vitro* was increased from 300 to 1200 μ g. There was a linear relation between the leucine (pmol) incorporated into protein and the microsomal RNA (μ g) present in the system. The amount of leucine incorporated into protein/mg of microsomal RNA remained constant. This was true whether the microsomal RNA was from biotin-deficient or biotin-injected rat livers. However, the

Table 1. *Yield and composition of rat liver microsomal fractions*

For details see the text. Values are means $\pm$ s.d. for six experiments.

Group	Yield (mg of RNA/g of fresh liver)	RNA/protein
Normal (Laboratory Chow-fed)	4.01 $\pm$ 0.24	0.335 $\pm$ 0.014
Biotin-deficient	3.55 $\pm$ 0.21	0.334 $\pm$ 0.032
Biotin-injected	3.86 $\pm$ 0.28	0.334 $\pm$ 0.018

Table 2. *Effect of biotin on the incorporation of leucine into total protein in vivo and in vitro*

Albumin concentration was determined by the method of Oudin (1952). For further details see the text.

Tissue	Incorporation of leucine (pmol/mg of protein)					
	<i>In vivo</i>			<i>In vitro</i>		
	With biotin	Without biotin	Ratio of with biotin/ without biotin	With biotin	Without biotin	Ratio of with biotin/ without biotin
Liver	21.92	9.27	2.36	78.21	43.75	1.79
Albumin	11.34*	5.64*	2.01*	—	—	—
Intestinal mucosa	29.42	6.99	4.21	104.22	66.21	1.57
Skin	10.21	3.51	2.91	28.91	18.75	1.54
Pancreas	27.91	5.79	4.82	—	—	—

* Data are expressed as pmol of leucine incorporated/mg of albumin.

amount of leucine incorporated into protein/mg of microsomal RNA from biotin-deficient rats was always about half of that incorporated by microsomal RNA from biotin-injected rats over a similar range of microsomal RNA concentrations studied. Thus at the same concentration of microsomal RNA in the incubation *in vitro*, the preparation from biotin-deficient rat livers could sustain only about half of the amino acid incorporation attained by the microsomal fractions from biotin-injected rat liver. Thus the impairment in amino acid incorporation is not due to any difference in the amount of total microsomal RNA between these preparations, but to a difference in the nature of the microsomal RNA species. Dakshinamurti & Litvak (1970) showed that the microsomal fractions from biotin-deficient rat livers gave a higher stimulation of polyphenylalanine synthesis by added poly(U) than similar preparations from biotin-injected or normal rat livers.

Table 2 compares the data from amino acid-incorporation experiments *in vitro* and *in vivo*. A higher stimulation ratio was observed in the experiments *in vivo*. Rat serum albumin isolated from liver homogenates also showed a stimulation ratio of 2. Leucine incorporated *in vitro* into total protein/mg of ribosomal RNA was calculated to be 26.2 pmol/mg of RNA for biotin-injected and 14.6 pmol/mg of RNA for biotin-deficient rat preparations.

To study the pattern of the biotin-mediated stimulation of amino acid incorporation, we analysed the products of amino acid incorporation *in vivo* by column chromatography. We used the dual-label method described in the Experimental section. Equal weights of [^{14}C]leucine-labelled (biotin-treated) and [^3H]leucine-labelled (control) livers were mixed and deoxycholate-treated, gel-filtered post-mitochondrial supernatants were prepared as described under 'Amino acid incorporation *in vivo*'. Samples of volume

10–15 ml of the Sephadex G-25 eluate containing 75 mg of protein, labelled *in vivo* as described above, were analysed on a column (1.5 cm $\times$ 100 cm) of DEAE-Sephadex A-50. The column was developed with a gradient of 0–0.5 M-NaCl in 0.05 M-Tris-HCl, pH 7.8. The elution profile is given in Fig. 1. The leading peak contains large amounts of protein and radioactivity. The stimulation ratio of this peak is 2.0 and it is probable that this large amount of protein contributes the greatest to the ratio of 2.0 which is observed in total protein experiments (Table 2). The proteins eluted by a NaCl concentration of 0.1–0.25 M also contain a relatively high amount of labelled leucine. Certain areas of the profile show a ratio as high as 8.0, and other areas show a ratio of 1.0, i.e. no stimulation at all. The minimal variation (between 0.70 and 1.35) of the control ratio indicates that the dual-label technique does not introduce any artifacts.

A similar lack of uniformity is observed when material from amino acid-incorporating experiments *in vitro* is analysed. Large-volume incorporation systems *in vitro* from biotin-treated and control rats were set up as described in the Experimental section. Each reaction mixture contained 50 mg of microsomal protein and 25 mg of pH 5.0-fraction protein. The final volume of the incubation mixtures was 25 ml. L-[^{14}C]leucine (25 μCi) was used in incubations with microsomal fractions from biotin-treated animals, and L-[4,5- ^3H]leucine (250 μCi) was used in control systems. The final concentration of added leucine in each case was 5 μM . The combined supernatants after passage through Sephadex G-25 had a total volume of approx. 100 ml and contained about 100 mg of protein. Samples of volume 25 ml of this ^{14}C - and ^3H -labelled preparation were applied to a column (2.5 cm $\times$ 45 cm) of DEAE-Sephadex A-50. The column was developed with a gradient of 0–0.5 M-NaCl in the starting buffer of 0.05 M-Tris-HCl,

Fig. 1. DEAE-Sephadex A-50 chromatography of proteins from experiments *in vivo*

A column (1.5cm $\times$ 100cm) of DEAE-Sephadex A-50 was used, and was developed with a gradient of 0–0.5M-NaCl in 0.05M-Tris-HCl, pH7.8, and fractions (5 ml) were collected. The labelled protein solution used was prepared as described in the text. $\blacksquare$, E_{280} ; $\bullet$, $[^{14}\text{C}]$ leucine incorporated (pmol/ml) of fraction; (----), concentration of NaCl; $\blacktriangle$, ratio of $[^{14}\text{C}]$ leucine incorporated (+biotin)/ $[^3\text{H}]$ leucine incorporated (+biotin); $\triangle$, ratio of $[^{14}\text{C}]$ leucine incorporated (+biotin)/ $[^3\text{H}]$ leucine incorporated (+biotin).

pH7.8. Fig. 2 shows the elution pattern obtained. The ratio, here as in Fig. 1, is the ratio of $[^{14}\text{C}]$ leucine incorporated/ $[^3\text{H}]$ leucine incorporated. As such it is a measure of the biotin-mediated stimulation of amino acid incorporation. Several areas of the elution profile show a more pronounced stimulation after biotin treatment, whereas other areas show no stimulation at all. The peak eluted at 0.025M-NaCl shows the highest stimulation. It is noteworthy that the peak showing the next highest amount of incorporation, i.e. that eluted at 0.175M-NaCl, shows no stimulation at all.

In a further experiment, L-[4,5- ^3H]leucine (100 μCi /

ml and 5 μM) was used in both biotin-treated and control incorporating systems *in vitro*, and the products of the two incubations were analysed separately by using the same procedure as described for the dual-labelling experiment. The elution patterns from DEAE Sephadex A-50 columns were almost identical with that obtained in Fig. 2.

Fractions 10–37 (A), 38–56 (B), 57–80 (C) and 80–100 (D) obtained from biotin-treated and control incubations were pooled, dialysed against 0.05M- NH_4HCO_3 and freeze-dried. The protein fractions A, B, C and D from biotin-treated incubations and A', B', C' and D' from control incubations were

Fig. 2. Analysis of proteins from amino acid incorporation *in vitro*

A column (2.5cm $\times$ 45cm) of DEAE-Sephadex A-50 was prepared and a gradient of 0–0.5M-NaCl in 0.05M-Tris-HCl, pH7.8, was used to develop the column. Fractions (5 ml) were collected. The labelled protein preparation used was prepared as described in the text. $\square$, [^3H]Leucine incorporated [pmol/ml of fraction (–biotin)]; $\blacksquare$, [^{14}C]leucine incorporated [pmol/ml of fraction (+biotin)]; (—), concentration of NaCl; (·····), E_{280} ; $\bullet$, ratio of [^{14}C]leucine incorporated (+biotin)/[^3H]leucine incorporated (–biotin).

analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, to compare fraction A with A', B with B', C with C', and D with D'.

Fig. 3 shows the sodium dodecyl sulphate–polyacrylamide-gel patterns obtained with each of these fractions. The ratio (pmol of [^3H] leucine incorporated by the biotin-treated system/pmol of [^3H]leucine incorporated by the control system) indicates the biotin-mediated stimulation of the incorporation of amino acid into protein. Each fraction shows some areas of high biotin stimulation. Fractions A and B show two and four such areas respectively, each with a stimulation ratio of approx. 5. Fraction C shows at least two of these areas, both with mol wts. between 60000 and 80000. The second of these peaks has a mobility identical with that of

serum albumin. Similarly fraction D shows peaks in the region of mol wt. greater than 50000.

Discussion

Biotin increases the activities of enzymes that do not contain biotin as the prosthetic group. The biotin-mediated increases of enzyme activities were abolished by inhibitors of protein and RNA synthesis (Dakshinamurti & Cheah-Tan, 1968, 1970; Dakshinamurti & Hong, 1970; Dakshinamurti *et al.*, 1970). Biotin administration seemed to have a general stimulatory effect on both cell-free amino acid incorporation into protein and orotic acid incorporation into nuclear RNA in the biotin-deficient rat *in vivo* (Dakshinamurti & Mistry, 1963;

Fig. 3. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of fractions obtained from DEAE-Sephadex chromatography of proteins from amino acid incorporation *in vitro*

Protein fractions from biotin-treated (A, B, C and D) and control (A', B', C' and D') incubations were analysed as described in the text. ●—●, [^3H]Leucine incorporated [pmol/fraction (+biotin)]; ●---●, [^3H]leucine incorporated [pmol/fraction (−biotin)]; ▲, ratio (+biotin/−biotin). (a)–(d), ratio of [^3H]leucine incorporated into fractions A and A', B and B', C and C', and D and D' respectively; (a')–(d'), [^3H]leucine incorporated into fractions A and A', B and B', C and C', and D and D' respectively. Mobility is expressed as distance moved relative to marker dye (Bromophenol Blue).

Dakshinamurti & Litvak, 1970). The stimulation of amino acid incorporation *in vitro* occurred as early as 8h after a single dose of 200 μg of biotin. The stimulation of orotate incorporation into nuclear RNA preceded the effect on amino acid incorporation, and could be observed as early as 2h after biotin administration (Dakshinamurti & Litvak, 1970). It was not clear whether the effect of biotin was general or

whether, as some evidence suggested, the synthesis of some proteins was preferentially stimulated. Although the activities of glucokinase, phosphofructokinase and pyruvate kinase were increased by biotin administration, the activity of hexose phosphate isomerase was not affected (Dakshinamurti & Cheah-Tan, 1968, 1970; Dakshinamurti & Hong, 1970; Dakshinamurti *et al.*, 1970).

In the present paper we show that amino acid incorporation in liver, intestinal mucosa and skin both *in vivo* and *in vitro* is stimulated by a single dose of biotin. Although experiments in pancreas *in vitro* were not possible we observed an effect of biotin *in vivo*. The synthesis of rat serum albumin, a major product of the liver protein-synthetic machinery, was studied specifically. Its synthesis was stimulated twofold. No difference in the microsomal concentrations of leucine could be demonstrated in our systems, *in vitro*, and the present data and earlier crossover experiments (Dakshinamurti & Litvak, 1970) indicate that the defect is primarily associated with the microsomal fraction.

The pattern of the biotin-mediated stimulation of amino acid incorporation is complex, however. The synthesis of different proteins is stimulated from eightfold to not at all. Some major products of the amino acid-incorporating systems, such as serum albumin, are stimulated by at least twofold. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of various fractions of the products of amino acid incorporation *in vitro* indicate that the synthesis of several proteins (at least 10) are stimulated about fivefold, thus establishing the marked heterogeneity in the stimulation of protein synthesis mediated by biotin.

In the experiments described above biotin-deficient rats were injected with 100–200 μ g of biotin. This is over 50 times the daily requirement of biotin by the rat. The amino acid incorporation of leucine into protein *in vitro* by the microsomal-pH 5 incorporation system of the biotin-injected rat liver was about 90% of the value obtained in incubations with microsomal fractions and pH 5 fraction from normal (Laboratory-Chow-fed) rat liver. Thus the injected biotin seems only to return the system to normal.

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