Biochemical Pharmacology, Vol. 17, pp. 1173–1180. Pergamon Press. 1968. Printed in Great Britain

ACTION OF GROWTH HORMONE ON THE METABOLISM OF COLLAGEN IN THE RAT

JUHANI AER, JOUKO HALME, KARI I. KIVIRIKKO and OSSI LAITINEN

Department of Medical Chemistry, University of Helsinki, Finland

(*Received* 15 *December* 1967; *accepted* 15 *February* 1968)

Abstract—The action of growth hormone on the metabolism of collagen was studied with ^{14}C-proline. (a) When ^{14}C-proline was injected 20 days after the beginning of growth hormone injections, the total activity of ^{14}C-hydroxyproline in the collagen fractions of the skin and in the nonfractionated collagen of the femurs was higher in the rats treated with growth hormone than in the controls. The distribution of ^{14}C-hydroxyproline between the soluble and insoluble collagen of the skin was similar in the treated rats and the controls. The total activity of urinary ^{14}C-hydroxyproline was higher in the treated rats than in the controls, and this increase was of the same magnitude as in the ^{14}C-hydroxyproline of the skin and bone. (b) When ^{14}C-proline was injected 12 days before the beginning of growth hormone injections, administration of growth hormone caused an increase in the total activity of urinary ^{14}C-hydroxyproline compared to the controls. The results of the present study suggest that the rate of collagen synthesis was increased by growth hormone, and this effect was followed by an increased urinary excretion of hydroxyproline. No marked changes were found in the rate of conversion of soluble collagen into insoluble collagen or in the rate of catabolism of soluble collagen when expressed per unit of soluble collagen. The rate of catabolism of insoluble collagen was increased by growth hormone, and this effect contributed to the increased urinary hydroxyproline excretion.

HYDROXYPROLINE is an amino acid found in the tissues of vertebrates almost exclusively in collagen, and studies on the biosynthesis of this protein have indicated that the hydroxyproline in collagen is synthesized by hydroxylation of proline in a large polypeptide precursor of collagen (for review, see ref. 1). Consequently, the presence of hydroxyproline in tissues, serum or urine can be used as a measure of collagen or of degradative products of collagen.[1] Administration of growth hormone increases the content of free hydroxyproline in the blood[2] and the excretion of hydroxyproline in the urine[3] of rats. Urinary excretion of hydroxyproline is also increased in patients with active acromegaly[4–8] and reduced in children with hypopituitarism.[4] Studies on tissue collagen have indicated that growth hormone increases the content of acid soluble collagen in the skin,[9] and the incorporation of labelled amino acids into collagen *in vitro*.[10–12] These findings are in agreement with the well known stimulatory effect of growth hormone on the synthesis of proteins.

Relatively little is known of the effect of growth hormone on the other steps involved in the metabolism of collagen. Studies with ^{14}C-proline in rats[13–15] and monkeys[16] have indicated that urinary hydroxyproline is derived partly from the catabolism of recently synthesized soluble collagens and partly from the catabolism of mature insoluble collagen fibres. Thus the increased excretion of hydroxyproline after administration of growth hormone could be due solely to changes in the rate of collagen

synthesis and increased pool sizes of soluble and insoluble collagen, or to changes in the rate of degradation of soluble or insoluble collagen, or to changes in the rate of conversion of soluble collagen into insoluble form. In the present study isotope techniques were used to study the mode of action of growth hormone on the metabolism of collagen *in vivo*. In these experiments, ^{14}C-proline was administered to rats, and the radioactivities of ^{14}C-hydroxyproline were determined in the collagen fractions of the tissues and in the urine.

MATERIAL AND METHODS

The experimental animals were female albino Wistar rats, 5 months of age at the beginning of the experiments. They were fed a commercial pelleted diet (Hankkija Oy) which had a hydroxyproline content of about 0·05 per cent of the dry wt. The hydroxyproline excretion with this diet and with a completely hydroxyproline–free diet differed by less than 10 per cent. During the urine collections the animals were fed a completely hydroxyproline-free diet consisting of cheese and water, and this diet was begun 3 hr before the start of urine collection. Subcutaneous injections of porcine pituitary growth hormone (Somacton, Ferring AB, Malmö, Sweden) were given daily, as indicated in Tables 1–6. Uniformly labelled ^{14}C-proline (8·2 μC/μM, The Radiochemical Centre, Amersham) was injected i.p. in 1·00 ml of 0·9 % sodium chloride solution. The ^{14}C-proline used in these experiments was assayed for ^{14}C-hydroxyproline radioactivity after chromatography in a Dowex 50-X8 column with carrier hydroxyproline and found to contain less than 0·01 % ^{14}C-hydroxyproline.

Urine was collected under toluene during the periods indicated under Results. It was stored at + 4° until hydrolyzed with an equal volume of 12 N HCl for 3 hr at 124°, and used for determination of the amount and specific activity of hydroxyproline excreted during the collection period.

The skin specimens were fractionated for 0·45 M sodium chloride soluble collagen hydroxyproline and insoluble collagen hydroxyproline, as described earlier.[17,18] The values for each animal were obtained by independent analyses of two specimens of dorsal skin. The procedures used in the determination of the total radioactivity of crude 0·45 M sodium chloride soluble protein fraction of the skin, and in the determination of the amount and specific activity of hydroxyproline in the bones have also been described earlier.[18]

The quantity of hydroxyproline was determined as described by Kivirikko *et al.*[19] and the specific activity of ^{14}C-hydroxyproline by the method of Peterkofsky and Prockop.[20] Because of the high radioactivity of ^{14}C-proline in the urine samples collected during the first 12 hr after administration of the radioisotope, hydroxyproline was preliminarily separated from proline in these samples in a Dowex 50-X8 column with 2 N HCl as eluting agent. The counting efficiency of the liquid scintillation counter was 80 per cent for the samples of ^{14}C-pyrrole in the ^{14}C-hydroxyproline assays, and 71 per cent for the samples of total radioactivity of crude 0·45 M sodium chloride soluble protein fraction. The observed cpm were corrected for aliquots and for loss of one carbon in the ^{14}C-hydroxyproline assay, and converted to dpm. The total activity of ^{14}C-hydroxyproline was calculated from the quantity and sp. act. of hydroxyproline. The content of free proline[21] and sp. act. of free ^{14}C-proline[20] in the serum were determined after deproteinization of the serum with trichloroacetic acid.

RESULTS

Effect of growth hormone on the synthesis of collagen

The effect of growth hormone on the synthesis of collagen was studied in experiments in which hormone administration was started 20 days before the injection of ^{14}C-proline, and incorporation of ^{14}C-proline into ^{14}C-hydroxyproline was determined in the nonfractionated collagen of the femurs and in the 0·45 M sodium chloride soluble collagen fraction of the skin.

Administration of growth hormone for 20 days had no effect on the total amount of hydroxyproline in the *nonfractionated collagen of the femurs* (Table 1). However,

TABLE 1. EFFECT OF GROWTH HORMONE ON THE AMOUNT OF HYDROXYPROLINE, AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE TOTAL COLLAGEN OF THE FEMURS AFTER ADMINISTRATION OF ^{14}C-PROLINE

Time (hr after ^{14}C-proline injection)	Group	Total femur collagen		
		Hydroxyproline (μg/femur)	Specific activity of hydroxyproline (dpm/μg)	Total activity of hydroxyproline (dpm/femur)
12	Controls	4270 (3670–5050)	0·93 (0·59–1·34)	4010 (2060– 5460)
	G.H.	4260 (3450–5350)	1·95 (1·08–2·36)	8600 (3880–11900)
120	Controls	4510 (3250–5200)	1·44 (1·25–1·59)	6480 (4750– 8300)
	G.H.	3790 (3000–4260)	2·36 (2·00–2·88)	8750 (8500– 9100)

Daily administration of 3 mg of growth hormone (G.H.) was begun 20 days before administration of 12 μC of ^{14}C-proline. Three rats in both control groups, and 4 rats in both G.H. groups. Mean wt. of rats when ^{14}C-proline was injected: Controls 208 g; G.H. 216 g.

the sp. act. and total activity of ^{14}C-hydroxyproline were considerably higher in the rats receiving growth hormone than in the controls 12 hr and 120 hr after the ^{14}C-proline injection. In the *soluble collagen of the skin* the content of hydroxyproline was higher in the rats receiving growth hormone than in the controls (Table 2). There was no difference in the sp. act.'s of ^{14}C-hydroxyproline between the controls and the animals receiving growth hormone, but the total activity of ^{14}C-hydroxyproline in the animals receiving growth hormone was about 150 per cent of that of the controls 12 hr and 120 hr after administration of ^{14}C-proline in both experiments.

In order to establish whether the differences in the collagen ^{14}C-hydroxyproline were due to differences in the rates of collagen synthesis or in the specific activities of the free ^{14}C-proline pools, the content and specific activity of *free ^{14}C-proline* was determined in the serum after injection of ^{14}C-proline. The content of free proline in the serum was 27·4 (22·6–29·6) μg/ml in four control rats, and 20·5 (18·7–23·6) μg/ml in four animals receiving growth hormone. When 3μC of ^{14}C-proline was injected into the rats, the sp. act. of serum free ^{14}C-proline was 16·0 (15·0; 16·9) dpm/μg in two control rats, and 12·5 (11·7; 13·3) dpm/μg in two rats receiving growth hormone 3 hr after ^{14}C-proline injection. Eight hr after ^{14}C-proline injection the corresponding values were 8·5 (8·4; 8·6) dpm/μg, and 7·9 (7·7; 8·1) dpm/μg. These values suggest that the sp. act. of serum free ^{14}C-proline was not higher in the rats receiving growth hormone than in the controls.

In order to establish whether growth hormone had a stimulatory effect on the synthesis of other skin proteins besides collagen, the total radioactivity of the crude

TABLE 2. EFFECT OF GROWTH HORMONE ON THE CONTENT OF HYDROXYPROLINE, AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE SOLUBLE COLLAGEN FRACTION OF THE SKIN AFTER ADMINISTRATION OF ^{14}C-PROLINE

Experiment	Time* (hr)	Group	Soluble collagen		
			Content of hydroxyproline (μg/g of skin)	Sp. act. of hydroxyproline (dpm/μg)	Total activity of hydroxyproline (dpm/g of skin)
I	12	Controls	370 (340– 450)	20·7 (17·3–25·1)	7900 (6000–11,200)
		G.H.	510 (480– 550)	24·4 (22·6–25·3)	12,600 (11,100–14,000)
	120	Controls	420 (390– 440)	2·2 (2·0– 2·3)	920 (820– 1030)
		G.H.	630 (590– 670)	2·3 (2·0– 2·7)	1450 (1270– 1690)
II	12	Controls	480 (380– 540)	10·4 (9·2–11·4)	4960 (4050– 6100)
		G.H.	710 (630– 790)	11·0 (8·9–15·2)	7650 (6460– 9620)
	120	Controls	480 (330– 670)	3·1 (2·7– 3·6)	1540 (1030– 2380)
		G.H.	1040 (830–1190)	2·2 (1·9– 2·3)	2250 (1930– 2800)

Daily administration of 3 mg of growth hormone (G.H.) was begun 20 days before administration of ^{14}C-proline. In experiment I the dose of ^{14}C-proline was 15 μC, and there were 4 rats in each group. Mean wt of the rats, when ^{14}C-proline was injected in experiment I: controls 210 g, growth hormone 227 g. The rats in exp. II were the same rats as were used for determinations of bone collagen (for details, see Table 1).

* Hr after ^{14}C-proline injection.

0·45 *M sodium chloride soluble protein fraction* of the skin was determined. No change was observed in the radioactivity of this fraction after administration of growth hormone (Table 3), even though in the same experiment the total activity of ^{14}C-hydroxyproline in the soluble collagen of the skin was considerably increased (Table 2, experiment II).

TABLE 3. EFFECT OF GROWTH HORMONE ON THE TOTAL RADIOACTIVITY OF THE CRUDE 0·45 M SODIUM CHLORIDE SOLUBLE PROTEIN FRACTION IN THE SKIN AFTER ADMINISTRATION OF ^{14}C-PROLINE

Time (hr after ^{14}C-proline injection)	Group	0·45 M NaCl soluble protein
		Total activity (dpm/g of skin)
12	Controls	49,900 (48,400–51,500)
	G.H.	53,600 (43,300–59,100)
120	Controls	18,200 (16,000–21,000)
	G.H.	20,200 (15,600–22,900)

Experiment II. Same rats as were used for determinations of skin soluble collagen (Table 2), and of bone collagen (for details see Table 1).

Effect of growth hormone on the conversion of soluble collagen into soluble collagen and on the catabolism of soluble collagen

The conversion of soluble collagen into insoluble collagen was studied by determining the ^{14}C-hydroxyproline in the *insoluble collagen of the skin* 12 hr and 120 hr after ^{14}C-proline injection. The content of hydroxyproline in the insoluble collagen was

similar in the control rats and in the rats treated with growth hormone (Table 4). The sp. act. and total activity of ^{14}C-hydroxyproline were considerably higher in the treated rats than in the controls (Table 4). When the total activities of ^{14}C-hydroxyproline in insoluble and soluble collagen were calculated as percentages of the total ^{14}C-hydroxyproline in soluble (Table 2) and insoluble collagen (Table 4) combined, the following values were obtained: At 12 hr about 18 per cent of the observed ^{14}C-hydroxyproline radioactivity was present in the insoluble collagen, and 82 per cent in the soluble collagen in the control rats. The corresponding values in the rats treated with growth hormone were about 20 per cent and 80 per cent. At 120 hr about 88 per cent of the observed ^{14}C-hydroxyproline radioactivity was in the insoluble collagen and 12 per cent in the soluble collagen both in the controls and in the treated rats.

TABLE 4. EFFECT OF GROWTH HORMONE ON THE CONTENT OF HYDROXYPROLINE, AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE INSOLUBLE COLLAGEN OF THE SKIN AFTER ADMINISTRATION OF ^{14}C-PROLINE

Time (hr after ^{14}C-proline injection)	Group	Insoluble collagen		
		Content of hydroxyproline (mg/g of skin)	Specific activity of hydroxyproline (dpm/μg)	Total activity of hydroxyproline (dpm/g of skin)
12	Controls	16·9 (15·3–18·5)	0·10 (0·06–0·13)	1700 (1000– 2300)
	G.H.	16·8 (15·3–19·3)	0·19 (0·16–0·22)	3200 (2500– 3800)
120	Controls	18·4 (17·5–19·3)	0·35 (0·28–0·41)	6400 (5300– 7200)
	G.H.	18·4 (15·3–19·4)	0·59 (0·50–0·63)	10,100 (9500–11,000)

Experiment I. Same rats that were used for determinations of skin soluble collagen (for details, see Table 2).

The catabolism of soluble collagen was studied by determining the ^{14}C-hydroxyproline excreted in the *urine* during the first 12 hr after ^{14}C-proline injection. The amount of hydroxyproline excreted by the rats receiving growth hormone was about 150 per cent, and the total activity of the urinary ^{14}C-hydroxyproline about 180 per cent of the values for the controls (Table 5). The mean increase in the total activity

TABLE 5. EFFECT OF GROWTH HORMONE ON THE URINARY EXCRETION OF HYDROXYPROLINE AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF URINARY ^{14}C-HYDROXYPROLINE DURING THE FIRST 12 HR AFTER ADMINISTRATION OF ^{14}C-PROLINE

Group	Urinary hydroxyproline		
	μg during collection	Sp. act. (dpm/μg)	Total activity (dpm)
Controls	256 ± 61	13·3 ± 3·8	3400 ± 710
G.H.	382 ± 84*	17·0 ± 3·2	6250 ± 1100*

Experiment II. Same rats as were used for the determinations of skin soluble collagen (Table 2) and of bone collagen (Table 1) partly at 12 hr and partly at 120 hr. Thus there were 6 rats in the control group and 8 rats in the growth hormone group. Values are expressed as mean ± S.D.
*$P < 0·01$.

of urinary ^{14}C-hydroxyproline was slightly greater than the mean increase in the total activity of ^{14}C-hydroxyproline in the soluble collagen of the skin (Table 2), and slightly smaller than that in the total nonfractionated collagen in the femurs (Table 1), but it seems that the magnitude of all these increases was similar.

Effect of growth hormone on the catabolism of insoluble collagen

The catabolism of insoluble collagen was studied in an experiment in which ^{14}C-proline was injected 12 days before the start of the growth hormone injections, and the urinary ^{14}C-hydroxyproline was followed during the growth hormone treatment. Before the start of growth hormone treatment the ^{14}C-hydroxyproline excretion was

TABLE 6. EFFECT OF GROWTH HORMONE ON THE URINARY EXCRETION OF HYDROXYPROLINE AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF URINARY ^{14}C-HYDROXYPROLINE AFTER ADMINISTRATION OF ^{14}C-PROLINE

Collection period*	Group	Urinary hydroxyproline		
		μg during collection	Sp. act. (dpm/μg)	Total activity (dpm)
16–17	Controls	332 (244–410)	2·08 (1·85–2·21)	650 (520–900)
(4–5)	G.H.	274 (263–294)	2·24 (1·87–2·62)	610 (510–690)
21–22	Controls	354 (282–424)	1·62 (1·25–2·31)	550 (440–650)
(9–10)	G.H.	460 (334–692)	1·31 (0·93–1·62)	570 (540–640)
26–27	Controls	346 (272–435)	1·32 (1·01–1·75)	460 (270–580)
(14–15)	G.H.	762 (570–874)	0·94 (0·83–1·10)	720 (470–880)
30–31	Controls	369 (263–570)	1·10 (0·90–1·24)	390 (310–510)
(18–19)	G.H.	669 (482–792)	0·95 (0·76–1·20)	620 (510–710)

Daily administration of 3 mg of growth hormone (G.H.) was begun 12 days after administration of 25 μC of ^{14}C-proline. Four rats in both groups. Mean wt of the rats, when ^{14}C-proline was injected: Controls 210 g, G.H. 208 g, and 31 days later: Controls 207 g; G.H. 228 g.

* Days after ^{14}C-proline injection; in brackets: days after the beginning of growth hormone injections.

similar in the two groups. After 14 and 18 growth hormone injections the urinary excretion of hydroxyproline was about 2-fold that of the controls, and the total activity of urinary ^{14}C-hydroxyproline had increased to about 160 per cent, suggesting an increase in the rate of the catabolism of insoluble collagen at this stage of the experiment.

DISCUSSION

Several factors, including the rate of collagen synthesis, the rate of conversion of soluble collagen into insoluble form, and the rates of catabolism of soluble and insoluble collagen, may affect the amount of hydroxyproline excreted in the urine (see ref. 1). However, with isotope techniques it is possible to use the combined measurements of hydroxyproline in the collagen fractions in the tissues, and of the urinary hydroxyproline excretion to determine the principal sites of action of various agents on the metabolism of collagen.[1,15–18] The synthesis of collagen can be studied by determination of the radioactivity of ^{14}C-hydroxyproline in the soluble collagen or total nonfractionated collagen after injection of ^{14}C-proline, and the conversion of soluble collagen into insoluble form by measuring the conversion of ^{14}C-hydroxy-

proline of soluble collagen into ^{14}C-hydroxyproline of insoluble collagen. The catabolism of soluble collagen can be studied by measuring the urinary ^{14}C-hydroxyproline shortly after ^{14}C-proline injection, and the catabolism of insoluble collagen can be studied by determination of the urinary ^{14}C-hydroxyproline in experiments in which ^{14}C-hydroxyproline is allowed to become incorporated into insoluble collagen before the start of administration of the agent studied.

In the present study, the experiments on the effect of growth hormone on the synthesis of collagen indicated an increase in the rate of collagen synthesis, for the total activity of ^{14}C-hydroxyproline was increased in the soluble collagen of the skin and in the total nonfractionated collagen of the bones after administration of ^{14}C-proline. Determinations of the sp. act. of free ^{14}C-proline in the serum suggested that the differences in collagen were due not to differences in the free ^{14}C-proline pools but to differences in the rates of collagen synthesis. These results are consistent with the well known stimulatory effect of growth hormone on protein synthesis, and with the results of earlier studies suggesting an increase caused by growth hormone in the rate of collagen synthesis *in vitro*.[10–12] In the present study, the incorporation of ^{14}C-proline into other proteins of the crude 0·45 M sodium chloride soluble protein fraction of the skin was not increased by growth hormone administration, which suggests that the effect of growth hormone on the synthesis of soluble collagen in the skin was a selective one compared to its effect on the synthesis of other soluble proteins in the skin.

Growth hormone did not have any direct effect on the conversion of soluble collagen into insoluble form. The increase in the radioactivity of insoluble collagen hydroxyproline was similar to that in the soluble collagen hydroxyproline, and the distribution of ^{14}C-hydroxyproline radioactivity between soluble and insoluble collagen was similar in controls and in treated rats. Neither did growth hormone seem to have any notable direct effect on the rate of catabolism of soluble collagen. During the first 12 hr after ^{14}C-proline injection, the urinary excretion of ^{14}C-hydroxyproline was higher in the rats receiving growth hormone than in the controls, but the increase was of the same magnitude as in the radioactivity of ^{14}C-hydroxyproline in the soluble collagen of the skin and in the total collagen of the bones. The results suggest, therefore, that a main effect of growth hormone on the metabolism of collagen is to increase the rate of synthesis of soluble collagen. This leads to increased pool sizes of newly-synthesized soluble collagen, and increased excretion of hydroxyproline in the urine, whereas there seem to be no marked changes in the rate of catabolism of newly-synthesized collagen expressed per unit of newly-synthesized collagen. The rate of catabolism of mature insoluble collagen was found to increase after administration of growth hormone, and thus hydroxyproline derived from insoluble collagen also contributed to the increased hydroxyproline excretion.

The present study was carried out with non-hypophysectomized animals receiving excessive doses of growth hormone, and the results do not necessarily represent the action of physiological doses of growth hormone on the metabolism of collagen. However, the changes described above may be related to those occurring in patients with hypersecretion of growth hormone, since greatly increased urinary excretion of hydroxyproline is found in patients with active acromegaly.[4–8]

Acknowledgement—This study was partly supported by a grant from the Sigrid Jusélius Foundation.

REFERENCES

1. D. J. PROCKOP and K. I. KIVIRIKKO, *Ann. int. Med.* **66**, 1243 (1967).
2. K. I. KIVIRIKKO, M. LIESMAA and T. LUUKKAINEN, *Acta endocr., Copenh.* **27**, 118 (1958).
3. K. KOWALEWSKI, *Acta endocr., Copenh.* **50**, 321 (1965).
4. H. E. JASIN, C. W. FINK, W. WISE and M. ZIFF, *J. clin. Invest.* **41**, 1928 (1962).
5. T. A. DULL and P. H. HENNEMAN, *New Engl. J. Med.* **268**, 132 (1963).
6. F. L. BENOIT, G. B. THEIL and R. H. WATTEN, *Metabolism* **12**, 1072 (1963).
7. C. A. LEE and H. M. LLOYD, *Med. J. Aust.* **1**, 992 (1964).
8. P. KOCHER, R. VUILLE, D. ROVARINO and B. COURVOISIER, *Helv. med. Acta* **32**, 480 (1965).
9. W. G. BANFIELD, *Proc. Soc. exp. Biol. Med.* **97**, 309 (1958).
10. W. H. DAUGHADAY and I. K. MARIZ, *J. Lab.clin. Med.* **59**, 741 (1962).
11. G. M. VAES and G. NICHOLS, JR., *Endocrinology* **70**, 890 (1962).
12. L. MIKKONEN, K. LAMPIAHO and E. KULONEN, *Acta endocr., Copenh.* **51**, 23 (1966).
13. S. LINDSTEDT and D. J. PROCKOP, *J. biol. Chem.* **236**, 1399 (1961).
14. D. J. PROCKOP, *J. clin. Invest.* **43**, 453 (1964).
15. O. LAITINEN, *Acta endocr., Copenh. suppl.* 120 (1967).
16. L. AVIOLI and D. J. PROCKOP, *J. clin. Invest.* **46**, 217 (1967).
17. K. I. KIVIRIKKO, O. LAITINEN, J. AER and J. HALME, *Biochem. Pharmac.* **14**, 1445 (1965).
18. K. I. KIVIRIKKO, O. LAITINEN, J. AER and J. HALME, *Endocrinology* **80**, 1051 (1967).
19. K. I. KIVIRIKKO, O. LAITINEN and D. J. PROCKOP, *Analyt. Biochem.* **19**, 249 (1967).
20. B. PETERKOFSKY and D. J. PROCKOP, *Analyt. Biochem.* **4**, 400 (1962).
21. W. TROLL and J. LINDSLEY, *J. biol. Chem.* **215**, 655 (1955).