

## Hepatic Collagen Production in the Rat Is Unaffected by Methotrexate<sup>1</sup> (42327)

LOREN LAINE AND MARIO CHOJKIER

*Division of Gastroenterology, Department of Medicine, Veterans Administration Medical Center and University of California, San Diego, California 92161*

---

*Abstract.* Methotrexate (MTX) has been implicated in the pathogenesis of hepatic fibrosis. However, no information exists regarding the effects of MTX on hepatic collagen metabolism. Therefore, we studied the role of MTX in hepatic collagen production *in vivo* in rats receiving an 8-week course of varying doses of MTX. Twenty-four hours prior to sacrifice animals received an injection of [5-<sup>3</sup>H]proline. Collagen was extracted with hot trichloroacetic acid and the protein-bound [<sup>3</sup>H]hydroxyproline was used as a measure of *de novo* collagen production. The hepatic collagen content was essentially the same in the control and treatment groups in spite of evidence of hepatotoxicity. Similarly, no significant differences were present among the control and MTX-treated groups in the *de novo* absolute collagen production. In summary, we found no evidence of increased hepatic fibrogenesis in small groups of animals after 8 weeks of treatment with MTX. Data clearly supporting the claim that MTX itself is responsible for hepatic fibrosis are lacking.

© 1986 Society for Experimental Biology and Medicine.

---

Methotrexate (MTX) is a folate antagonist with clinical utility in the treatment of neoplastic disorders, rheumatologic diseases, and psoriasis (1, 2). However, MTX treatment has been implicated in the pathogenesis of hepatic fibrosis. Originally described in children with leukemia (3, 4), this association between MTX and hepatic fibrosis has been best studied in patients with psoriasis. Although some authors have reported the development of hepatic fibrosis in patients on MTX treatment (4-8), others have not confirmed this association (9-12). Other factors, including alcohol intake and the underlying illness itself, may have contributed to the hepatotoxicity observed (5, 8, 9, 13).

Although guidelines concerning MTX therapy for psoriasis have been recommended (14), no evidence showing that MTX affects hepatic collagen metabolism is available. An animal model would allow us to study the actions of MTX on hepatic collagen production independently of the other factors present in

patients. However, no such animal model has been developed.

Custer *et al.* (15) administered varying doses of MTX to rats for periods of up to 24 months and found fatty infiltration of the liver as well as a significant increase in hepatic triglyceride content when compared to control animals. These workers also noted that an unspecified number of rats given MTX had hepatic fibrosis, although the authors felt that this usually was due to "collapse." In a follow-up study this group reported that ~20% of MTX-treated rats developed hepatic fibrosis (16). These experiments were not designed to study hepatic fibrosis primarily, and no specific conclusions relating to MTX's effect on hepatic fibrogenesis were reached. It also should be pointed out that histologic examination is an insensitive method of following the development of hepatic fibrosis. Animal models of hepatotoxicity have revealed that increased fibrogenesis may be detected biochemically by light microscopy even when fibrosis is lacking (17, 18).

Since no further experimental data regarding MTX's effect on hepatic fibrogenesis are available, the question of whether MTX itself induces hepatic fibrosis remains unsettled. Therefore, we initiated studies to clarify the

---

<sup>1</sup> This study was supported by the Research Service, Veterans Administration. Loren Laine was supported by a National Institutes of Health Training Program Grant (AM-07202).

role of MTX in hepatic collagen production in rats.

**Materials and Methods.** [ $^3\text{H}$ ]proline (19 mCi/ $\mu\text{mole}$ ), DL-2-hydroxy[2- $^{14}\text{C}$ ]proline (15 mCi/mole), and ACS scintillation fluid were obtained from Amersham/Searle Corporation (Arlington Heights, Ill.); [ $^3\text{H}$ ]toluene and [ $^{14}\text{C}$ ]toluene standards and [*methyl*- $^{14}\text{C}$ ]collagen (9.9  $\mu\text{Ci}/\text{mg}$ ) were purchased from New England Nuclear (Boston, Mass.). Sources of other chemicals were AG50W-X8 resin (100–200 mesh) from Bio-Rad Laboratories (Richmond, Calif.); L-proline, hydroxy-L-proline, L-aspartic acid, Trizma buffer solution, and trichloroacetic acid from Sigma Chemical Company (St. Louis, Mo.); methanol (glass distilled) and chloroform (glass distilled) from EM Science (Gibbstown, N.J.); ethyl ether anhydrous from Mallinkrodt (Paris, Ky.); and sodium citrate buffers for amino acid analysis from Benson Company (Reno, Nev.). Methotrexate sodium from Lederle Parenterals, Inc. (Carolina, Puerto Rico) was used. Male Fischer rats (strain F344) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.).

**Animals.** Animals weighing approximately 80 g received one of four experimental regimens: (i) control—normal saline (volume equal to that of Group iii) ip qd (“qd” defined as 5 consecutive days per week); (ii) MTX, 250  $\mu\text{g}/\text{kg}$  ip qd; (iii) MTX, 400  $\mu\text{g}/\text{kg}$  ip qd; (iv) MTX, 600  $\mu\text{g}/\text{kg}$  ip qod (“qod” defined as every other day). Dosages were chosen in an attempt to induce maximal hepatotoxicity without mortality in the first 2 months of drug administration based on the data of Custer *et al.* (15). MTX-induced hepatotoxicity, as measured by hepatic triglyceride content, has been shown to be dose-dependent and reproducible (15, 16). Animals were weighed each week and doses adjusted accordingly. The rats were allowed free access to standard laboratory chow and water. The animal facility was temperature and humidity controlled.

Animals receiving 400  $\mu\text{g}/\text{kg}$  qd and 600  $\mu\text{g}/\text{kg}$  qod began to die after 8 weeks of treatment. Therefore, the rats were sacrificed at 8 weeks of therapy.

**Determination of de novo collagen production.** Twenty-four hours prior to sacrifice the animals received an intraperitoneal injection of 100  $\mu\text{Ci}$  of [ $^3\text{H}$ ]proline in 0.5 ml of sterile

saline. After sacrifice by ether overdose the livers were removed, washed in ice-cold 0.05 *M* Tris/0.11 *M* NaCl, pH 7.6, dried on filter paper, and frozen at  $-20^\circ\text{C}$ .

Portions of liver (approximately 3 g) were homogenized at  $4^\circ\text{C}$  in 0.05 *M* Tris, pH 7.6. Trichloroacetic acid (TCA) was added to yield a final concentration of 100 mg/ml. The samples were centrifuged at 1000*g* for 10 min at  $4^\circ\text{C}$ , and the supernatants were collected and stored at  $4^\circ\text{C}$ . The precipitates were resuspended in TCA (50 mg/ml) and collected by centrifugation (19, 20).

Collagen extraction was then undertaken by resuspension of the precipitate with TCA (50 mg/ml) followed by heating at  $90^\circ\text{C}$  for 1 hr (21). The samples were centrifuged at 500*g* for 5 min, and the supernatant was collected. The precipitate was resuspended in TCA (50 mg/ml), again heated at  $90^\circ\text{C}$  for 1 hr, and the supernatant added to the former collection. The precipitate was stored at  $-20^\circ\text{C}$ .

Two volumes of ether was added to the supernatant, and the solution was mixed at 100 oscillations/min for 5 min. The upper layer (TCA and ether) was removed and the procedure was repeated two times. The lower, aqueous layer was heated in a  $50^\circ\text{C}$  water bath for 2 min in order to evaporate any residual ether and then lyophilized.

The extract was hydrolyzed with 6 *N* HCl at  $120^\circ\text{C}$  for 3 hr in order to release [ $^3\text{H}$ ]HYP (19). There was a negligible amount of tritium exchange during the acid hydrolysis, as previously described (22, 23). Activated charcoal was added, and the supernatant was collected after centrifugation and washing with distilled water. The solution was then filtered, lyophilized, and reconstituted with 325  $\mu\text{l}$  of 0.2 *N* sodium citrate, pH 2.2. Recovery of [ $^{14}\text{C}$ ]collagen internal standards by this method of extraction was 90%.

[ $^3\text{H}$ ]HYP was eluted on a 119 Beckman amino acid analyzer as previously described (22). Fractions were counted by liquid scintillation spectrometry. The amino acid analyzer yielded an 82% recovery of [2- $^{14}\text{C}$ ]HYP. No radioactivity was detected in the region in which *cis*-HYP elutes. The protein-bound [ $^3\text{H}$ ]HYP was used as a measure of collagen.

The noncollagen protein precipitates were dissolved in 0.3 *N* NaOH, and the radioactivity was counted and expressed as disintegrations

per minute per gram of liver. Ninety-five percent of the protein-bound [ $^3\text{H}$ ]proline was present in the noncollagen protein fraction, while 5% was in the hot-TCA-extractable fraction.

The radioactivity in collagen and noncollagen proteins was used to calculate the relative collagen production (19, 20):

$$\frac{\text{dpm in collagen}}{\text{dpm in noncollagen protein}} \times 5.4 + \text{dpm in collagen}$$

*Specific radioactivity in the free proline pool.* The TCA-soluble fractions of liver homogenates were desalted on a 1.5-ml AG50 column, and the proline eluted with 4 ml of 6 N HCl. The eluant was lyophilized and reconstituted with 400  $\mu\text{l}$  of 0.2 N sodium citrate, pH 2.2. Separate portions of the solutions were used for quantitation of proline by ninhydrin reaction on the amino acid analyzer and for measurement of radioactivity. The absolute amount of collagen production was calculated as follows (19, 20):

$$\frac{\text{radioactivity in collagen (dpm)}}{\text{proline specific activity (dpm/nmole)}}$$

This value was expressed as nanomoles of protein-bound HYP formed per gram of liver. The absolute noncollagen protein production was calculated similarly from the radioactivity in noncollagen protein and the specific activity of the free proline pool. This value was expressed as nanomoles of proline incorporated into noncollagen protein per gram of liver.

*Unlabeled collagen content in liver.* The HYP content of the hydrolyzed hot-TCA-extractable fraction was determined by the ninhydrin reaction in the amino acid analyzer (22). The value of unlabeled collagen content was expressed as nanomoles of HYP per gram of liver.

*Triglyceride content.* Triglyceride content was determined by a modification of the methods of Folch *et al.* (24). Portions of liver (approximately 0.5 g) were weighed and homogenized with a 2:1 chloroform-methanol mixture to a final dilution 20 times the volume of the liver sample. After 1 hr the crude extract was mixed thoroughly with 0.2 vol water, and the mixture was allowed to separate into two phases over 15 hr at room temperature. The

lower phase was removed and dried in a 37°C water bath under nitrogen. Triglyceride content was measured on a Technicon Autoanalyzer II (25). The extract was reconstituted with isopropanol and hydrolyzed to free glycerol. Glycerol was oxidized to formaldehyde, coupled with acetylacetone to give the fluorescent product 3,5-diacetyl-4-dihydroxylutidine, and measured fluorometrically. Values were expressed as milligrams of triglyceride per gram of liver.

*Statistics.* All the results were expressed as means  $\pm$  SE. A two-tailed, unpaired Student *t* test was used to evaluate the differences of the means between groups, accepting  $P < 0.05$  as significant.

**Results.** The unlabeled hepatic collagen content was essentially the same in the control and MTX treatment groups (Table I). Thus, no net deposition of collagen occurred in the liver after 2 months of MTX administration. We then studied the effect of MTX on hepatic *de novo* collagen production, a more sensitive parameter of fibrogenesis.

The collagen production relative to total protein production was similar in the control animals and in the MTX treatment groups (Table II). Expressing collagen production in relation to total protein production eliminates consideration of changes in amino acid transport or other factors that could modify the specific activity of the free proline pool. How-

TABLE I. NONRADIOACTIVE HEPATIC COLLAGEN CONTENT IN METHOTREXATE-TREATED RATS

Experimental <sup>a</sup> group	Collagen <sup>b</sup> (nmole/g liver)
Control ( $n = 5$ )	500 $\pm$ 40
MTX, 250 $\mu\text{g}/\text{kg}$ qd ( $n = 4$ )	520 $\pm$ 60
MTX, 400 $\mu\text{g}/\text{kg}$ qd ( $n = 3$ )	510 $\pm$ 50
MTX, 600 $\mu\text{g}/\text{kg}$ qod ( $n = 2$ )	480 (110)
MTX, all doses ( $n = 9$ )	510 $\pm$ 30

*Note.* Results are means  $\pm$  SE. Values shown in parentheses are one-half the range. The differences between groups are not statistically significant.

<sup>a</sup> Same as those described under Materials and Methods.

<sup>b</sup> Protein-bound hydroxyproline was determined by amino acid analysis of the hydrolyzed hot-TCA-extractable fraction.

TABLE II. RELATIVE HEPATIC COLLAGEN PRODUCTION IN METHOTREXATE-TREATED RATS

Experimental <sup>a</sup> group	Collagen <sup>b</sup>	Noncollagen <sup>c</sup>	Relative collagen <sup>d</sup>
Control (n = 5)	2.2 ± 0.7	1.4 ± 0.4	2.6 ± 0.3
MTX, 250 µg/kg qd (n = 3)	1.9 ± 0.2	1.8 ± 0.2	1.9 ± 0.1
MTX, 400 µg/kg qd (n = 4)	2.2 ± 0.3	2.0 ± 0.1	2.1 ± 0.4
MTX, 600 µg/kg qod (n = 2)	1.9 (0.3)	1.3 (0.2)	2.7 (0.8)

*Note.* Results are means ± SE. Values shown in parentheses are one-half the range. The differences between groups are not statistically significant.

<sup>a</sup> Animals were injected with 100 µCi of [5-<sup>3</sup>H]proline in 0.5 ml of sterile saline ip 24 h prior to sacrifice.

<sup>b</sup> Determined as protein-bound [<sup>3</sup>H]hydroxyproline in the hot-TCA-extractable fraction and expressed as 10<sup>3</sup> × dpm/g liver.

<sup>c</sup> Determined as <sup>3</sup>H radioactivity in the TCA-nonextractable fraction and expressed as 10<sup>6</sup> × dpm/g liver.

<sup>d</sup> Calculated from the <sup>3</sup>H radioactivity in collagen and noncollagen protein as described under Materials and Methods and expressed as 10<sup>-2</sup>%.

ever, alterations in collagen production could be masked by parallel changes in noncollagen protein production. To eliminate this possibility the absolute protein production was determined using the specific activity of [<sup>3</sup>H]proline in the free amino acid pool.

No significant differences were present among the specific activities of the free [<sup>3</sup>H]proline pool in control and MTX-treated animals (Table III), suggesting that MTX did not affect free proline transport or metabolism. Although some studies have suggested that measurement of the aminoacyl-tRNA pool is a more accurate method of determining precursor specific activity (26), at least for leucine (27, 28), there is much convincing evidence to the contrary. After the intravenous injection of [<sup>14</sup>C]leucine to both control and ethanol-

fed rats, the leucine specific activities were similar in the free hepatic pool and hepatic rRNA (29). Also, it has been shown that, for various rat tissues labeled with several different amino acids, the specific activities in the free amino acid pool and in specific proteins were almost identical (30, 31). The absolute *de novo* collagen production was not increased in livers from animals treated with MTX (Table III). Calculation of absolute noncollagen protein production also revealed no significant differences among the groups (Table III).

We have shown that weight loss is correlated with a selective decrease of hepatic collagen production in the rat (32). Therefore, we compared the weight changes in control and treatment groups over the entire 8-week period of study as well as during the final 48 hr (*de novo*

TABLE III. FREE PROLINE SPECIFIC ACTIVITY AND ABSOLUTE HEPATIC PROTEIN PRODUCTION IN METHOTREXATE-TREATED RATS

Experimental <sup>a</sup> group	Free [ <sup>3</sup> H]proline <sup>b</sup> specific activity (dpm/nmole)	Collagen <sup>c</sup> (nmole/g liver)	Noncollagen <sup>d</sup> (µmole/g liver)
Control (n = 5)	70 ± 22	31 ± 3	23 ± 3
MTX, 250 µg/kg qd (n = 3)	75 ± 3	25 ± 2	25 ± 1
MTX, 400 µg/kg qd (n = 4)	75 ± 6	30 ± 3	27 ± 3
MTX, 600 µg/kg qod (n = 2)	53 (3)	36 (8)	25 (2)
MTX, all doses (n = 9)	70 ± 2	30 ± 2	26 ± 1

*Note.* Results are means ± SE. Values shown in parentheses are one-half the range. The differences between groups are not statistically significant.

<sup>a</sup> Animals were injected with 100 µCi of [5-<sup>3</sup>H]proline in 0.5 ml of sterile saline ip 24 hr prior to sacrifice.

<sup>b</sup> Proline was purified by ion-exchange chromatography of the acid-soluble fraction of liver homogenates. Separate portions were used for quantitation of proline on amino acid analyzer and for measurement of radioactivity.

<sup>c</sup> Calculated from the protein-bound [<sup>3</sup>H]hydroxyproline and the specific activity of the free proline pool.

<sup>d</sup> Calculated from the <sup>3</sup>H radioactivity in noncollagen protein and the specific activity of the free proline pool.

collagen production was determined in the last 24 hr of life). No significant differences were found in the weight changes over 8 weeks (control,  $220 \pm 11\%$  vs MTX, all doses,  $204 \pm 7\%$ ) or over the last 48 hr of life (control,  $0.7 \pm 0.4\%$  vs MTX, all doses,  $-0.5 \pm 1.1\%$ ).

In summary, there was no significant difference in any measurement of hepatic collagen metabolism when comparing the control group with each of the MTX groups ( $250 \mu\text{g}/\text{kg}$  qd,  $400 \mu\text{g}/\text{kg}$  qd,  $600 \mu\text{g}/\text{kg}$  qod). Nor did any significant differences exist among the three MTX treatment groups. Because of this lack of dose response to MTX we combined the three groups given MTX for comparison with the control group. Once again, no measurement of hepatic collagen metabolism was significantly different in the control and MTX groups (Tables I and II).

An elevation in the free proline pool has been associated with increased hepatic collagen content in human cirrhosis (33) and has been reported to stimulate collagen synthesis in the rat liver (34), although conflicting results also have been presented (35, 36). Measurement of the free proline pool in our animals revealed an increase in each MTX treatment group when compared to the control group. These increases did not reach statistical significance. However, when the MTX-treated animals were combined, the free proline pool was significantly greater in the MTX treatment group ( $283 \pm 19$  vs  $198 \pm 24$  nmole proline/g liver,  $P < 0.02$ ). No evidence of an increase in collagen production was found in spite of this MTX-induced rise in free hepatic proline pool. Thus, our data fail to support the association between free proline and collagen production in the rat liver.

As expected, measurement of hepatic triglyceride did reveal a significant elevation in the MTX-treated animals ( $7.9 \pm 3.1$  vs  $4.8 \pm 1.0$  mg triglyceride/g liver,  $P < 0.05$ ), confirming past observations of this MTX effect (15, 16).

**Discussion.** MTX treatment has been associated with hepatic fibrosis and cirrhosis in the human (4–8). However, whether MTX is responsible for this effect remains to be established. We found no evidence of increased collagen production or increased collagen content in the livers of rats treated with MTX. A hepatotoxic effect of MTX in the rat was

reconfirmed in the present study: The liver triglyceride content was significantly elevated in rats treated with MTX when compared with that of the control group. Others have noted an elevation in triglyceride content and pronounced fatty infiltration in the livers of rats given chronic MTX treatment (15, 16). Although the increased triglyceride content may be a precursor to the later development of fibrosis, sensitive methods of measurement detected no increase in hepatic collagen production or collagen content in rats treated with MTX. The hepatic collagen content has been reported to increase in alcohol-induced fatty liver in rats, in the absence of fibrosis (17, 18). Presumably, we would have detected a similar effect of MTX in our animals, if present. It is conceivable that total collagen content only increases after MTX treatment for periods greater than 2 months. However, the determination of *de novo* hepatic collagen production measures collagen production over 24 hr in response to a single dose of MTX. The lack of stimulation of *de novo* hepatic collagen production by MTX argues strongly against an effect of MTX on hepatic fibrogenesis. Of course, we cannot completely exclude the possibility of an alteration in the effect of MTX on *de novo* collagen production when MTX is administered for even longer periods of time.

What are the possible explanations for the absence of MTX-induced fibrosis in the rat liver? Other factors may play a major role in MTX-associated hepatic fibrosis in man. A significant number of patients with psoriasis have abnormalities noted on liver biopsy before MTX treatment (13, 37–40). Leukemic children also frequently have evidence of liver pathology prior to chemotherapy (41). Thus, the underlying disease for which MTX is used may contribute to the development of hepatotoxicity. Alcohol intake repeatedly has been postulated to contribute to the hepatic fibrosis seen with MTX treatment in psoriasis (5, 8, 9, 13). Other, less well-characterized factors may also be involved in the induction of hepatic fibrosis in MTX-treated patients.

MTX-induced hepatic fibrosis could be a species-specific effect not seen in the rat. A similar difference among species may exist in alcohol-fed animals. Baboons fed ethanol for a prolonged period developed steatosis followed later by fibrosis on serial liver biopsies

(42). Chronic ethanol feeding in monkeys also led to fatty infiltration but caused no alteration in hepatic collagen metabolism or deposition (43).

Barak and Kemmy (44) reported that hepatic levels of betaine, an oxidative product of choline used as a secondary source of methyl groups in methionine metabolism, are decreased by MTX treatment in the rat. MTX inhibits the production of tetrahydrofolate and tetrahydrofolate cofactors which function as primary sources of methyl groups. The authors suggest that betaine may compensate for the MTX-induced reduction in tetrahydrofolate cofactors in the rat. Since the rat liver contains high levels of choline oxidase activity (45) for betaine production, Barak and Kemmy further postulate that the rat may be more resistant to the hepatotoxicity of MTX than other species, including man, which possess much less hepatic choline oxidase activity. However, the MTX-induced decrease in betaine could be considered paradoxical in the face of high choline oxidase activity in the rat, and any relationship between hepatic betaine levels or choline oxidase activity and hepatic fibrogenesis is highly speculative.

Data clearly supporting the claim that MTX itself is responsible for hepatic fibrosis are lacking. Confirmation of MTX-induced hepatic fibrosis will require evidence of increased hepatic fibrogenesis in a MTX-treated animal model, thereby isolating the MTX effect and removing potentially confounding factors. Such a model has been established for alcohol-induced liver fibrosis (17, 18, 42).

The present study provides the first assessment of hepatic collagen metabolism in MTX treatment. We found no alteration in hepatic collagen production or content in small groups of animals after 8 weeks of treatment with MTX. MTX clearly requires study in other species, such as subhuman primates, in order to characterize better its hepatic effect.

We are grateful to Dr. William G. M. Hardison for his critical review of this manuscript. We thank J. Juliano for his assistance with triglyceride determinations and Gary Deming for his skillful preparation of this manuscript.

- 
1. Bleyer WA. Methotrexate: Clinical pharmacology, current status and therapeutic guidelines. *Cancer Treat Rep* **4**:87-101, 1977.
  2. Willkens RF, Watson MA. Methotrexate: A perspec-

3. Colsky J, Greenspan EM, Warren TN. Hepatic fibrosis in children with acute leukemia after therapy with folic acid antagonists. *Arch Pathol* **59**:198-205, 1955.
4. McIntosh S, Davidson DL, O'Brien RT, Pearson HA. Methotrexate hepatotoxicity in children with leukemia. *J Pediatr* **90**:1019-1021, 1977.
5. Nyfors A, Poulsen H. Liver biopsies from psoriatics related to methotrexate therapy 2: Findings before and after methotrexate therapy in 88 patients; A blind study. *Acta Pathol Microbiol Immunol Scand Sect A* **84**:262-270, 1976.
6. Nyfors A. Liver biopsies from psoriatics related to methotrexate therapy 3: Findings in post-methotrexate liver biopsies from 160 psoriatics. *Acta Pathol Microbiol Immunol Scand Sect A* **85**:511-518, 1977.
7. Robinson JK, Baughman RD, Auerbach R, Cimisi RJ. Methotrexate hepatotoxicity in psoriasis. *Arch Dermatol* **116**:413-415, 1980.
8. Ashton RE, Millward-Sadler GH, White JE. Complications in methotrexate treatment of psoriasis with particular reference to liver fibrosis. *J Invest Dermatol* **79**:229-232, 1982.
9. Almeyda J, Barnardo D, Baker H, Levene GM, Landells JW. Structural and functional abnormalities of the liver in psoriasis before and during methotrexate therapy. *Brit J Dermatol* **87**:623-630, 1972.
10. Reese LT, Grisham JW, Aach RD, Eisen AZ. Effects of methotrexate on the liver in psoriasis. *J Invest Dermatol* **62**:597-602, 1974.
11. Shapiro HA, Trowbridge JO, Lee JC, Maibach HI. Liver disease in psoriatics—An effect of methotrexate therapy. *Arch Dermatol* **110**:547-551, 1974.
12. Zachariae H, Grunnet E, Sogaard H. Liver biopsy in methotrexate-treated psoriatics—A re-evaluation. *Acta Derm Venereol* **55**:291-296, 1975.
13. Lanse SB, Arnold GL, Gowans JDC, Kaplan MM. Low incidence of hepatotoxicity associated with long-term, low-dose oral methotrexate in treatment of refractory psoriasis, psoriatic arthritis and rheumatoid arthritis. *Dig Dis Sci* **30**:104-109, 1985.
14. Roenigk HH Jr, Maibach HI, Weinstein GP. Methotrexate therapy for psoriasis, guideline revisions. *Arch Dermatol* **108**:35, 1973.
15. Custer RP, Freeman-Narrodd M, Narrodd SA. Hepatotoxicity in Wistar rats following chronic methotrexate administration: A Model of human reaction. *J Natl Cancer Inst* **58**:1011-1015, 1977.
16. Freeman-Narrodd M, Narrodd SA, Custer RP. Chronic toxicity of methotrexate in rats: Partial to complete protection of the liver by choline: Brief communication. *J Natl Cancer Inst* **59**:1013-1017, 1977.
17. Mezey E, Potter JJ, Slusser RJ, Abdi W. Changes in hepatic collagen metabolism in rats produced by chronic ethanol feeding. *Lab Invest* **36**:206-214, 1977.
18. Feinman L, Lieber CS. Hepatic collagen metabolism: Effect of alcohol consumption in rats and baboons. *Science* **176**:795, 1972.

19. Peterkofsky B, Chojkier M, Bateman J. Determination of collagen synthesis in tissue and cell culture systems. In: Furthmayr H, ed. *Immunochemistry of the Extracellular Matrix*. Boca Raton, Fla., CRC Press, Vol 2:pp19-47, 1982.
20. Chojkier M, Spanheimer R, Peterkofsky B. Specifically decreased collagen biosynthesis in scurvy dissociated from an effect on proline hydroxylation and correlated with body weight loss. *J Clin Invest* 72:826-835, 1983.
21. Rojkind M. Inhibition of liver fibrosis by L-azetidine-2-carboxylic acid in rats treated with carbon tetrachloride. *J Clin Invest* 52:2451-2456, 1973.
22. Chojkier M, Bateman J, Phang JM, Peterkofsky B. Formation of proline metabolites in chick embryo bone: Interference with the measurement of free hydroxyproline by ion-exchange chromatography. *Anal Biochem* 120:330-338, 1982.
23. Chojkier M, Peterkofsky B, Bateman J. A new method for determining the extent of proline hydroxylation by measuring changes in the ratio of [4-<sup>3</sup>H]:[<sup>14</sup>C] proline in collagenase digests. *Anal Biochem* 108:385-393, 1980.
24. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497-509, 1957.
25. *Manual of laboratory operations, lipid research clinics program Volume 1: lipid and lipoprotein analysis*. Bethesda, Md., DHEW Publication No. (NIH) 75-628, 1975.
26. Hildebran JN, Airhart J, Stirewalt WS, Low RB. Prolyl-tRNA-based rates of protein and collagen synthesis in human lung fibroblasts. *Biochem J* 198:249-258, 1981.
27. Van Venrooij WJ, Moonen H, Van Loon-Klaassen L. Source of amino acids used for protein synthesis in HeLa cells. *Eur J Biochem* 50:297-304, 1974.
28. Schneible PA, Airhart J, Low RB. Differential compartmentation of leucine for oxidation and for protein synthesis in cultured skeletal muscle. *J Biol Chem* 256:4888-4894, 1981.
29. Baraona E, Leo MA, Borowsky SA, Lieber CS. Pathogenesis of alcohol-induced accumulation of protein in the liver. *J Clin Invest* 60:546-554, 1977.
30. Lofffield RB, Harris A. Participation of free amino acids in protein synthesis. *J Biol Chem* 219:151-159, 1956.
31. Fern EB, Garlick PJ. The specific radioactivity of the tissue free amino acid pool as a basis for measuring the rate of protein synthesis in the rat *in vivo*. *Biochem J* 142:413-419, 1974.
32. Flaherty M, Laine L, Chojkier M. Selective decrease of hepatic collagen production correlated with body weight loss in the rat. *Hepatology* 4:1064, 1984. [Abstract]
33. Kershenovich D, Fierro FJ, Rojkind M. The relationship between the free pool of proline and collagen content in human liver cirrhosis. *J Clin Invest* 49:2246-2249, 1970.
34. Tyopponen J, Forsander OA, Kulonen E. Influence of extracellular proline on collagen synthesis in rat liver slices. *Scand J Gastroenterol* 15:373-376, 1980.
35. Forsander OA, Pikkarainen JAJ, Salaspuro MP. A high hepatic concentration of free proline does not induce collagen synthesis in rat liver. *Hepatogastroenterology* 30:6-8, 1983.
36. Devenji R, Tiefenbach H, Orrego H, Varghese G, Israel Y. Does an excess in liver proline increase the accumulation of collagen induced by carbon tetrachloride? *Experientia* 35:1641-1642, 1979.
37. Berge G, Lundquist A, Rorsman H, Akerman M. Liver biopsy in psoriasis. *Brit J Dermatol* 82:250-253, 1970.
38. Weinstein G, Roenigk H, Maibach H, Cosmides J, Halprin K, Millard M. Psoriasis-liver-methotrexate interactions. *Arch Dermatol* 108:36-42, 1973.
39. Tobias H, Auerbach R. Hepatotoxicity of long-term methotrexate therapy for psoriasis. *Arch Intern Med* 132:391-396, 1973.
40. Nyfors A, Poulsen H. Liver biopsies from psoriasis related to methotrexate therapy 1: Findings in 123 consecutive non-methotrexate treated patients. *Acta Pathol Microbiol Immunol Scand [A]* 84:253-261, 1976.
41. Hutter RVP, Shipkey FH, Tan CTC, Murphy ML, Chowdhury M. Hepatic fibrosis in children with acute leukemia, a complication of therapy. *Cancer* 13:288-307, 1960.
42. Popper H, Lieber CS. Histogenesis of alcoholic fibrosis and cirrhosis in the baboon. *Amer J Pathol* 98:695-709, 1980.
43. Mezey E, Potter JJ, French SW, Tamura T, Halsted CH. Effect of chronic ethanol feeding on hepatic collagen in the monkey. *Hepatology* 3:41-44, 1983.
44. Barak AJ, Kemmy RJ. Methotrexate effects on hepatic betaine levels in choline-supplemented and choline deficient rats. *Drug-Nutr Interact* 1:275-278, 1982.
45. Sidransky H, Farber E. Liver choline oxidase activity in man and in several species of animals. *Arch Biochem Biophys* 87:129-133, 1960.

---

Received October 16, 1985. P.S.E.B.M. 1986, Vol. 182.  
Accepted February 18, 1986.