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The effect of paraquat on the incorporation of radiolabelled proline into acid-extractable lung proteins and collagens

F. M. ENGELBRECHT, M.-L. WINDT, E. MAREE, M. W. P. NIENABER

Summary

In this comparative study we describe the influence of paraquat on the rate of L-2,3-^{3}H-proline incorporation into the acid-extractable proteins and into newly synthesized protropocollagen molecules from the lungs of rats and rabbits. Exposure to paraquat took two forms: (*a*) addition of paraquat *in vitro* to lung tissue taken from rats and rabbits, and (*b*) intraperitoneal injection of paraquat prior to death.

Paraquat (0,5 - 1,0 mM) added *in vitro* significantly slowed the rates of ^{3}H-proline incorporation into the acid-extractable proteins and into newly synthesized protropocollagen in both rat and rabbit lung tissue.

Paraquat administered intraperitoneally (27 mg/kg) to rabbits did not markedly influence the rate of ^{3}H-proline incorporation into acid-extractable proteins and collagen assessed *in vitro* 24, 48 and 96 hours after injection.

Paraquat injected intraperitoneally into rats induced no significant difference in synthesis rates of acid-soluble proteins up to 48 hours after injection. During the same period, the collagen synthesis rate of rat lung tissue was reduced. At 96 hours an increase was found when the rate of synthesis was expressed as cpm/mg DNA and as cpm/μg hydroxyproline.

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MRC Lung Metabolism Research Group, Department of Medical Physiology and Biochemistry, University of Stellenbosch, Parowvallei, CP
F. M. ENGELBRECHT, D.SC.
M.-L. WINDT, B.SC.
E. MAREE, B.SC.
M. W. P. NIENABER, M.B. CH.B., M.MED. (ANAES.)

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Collagen has an important role in the development, structure and mechanical properties of lung tissue and in the pathogenesis of a wide spectrum of lung diseases, including fibrotic disorders.[1] Collagen is distributed throughout the lung, including the tracheobronchial tree, the blood vessels and the alveolar interstitium and constitutes about 20% of the dry weight of the adult human lung.[2] In the normal adult lung, collagen synthesis proceeds at a constant level, representing 4-5% of the total amino acid incorporation into lung proteins.[2] Collagen constitutes 10-15% of the proteins synthesized by cultures of rabbit lung cells and fibroblasts.

Five major types of collagen have been identified in lung tissue. It appears that almost all tissues contain several types of collagen, although in different proportions.[1] There are over 40 cell types in the lung and it is therefore extremely difficult to identify the types of collagen in lung tissue and specifically the cells responsible for synthesis of each type.[2] Fibroblasts are involved in the synthesis and secretion of type I and type III collagen: the latter accounts for a small proportion varying from 5% to 30% of the total secreted procollagen. Type II collagen is mostly produced by tracheal and bronchial chondrocytes. Smooth-muscle cells synthesize a higher proportion of type III compared with type I collagen than do fibroblasts. Small amounts of type V and a pepsin-resistant fragment, probably

derived from type IV, are also synthesized by smooth-muscle cells.[3] The latter two types of collagen are also synthesized by alveolar and capillary basement membranes.[3]

In fibrotic lung disorders, histological evidence suggests that there is either an overall abnormal accumulation of collagen or an accumulation in abnormal regions of the lung.[3,4] The accumulation of collagen could be due to an imbalance between synthesis and proteolysis; synthesis rates may be normal while proteolysis rates may be decreased, thus leading to collagen accumulation and lung dysfunction.[5] Recently, defects in the regulation of the types of collagen synthesized and in the enzymes involved in the post-translational modifications have been found in heritable diseases of connective tissue. However, increased synthesis rather than decreased degradation appears to be the critical factor leading to excessive accumulation of collagen in affected tissues.[6]

Pulmonary fibrosis therefore does not merely represent an acceleration of a normal synthetic process or an abnormal distribution of the collagen fibres, but the proportion of the various types of collagen is also altered.[7] For instance, in pulmonary fibrosis the relative content of type III collagen is markedly diminished and the degree of hydroxylation of lysyl residues in type I and type III collagen is reduced.[7] An immunofluorescence study confirmed a marked increase in type I and type V collagen in alveolar septa, whereas type III collagen was diminished.[3]

It is well known that animals surviving a LD_{50} dose of paraquat (PQ), a very toxic herbicide accumulated by active transport in lung cells, develop lung fibrosis in time.[8] Greenberg *et al.*[9] showed a marked increase in the rate of collagen synthesis in rat lung tissue as early as 2 days after the intraperitoneal injection of PQ (24 mg/kg body mass), accompanied by increased protein synthesis and a rise in the proline pool from day 3 onward. However, Kuttan *et al.*[10] found no change in the rate of collagen synthesis or in the total hydroxyproline content after intraperitoneal PQ administration (25 mg/kg). When PQ was added to the culture medium and incubated with lung slices, marked decreases in collagen and protein synthesis were found. There was also a concomitant decrease in activity of prolylhydroxylase, an enzyme necessary for the hydroxylation of proline, measured at 3 and 48 hours.[10]

Morphological evidence exists that PQ administered intravenously (20 mg/kg) into rats caused alveolar damage, haemorrhage and oedema within 24 hours, followed by consolidation of lung areas at 48-72 hours and some fibrosis between 7 and 10 days. Intravenous injection of PQ into rabbits, in doses up to 80 mg/kg, induced no characteristic lung lesions.[11]

In view of the controversy regarding the role of PQ in fibrogenesis, this investigation was undertaken to determine the effect of PQ added to tissue slices *in vitro* and after intraperitoneal injection of PQ into rats and rabbits respectively on acid-soluble protein and collagen biosynthesis in lung tissue.

Materials and methods

New Zealand White rabbits (1-2 kg) and Wistar Albino rats (250 ± 20 g) were used. The rats were anaesthetized by intraperitoneal injection of pentobarbitone sodium (50 mg/kg) and the rabbits by intravenous injection of thiopentone sodium (20 mg/kg).

The lungs were immediately perfused *in situ* with 15 ml (rat) and 50 ml (rabbit) ice-cold isotonic saline,w hile artificial ventilation was applied. The lungs were then removed and transferred into ice-cold phosphate-buffered saline (PBS) solution.

The major airways and blood vessels were removed and the peripheral lung was cut into smaller pieces, blotted and sectioned into 0,7 mm slices with a McIlwain tissue chopper. The slices were blotted and weighed accurately. Samples of 250 mg were used for incubation and also for dry-weight determination.

Two different procedures were used to assess the effects of PQ on the synthesis of (*a*) total acid-extractable protein, and (*b*) collagen.

Incubation medium

Incubation was performed in Dulbecco's modified Eagle's medium, to which was added 0,026M $NaHCO_3$, 0,7 mM ß-aminoproprionitrile, 0,5 mM ascorbic acid and 0,5 mM L-lysine (this medium contains no proline, making it suitable for isotope studies with this amino acid).[12] The medium was equilibrated beforehand with 95% O_2/5% CO_2 for 1 hour, during which time the pH was regularly adjusted to 7,4.

Pre-incubation

Samples of slices (250 mg each) were pre-incubated in 5 ml of the above medium for 1 hour at 37° C in metabolic flasks in a 95% O_2/5% CO_2 gas phase.[12]

Final incubation

After pre-incubation, the slices were filtered and transferred carefully to the original metabolic flasks. When total acid-extractable proteins were to be determined, 5 ml of the above medium, containing 18 µM cold proline plus radioactive L-2,3-^{3}H-proline (4 µl) (specific activity 23,7 Ci/mmol and 1 mCi/ml, giving about 1,75 x 10^6 cpm/5 ml, was added. The flask was then gassed for 15 seconds, stoppered and incubated for 4 hours at 37° C.

For collagen synthesis, 1 ml of medium containing L-2,3-^{3}H-proline (same specific activity as above), giving 3,5 x 10^6 cpm/ml, was used. The slices were incubated for 3 hours at 37° C.

Paraquat administration

To determine the *in vitro* effect of PQ on total acid-extractable protein synthesis, PQ was added to the final incubation medium of the experimental flasks to concentrations of 0,5 and 1,0 mM. For *in vitro* collagen synthesis the PQ concentration was 1,0 mM.

For the *in vivo* experiments, PQ dissolved in isotonic saline was administered intraperitoneally (27 mg/kg)[9,10] to rats and rabbits. After 24, 48 and 96 hours they were anaesthetized and lung samples were prepared as described above. Incorporation of ^{3}H-proline into the acid-extractable protein and collagen of lung slices from these animals was compared with the incorporation into lung slices from normal controls.

Extraction of protein and collagen

After incubation the slices were filtered and rinsed 3 times with 10 ml cold PBS at pH 7,4.[12] The slices were then transferred to 30 ml Corex tubes, containing 2,5 ml 0,5M acetic acid and homogenized in an Ultra Turrax for 1 minute. The tip of the homogenizer was washed with an additional 2,5 ml cold 0,5M acetic acid to give a final volume of 5 ml.

Extraction was carried out overnight (16 hours) at 4° C in a cold room, with continuous stirring. The tubes were centrifuged at 24 000 *g* for 20 minutes. The supernatant was decanted into clean Corex tubes and the pellet used for DNA determination.[13]

The extracted collagen and protein in the supernatant were precipitated by the addition of NaCl to a final concentration of 5%,[12] allowed to stand for 1 hour at 4° C, and centrifuged (24 000 *g* for 20 minutes). The supernatant was discarded, the pellet rinsed once with 5 ml 5% cold NaCl and pelleted again (24 000 *g* for 20 minutes).

When total acid-extractable proteins were determined, the pellets were washed 3 times with 10 ml 5% ice-cold NaCl and centrifuged at 24 000 *g*. Half of the washed pellets were hydrolysed in 1M NaOH at 37°C overnight and used for protein determinations.[14] The other pellets were hydrolysed in 0,5 ml 73% perchloric acid for 2 hours at 110°C.[15]

For collagen determinations, the pellets were dissolved in 5 ml 0,5M acetic acid and 5 mg pepsin was added to each pellet. Hydrolysis of the protein fraction of the pellet was carried out for 16 hours at 4°C, with continuous stirring. The remaining collagen fraction was precipitated with sodium chloride (final concentration 5%) at 4°C for 1 hour and centrifuged. The pellets were washed once with 10 ml cold 5% NaCl and centrifuged again (24 000 *g*). The supernatants were discarded and the pellets hydrolysed in 0,5 ml 73% perchloric acid for 2 hours at 110°C.[15]

The perchloric acid hydrolysates obtained with both procedures were neutralized by the addition of 0,5 ml 5M NaOH to each, and filtered into 5 ml volumetric flasks. The tubes were rinsed 3 times with 1 ml distilled water and the washings used to rinse the filter.

The flask was brought to volume with distilled water, mixed well and a 100 μl sample was transferred into counting vials containing 10 ml Instagel (Packard) for determining the radioactivity of the hydroxyproline in a Beckmann liquid scintillation counter with 40% counting efficiency.

Hydroxyproline, DNA and dry weight determination

Hydroxyproline was determined in the perchloric acid hydrolysate, according to the method of Baily,[15] and DNA was determined in the tissue pellets after acid extraction of the proteins, according to the method of Burton.[13] Dry weight was determined on slices from the perfused lungs, which were chopped with a McIlwain chopper into 0,7 mm slices, and blotted with Whatman No. 1 filter paper to absorb excess fluid. Accurately weighed 250 mg slices were separately transferred to preweighed watchglasses and dried in an oven for 48 hours at 110°C, or to a constant weight.

Each experiment was repeated 5 times in quadruplicate and standard methods were used to compute the mean and standard error of the mean. Pairwise comparisons were made, using a two-sided Student's *t* test.

Results

The results recorded here were obtained over 1 year. Since no specific-pathogen-free animals were available, the animals in each experiment were carefully matched for age, weight and general health.

In the first series of experiments, in which total acid-extractable protein synthesis was determined, two baselines were used to express the results, i.e. the total protein (protein plus collagen) extracted with 0,5M acetic acid and the hydroxyproline content of the same extract.

In Table I baseline values are recorded to demonstrate the effects of PQ *in vitro* (0,5 and 1,0 mM) and *in vivo* (27 mg/kg injected intraperitoneally 24 and 48 hours previously) on these parameters. The amount of acid-soluble protein (determined as

TABLE I. PROTEIN AND HYDROXYPROLINE VALUES OF THE ACID-SOLUBLE PROTEINS EXTRACTED FROM RABBIT AND RAT LUNG SLICES WITH 0,5M ACETIC ACID FOR 16 HOURS AT 4° C AFTER EXPOSURE TO PQ (0,5 - 1,0mM FOR 4 HOURS *IN VITRO*) AND 24 - 48 HOURS AFTER INTRAPERITONEAL INJECTION (27 mg/kg *IN VIVO*)*

		In vitro			*In vivo*		
		Control	0,5 mM PQ	1,0 mM PQ	Control	24 h	48 h
Rabbits	Protein mg/100 mg wet tissue	1,93 ± 0,17	1,96 ± 0,08 $P > 0,05$	1,82 ± 0,31 $P > 0,05$	1,81 ± 0,27	1,73 ± 0,27 $P > 0,05$	1,62 ± 0,24 $P > 0,05$
	Hydroxyproline μg/100 mg wet tissue	6,44 ± 0,24	6,70 ± 0,48 $P > 0,05$	5,86 ± 0,60 $P > 0,05$	6,63 ± 0,32	5,77 ± 0,46 $P > 0,05$	5,84 ± 0,36 $P > 0,05$
Rats	Protein mg/100 mg wet tissue	1,74 ± 0,15	1,67 ± 0,30 $P > 0,05$	1,65 ± 0,06 $P > 0,05$	1,75 ± 0,22	1,66 ± 0,37 $P > 0,05$	0,97 ± 0,13 $P < 0,05$
	Hydroxyproline μg/100 mg wet tissue	5,58 ± 0,41	5,19 ± 0,48 $P > 0,05$	4,92 ± 0,65 $P > 0,05$	4,84 ± 0,98	3,94 ± 0,28 $P > 0,05$	3,45 ± 0,32 $P > 0,05$

*Results are the mean of at least 5 experiments each consisting of quadruple estimations.

TABLE II. THE *IN VITRO* AND *IN VIVO* EFFECTS OF PQ ON THE BIOSYNTHESIS OF ACID-SOLUBLE PROTEINS BY LUNG TISSUE OF RATS AND RABBITS*

		In vitro			*In vivo*		
		Control	0,5 mM PQ	1,0 mM PQ	Control	24 h	48 h
Rabbits	cpm/100 mg wet tissue	22 615 ± 1 508	20 407 ± 1 537 $P < 0,05$	14 035 ± 1 378 $P < 0,01$	22 008 ± 2 028	17 064 ± 4 033 $P < 0,05$	15 298 ± 2 513 $P < 0,01$
	cpm/mg protein	11 757 ± 818	10 456 ± 954 $P > 0,05$	7 883 ± 1 489 $P < 0,01$	12 231 ± 789	9 818 ± 1 636 $P > 0,05$	9 498 ± 1 277 $P > 0,05$
	cpm/μg hydroxyproline	3 521 ± 325	3 047 ± 106 $P < 0,05$	2 408 ± 274 $P < 0,05$	3 326 ± 331	2 983 ± 781 $P > 0,05$	2 740 ± 736 $P > 0,05$
Rats	cpm/100 mg wet tissue	24 908 ± 690	16 625 ± 2 457 $P < 0,05$	14 393 ± 852 $P < 0,01$	24 384 ± 1 983	20 431 ± 1 084 $P > 0,05$	20 749 ± 613 $P > 0,05$
	cpm/mg protein	15 955 ± 1 925	10 029 ± 1 325 $P < 0,05$	8 746 ± 553 $P < 0,05$	14 188 ± 2 756	12 639 ± 2 352 $P > 0,05$	21 759 ± 3 249 $P > 0,05$
	cpm/μg hydroxyproline	4 475 ± 226	3 225 ± 582 $P < 0,05$	2 951 ± 333 $P < 0,01$	5 164 ± 1 020	5 194 ± 265 $P > 0,05$	6 089 ± 559 $P > 0,05$

*Results are the mean of at least 5 experiments each consisting of quadruple estimations.

protein) extracted with 0,5M acetic acid from rabbit lung did not differ significantly in the *in vitro* and *in vivo* experiments. In rats, however, the amount of proteins extracted from lung slices 48 hours after PQ administration was significantly lower than the control value. PQ had no significant effect on the hydroxyproline content of the extracted proteins from rat and rabbit lung tissue either *in vitro* or *in vivo*.

In Table II, the *in vitro* effects of PQ on the incorporation of radiolabelled proline into the acid-extractable proteins of lung slices over a 4-hour period are recorded. The results are expressed in terms of cpm/100 mg wet weight, cpm/mg protein and cpm/μg hydroxyproline. The effect of PQ (0,5 and 1,0 mM) on the rate of synthesis of acid-extractable proteins by rat and rabbit lung slices *in vitro* in terms of the above baselines is one of significant inhibition (except with 0,5 mM PQ in rabbit lung) when expressed in terms of cpm/mg protein.

The results obtained with lung slices from rats and rabbits exposed to a LD_{50} dose of PQ (27 mg/kg intraperitoneally) and expressed against the same baselines are confusing. In terms of cpm/100 mg wet weight of lung tissue, a significant inhibition was found after 24 and 48 hours in rabbit lung, whereas rat lung treated similarly showed no significant effect at either 24 or 48 hours. Judged by the cpm/mg acid-extractable protein and the cpm/μg hydroxyproline, PQ had no significant effect on the rate of protein synthesis by rat and rabbit lungs up to 48 hours after administration.

In the second series of experiments, the *in vitro* and *in vivo* effects of PQ were investigated on collagen biosynthesis only. The results are expressed in terms of mg DNA, mg dry weight and μg hydroxyproline (Table III).

The baseline parameters used are summarized in Table IV. In the *in vitro* experiments the baseline values of the experimental groups were not significantly different from the control values for both species. On the other hand, PQ injected intraperitoneally induced a significant increase in the DNA content of rabbit lung tissue after 96 hours. A significant increase also occurred in the hydroxyproline content after both 48 and 96 hours, whereas the dry weight values were not significantly affected. PQ injected intraperitoneally into rats caused no significant change in any of the abovementioned parameters at either 48 or 96 hours.

The rate of collagen biosynthesis by rat and rabbit lung *in vitro* was significantly reduced by 1 mM PQ (Table III), except when collagen synthesis by rabbit lung was expressed as cpm/μg hydroxyproline. The rate of collagen synthesis in the lungs of the two species showed different tendencies 48 and 96 hours after intraperitoneal PQ administration. The rate of collagen synthesis by rabbit lung tissue 48 hours after injection of PQ was not

TABLE III. *IN VITRO* AND *IN VIVO* EFFECTS OF PQ ON COLLAGEN BIOSYNTHESIS BY LUNG TISSUE OF RATS AND RABBITS*

		In vitro		*In vivo*		
		Control	1,0 mM PQ	Control	48 h	96 h
Rabbits	cpm/100 mg wet tissue	17 769 ± 1 372	14 217 ± 716 $P < 0,05$	16 821 ± 1 892	17 493 ± 3 759 $P > 0,05$	15 571 ± 1 719 $P > 0,05$
	cpm/mg DNA	30 325 ± 2 687	22 625 ± 1 452 $P < 0,01$	27 250 ± 3 700	26 250 ± 5 724 $P > 0,05$	21 375 ± 2 341 $P < 0,05$
	cpm/μg hydroxyproline	8 501 ± 1 408	6 949 ± 999 $P > 0,05$	6 834 ± 577	5 983 ± 534 $P > 0,05$	5 016 ± 342 $P < 0,01$
	cpm/mg dry weight	1 003 ± 85	804 ± 89 $P < 0,05$	952 ± 109	952 ± 194 $P > 0,05$	844 ± 80 $P > 0,05$
Rats	cpm/100 mg wet tissue	23 192 ± 3 074	16 736 ± 504 $P < 0,05$	23 548 ± 2 078	17 796 ± 985 $P > 0,05$	26 828 ± 3 339 $P > 0,05$
	cpm/mg DNA	42 825 ± 6 018	30 975 ± 2 609 $P < 0,05$	43 600 ± 5 543	32 875 ± 3 331 $P > 0,01$	58 850 ± 4 118 $P < 0,05$
	cpm/μg hydroxyproline	7 728 ± 1 384	5 325 ± 517 $P < 0,05$	8 399 ± 1 148	6 658 ± 479 $P < 0,05$	11 083 ± 423 $P < 0,05$
	cpm/mg dry weight	1 234 ± 162	897 ± 37 $P < 0,05$	1 332 ± 98	998 ± 37 $P < 0,05$	1 518 ± 161 $P > 0,05$

*Results are the mean of at least 5 experiments each consisting of quadruple estimations.

TABLE IV. DNA, HYDROXYPROLINE AND DRY WEIGHT VALUES OF LUNG TISSUE OF RATS AND RABBITS AFTER EXPOSURE TO PQ (1,0 mM FOR 3 HOURS *IN VITRO*) AND 48 - 96 HOURS AFTER INTRAPERITONEAL INJECTION (27 mg/kg *IN VITRO*)*

		In vitro		*In vivo*		
		Control	1,0 mM PQ	Control	48 h	96 h
Rabbits	μg DNA/100 mg wet tissue	588 ± 54	630 ± 36 $P > 0,05$	619 ± 17	659 ± 8 $P > 0,05$	729 ± 19 $P < 0,01$
	mg dry weight/100 mg wet tissue	17,7 ± 0,38	17,8 ± 1,13 $P > 0,05$	17,7 ± 0,15	18,4 ± 0,41 $P > 0,05$	18,5 ± 0,82 $P > 0,05$
	μg hydroxyproline/100 mg wet tissue	2,12 ± 0,23	2,08 ± 0,28 $P > 0,05$	2,47 ± 0,27	2,90 ± 0,36 $P < 0,05$	3,10 ± 0,19 $P < 0,05$
Rats	μg DNA/100 mg wet tissue	543 ± 37	543 ± 37 $P > 0,05$	543 ± 37	544 ± 40 $P > 0,05$	456 ± 52 $P > 0,05$
	mg dry weight/100 mg wet tissue	18,8 ± 0,14	18,7 ± 0,51 $P > 0,05$	17,7 ± 0,40	17,8 ± 0,62 $P > 0,05$	17,5 ± 0,52 $P > 0,05$
	μg hydroxyproline/100 mg wet tissue	3,04 ± 0,46	3,17 ± 0,19 $P > 0,05$	2,83 ± 0,29	2,68 ± 0,14 $P > 0,05$	2,42 ± 0,21 $P > 0,05$

*Results are the mean of at least 5 experiments each consisting of quadruple estimations.

significantly different from control values, whereas at 96 hours a decrease in the rate of collagen synthesis in terms of all baselines was found. This decrease was only significant when expressed as cpm/mg DNA or cpm/μg hydroxyproline.

Collagen synthesis was depressed 48 hours after exposure of rats to PQ *in vivo*, but this was only significant when expressed as cpm/μg hydroxyproline or cpm/mg dry weight. After 96 hours, collagen synthesis in rat lungs was increased, reaching significance when expressed in terms of cpm/mg DNA and of cpm/μg hydroxyproline. This increased rate of collagen synthesis in rat lungs 96 hours after PQ administration is in contrast to that found in rabbit lung in which an inhibitory tendency was found at this time interval.

Discussion

A great deal of controversy exists regarding the *in vivo* effects of PQ on collagen biosynthesis in the lungs of animals. Greenberg *et al.*[9] found substantial increases in the rate of collagen synthesis when the hydroxyproline content of trichloracetic acid-precipitable proteins from lungs exposed to PQ was measured. Autor and Schmitt,[16] however, found no significant change in the rate of collagen synthesis in terms of prolylhydroxylase activity. The results of Kuttan *et al.*,[10] who determined the ^{14}C-proline incorporated into collagenase-digestible proteins of lung tissue from animals exposed to PQ, are in agreement with those of Autor and Schmitt.[16]

Previously reported work[17] from this laboratory showed that PQ added *in vitro* in a concentration of 1,0 mM significantly inhibited protein biosynthesis by rat and rabbit lung slices. Paraquat injected intraperitoneally (27 mg/kg) also caused a significant inhibition of protein synthesis in both species after 24 hours, but after 48 hours the rate of protein synthesis was increased in rat lung tissue, whereas in rabbit lung it had no significant effect.

In the first series of our present experiments, we investigated the effect of PQ added *in vitro* and PQ injected intraperitoneally on the synthesis of acid-extractable proteins, i.e. proteins soluble in 0,5M acetic acid, in contrast with the experiment described in the previous paragraph where proteins soluble in low-ionic-strength buffers were extracted. Acetic acid extraction is generally used to dissolve the acid-soluble, newly synthesized proteins and protropocollagen quantitatively.[1,12] Only about 1,0% or less of the pre-existing tissue collagen is extracted with this solvent.[2] To ensure that no cross-linking and helix formation took place during incubation, ß-aminopropionitrile was included[12] in the medium and ascorbate was also provided for optimal hydroxylation of proline and lysine. With this extraction method for newly synthesized acid-soluble proteins, readily reproducible results were obtained. *In vitro* PQ in concentrations of 0,5 and 1,0 mM inhibited the incorporation of L-2,3-^{3}H-proline into newly synthesized proteins when expressed in terms of cpm/100 mg wet tissue and in cpm/mg acid-extractable protein and cpm/μg hydroxyproline of the extracted proteins (Table II). This finding is in general agreement with the observations of Kuttan *et al.*[10] who claimed that PQ inhibited not only newly formed proteins but also prolylhydroxylase activity.

Paraquat, 24 and 48 hours after intraperitoneal injection (27 mg/kg), had no significant effect on the ability of rabbit lung slices to synthesize acid-extractable proteins when results were expressed in terms of cpm/mg acid-extractable protein and cpm/μg hydroxyproline. However, rat lung slices showed a non-significant increase in the incorporation of radiolabelled proline 48 hours after exposure to PQ in terms of the same baselines.

A similar observation[16] was made regarding the *in vivo* effect of PQ injected 48 hours previously on the synthesis of proteins soluble in low-ionic-strength buffers. Since the acid-soluble proteins consist of protein and collagen, this increase might be due to an increase in synthesis rate of either the acid-extractable protein component or the collagen fraction. Judging by the hydroxyproline content of the PQ-treated lungs *in vitro* and *in vivo* (Table I) it would appear that PQ decreased the hydroxylation of the proline residues up to 48 hours in newly synthesized protropocollagen. This finding is supported by the results of Kuttan *et al.*,[10] which showed that prolylhydroxylase activity is inhibited by PQ. The PQ-induced inhibition of prolylhydroxylase could be due to an interaction between PQ and the added ascorbic acid in the presence of tissue cytochrome *c* by which PQ is kept in the reduced form while ascorbic acid is oxidized (unpublished data).

In the second series of experiments the effect of PQ on collagen synthesis only was investigated. To elucidate the effect of PQ administration on collagen synthesis, additional parameters, i.e. μg DNA/100 mg lung tissue and mg dry weight/100 mg tissue, was also estimated. To separate the collagen fraction from the other acid-soluble proteins extracted with 0,5M acetic acid, pepsin was added and hydrolysis of the protein fraction[3] allowed to proceed for 16 hours at 4°C with continuous stirring. Pepsin could only be used after the acid-soluble proteins had been precipitated and washed. If it is used simultaneously with the extraction process, the amount of collagen extracted in terms of hydroxyproline content is more than doubled, whereas the newly synthesized collagen (in terms of cpm) is halved (unpublished data). It would therefore appear as if pepsin, together with the lysosomal enzymes of the tissues, has a very drastic hydrolytic effect on the protropocollagen molecules.

It is well known that pepsin removes the non-helical telopeptides from the procollagen molecule, hydrolyses the pro-region of collagen precursors but leaves the triple helical region of the tropocollagen molecule intact.[2,3] With our procedure, reasonably reproducible results were obtained as regards the amount of collagen as well as the hydroxyproline content of the collagen separated by pepsin hydrolysis at 4°C.

In rabbit lung tissue a significant increase in DNA content and in hydroxyproline content of the extracted collagen was found 96 hours after exposure to PQ. This might be due to a limited cell mobilization at this stage because the PQ concentration is much lower and the retention time shorter in rabbit lung than in rat lung.[18] This observation could indicate that the lungs were at this stage already in a repair phase. In contrast with rabbit lung, no significant differences were found between the baseline values at the different time intervals after exposure of rats to PQ. It should also be stressed that rat and rabbit lung dry weights differed from those reported in the literature.[9] Although these lungs were oedematous, with haemorrhagic areas, the lung slices were blotted before the samples were weighed. With this technique, no significant difference in dry weight was found between control and experimental samples.

Table IV shows that adding 1,0 mM PQ *in vitro* inhibited collagen synthesis by rat and rabbit lung slices. This finding supports our observation regarding the effect of PQ on acid-extractable proteins in the previous experiment (Table II).

Injected intraperitoneally 48 hours previously, PQ had no clear-cut effect on the ability of rabbit lung tissue to synthesize collagen. After 96 hours there appeared to be an inhibition when collagen synthesis was expressed as cpm/mg DNA and as cpm/μg hydroxyproline but this apparent inhibition is due to the slight increase in the baseline parameters concomitant with the early repair phase in rabbit lung. It seems reasonable to conclude that PQ (27 mg/kg intraperitoneally) has no significant effect on collagen synthesis in rabbit lung tissue.

PQ inhibited collagen synthesis by rat lung tissue up to 48 hours after exposure. This inhibition is apparently due to retention of PQ, reaching a peak concentration at 30 hours in rat lung tissue.[16] The inhibitory effect observed at 48 hours was reversed to one of stimulation 96 hours after intraperitoneal administra-

tion. This increase in collagen synthesis in rat lungs appeared to be real, although it was partially due to a decrease in the baseline values of DNA and hydroxyproline at this stage. Greenberg *et al.*[9] found an increase in the rate of collagen synthesis by rat lung tissue from 2 days onwards whereas our results show a definite increase only from day 4. This increase could not be ascribed to a direct stimulatory effect of PQ on collagen synthesis, as was claimed by Greenberg *et al.*[9] The small change in the rate of collagen synthesis in lung tissue after intraperitoneal administration of PQ could be due to the fact that pulmonary fibrosis is a disorder associated with a change in the distribution, ultrastructural organization and proportion of the different types of collagen rather than an increase in the amount of collagen. It is still uncertain whether this rearrangement is related to an increase in type I collagen or to an accelerated degradation of type III collagen or to both.[7,19]

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News and Comment/Nuus en Kommentaar

Acute appendicitis

Acute appendicitis continues to present a problem not only for the family doctor but also for the hospital surgeon. This was once more confirmed by a recent thesis on acute appendicitis by R. Pieper of Stockholm, which is discussed in the *Tidsskrift for den Norske Laegeforening* (1982; **102:** 894) by K. Solheim. Pieper has analysed 1 018 patients with a primary appendicectomy performed in a Stockholm hospital. His analysis showed that the surgeon had made the correct diagnosis in only 68% of the cases and that the frequency of perforation was 20%. Perforation was much more common among two classes of patients, children and elderly people, and the author suggests that the disease may be more aggressive in these two age groups. In cases of perforation the patient had frequently been in hospital for over 6 hours before operation, usually because the diagnosis was still in doubt. Only 2 out of the 1 000 patients died; both of them were over 70 years old and died as a result of cardiovascular complications. A comparison of the microscopic findings with the surgical diagnosis showed that a false-positive diagnosis of acute appendicitis had been made in 6,9% of 659 cases and a false-negative one in 12% of 291 cases.

Although there were only 2 deaths, complications were not uncommon; infective complications were commoner than non-infective complications (11,5% *v.* 8,1%) and were of course commonest in cases where perforation had taken place. Pieper concluded that whenever acute appendicitis is either diagnosed or strongly suspected, operation should take place without delay.

The age of menarche

The general impression is that in developed countries girls menstruate earlier than they used to, although this has recently been questioned. However, from extensive data presented by Wyshak and Frisch (*N Engl J Med* 1982; **306:** 1033) it looks as if the onset of menarche is truly taking place earlier, but only in relation to acceleration in growth and height. Therefore, in the USA in 1877 the average age at menarche was 14,75 years, in 1900 about 14 years, and in 1947 it was down to 12,8 years. But there are limits to the effect of environmental factors, and it seems as if the age of menarche has levelled off at 12,8 years in American girls.

There is also evidence that menarche is delayed by undernutrition and strenuous physical activity. In Bangladesh, for example, the age of menarche is rising, and delay in the onset of menstruation in dancers and athletes has also been reported. It therefore seems unlikely that future generations of girls will become more precocious as the decades go by.