Table II—Blood Concentrations of W-1372 in Several Species after Intravenous Administration[a]

Time, min.	W-1372, mcg./ml.
Dog, 20 mg./kg.	
1	11.9
5	6.1
15	1.1
30	Trace
60	Trace
Squirrel Monkey, 25 mg./kg.	
1	28.9
10	3.9
30	2.0
Cebus Monkey, 25 mg./kg.	
1	9.2
10	3.8
30	Trace

[a] Values are the average of two animals. The amount of drug given is as indicated.

was determined by the relative peak area method, using dibutyl phthalate as the internal standard.

RESULTS AND DISCUSSION

W-1372 can be quantitated gas chromatographically when the relative peak area is used as an index of concentration. The relationship between relative peak area and drug concentration in the range of 1–10 mcg./ml. of plasma is illustrated in Fig. 2. The reproducibility of the procedure, as indicated by the standard error of quintuplicate determinations, is also shown in Fig. 2. The recovery of W-1372 was 98–99%, as shown in Table I.

The extraction procedure effectively separates W-1372 from normal interfering plasma constituents, since determinations in normal plasma of humans (Fig. 1), dogs, or monkeys give little or no blank (<0.1 mcg./ml.). The known major metabolites of W-1372—benzoic acid, hippuric acid, and *N*-γ-phenylpropyl-*N*-benzyloxyamine (3)—do not interfere.

The blood-depletion pattern of W-1372 following intravenous administration was studied in the dog, squirrel monkey, and Cebus monkey. The results (Table II) indicate that the drug is rapidly removed from the blood, with only trace amounts present after 30 min. in the dog and Cebus monkey and a low level present at this time in the squirrel monkey. A previous study (3) has shown that the plasma half-life of radioactivity following oral administration of W-1372-benzyl-^{14}C is 4 hr. in the squirrel monkey and 12 hr. in the dog.

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Mechanism of Action of Retinyl Compounds on Wound Healing I: Structural Relationship of Retinyl Compounds and Wound Healing

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Abstract □ Retinol, retinyl acetate, and retinoic acid promote wound healing. Retinoic acid is most active. Beta-carotene is active, while lycopene is inactive; β-ionone is active and α-ionone is inactive. For full activity, the compound should contain a β-ionone ring, a conjugated double-bond side chain, and a terminal carboxylic acid side chain.

Keyphrases □ Retinyl compounds, activity—structural relationship □ Wound healing—retinyl compound effect □ GLC—separation □ NMR spectroscopy—identity

In a previous report, it was shown that local application of retinol, retinyl acetate, or retinoic acid, dissolved in a nonionic base (NIB), promotes skin wound healing (1). Retinoic acid is relatively more effective than retinol or retinyl acetate. The fact that retinol, retinyl acetate, and retinoic acid are all effective when applied locally on the skin wound indicates that the primary alcohol group is not essential in promoting wound healing. It is of interest, therefore, to study the structural relationship of the other part of the retinol molecule for wound-healing activity. The compounds evaluated in this investigation are β-carotene, lycopene, β-ionone, and α-ionone.

Beta-carotene has the same ring structure and conjugated double-bond hydrocarbon side chain as retinol. It should show activity on wound healing as retinol. Lycopene has essentially the same structure as β-carotene but differs from the latter in not having the closed ring structure at either end of the molecule. Beta-ionone, on the other hand, has the same cyclohexene ring but does not have the same length of hydrocarbon side chain as retinol or β-carotene. Alpha-ionone differs from β-ionone by the position of the double bond in the cyclohexene ring and does not conjugate with the side chain. The structure–activity relationships of these compounds to wound healing are discussed.

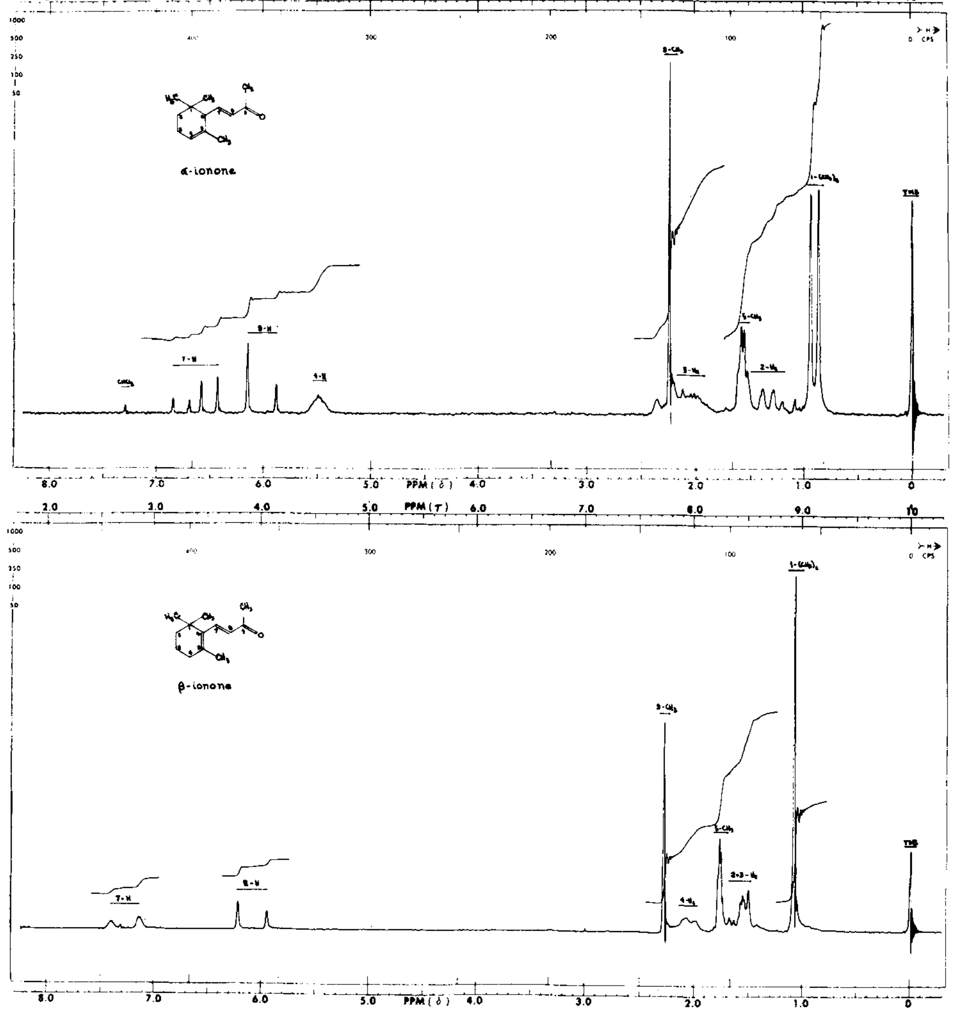

Figure 1—*NMR spectra of α- and β-ionones.*

EXPERIMENTAL

Materials and Chemicals—Synthetic crystalline, β-carotene, Sigma grade, Type 1, and crystalline lycopene, Blakeslea trispora origin, were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium salicylate and salicylic acid, reagent grade, were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. The prednisone used was a product of The Upjohn Co., Kalamazoo, Mich. Nonionic base and 1% hydrocortisone in NIB were prepared by the Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif. Beta-ionone (n_D^{20} 1.584) and α-ionone (n_D^{20} 1.5030), 77%, were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Purification of α-Ionone—The purities of the ionones were measured by their spectra (Analytical NMR Spectrometer, model A-60A, Varian Associates, Palo Alto, Calif.). Beta-ionone, as purchased, was a pure preparation, while α-ionone contained 31% of the β-isomer. The isomers were separated by using a preparative gas chromatograph (Varian Aerograph, model 700, Walnut Creek, Calif.). A FFAP column, 3.04 m. × 0.95 cm. (10 ft. × 0.37 in.), at 190° was used. The separation was complete and satisfactory. Both fractions collected were pure as measured by an NMR spectrometer. The NMR spectra are shown in Fig. 1.

Application of NIB Preparations—NIB preparations were applied, with gentle rubbing, directly on the sutured wound right after wounding. The application was repeated, once a day, on the 1st and 2nd days after wounding. For the control, only NIB was applied.

Administration of Drugs—Sodium salicylate, dissolved in a small amount of water and prednisone suspended in corn syrup, was fed to the rats daily for 4 days through a short stomach tube connected to a blunt hypodermic needle attached to a 50-ml. syringe, starting 1 day before operation. The dosage levels for sodium salicylate and prednisone were 50 and 2.5 mg. per rat per day, respectively.

Wound Procedure—Sprague-Dawley male rats, weighing 230–240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and underlying musculature at a distance about 1.5 cm. from the midline on each side; no ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread and a curved needle were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing (2). On the 7th day after wounding, the tensile strength of the wound was measured with a simple laboratory-made tensiometer. The tensiometer consisted of a 15.24 × 30.48-cm. (6 × 12-in.) board with one post, 10.16 cm. (4 in.) long, fixed on each side of the long ends. The board was placed at the end of a table. A pulley, with bearing, was mounted on the top of one post. A battery clamp, with 1-cm. clamp width, was tied to the tip of the post without a pulley by a piece of 20-lb. test monofilament fishing line so that the clamp could reach the middle of the board. Another battery clamp was tied to a long piece of fishing line with a 1-l. polyethylene bottle tied to the other end. Before testing, the animal was anesthetized with ethyl ether in an open mask. The sutures of the wound were cut out with a pair of scissors. The animal was then placed on a stack of paper towels on the middle of the board. The amount of the towels could be adjusted so that the wound was on the same level as the tips of the posts. The clamps were then carefully clamped on the skin at the

Table I—Structural Formulas of the Retinyl Compounds Studied

Compound	Formula
I. Retinol (vitamin A)	CH_3 CH_3 —CH=CHC=CHCH=CHC=CHCH$_2$OH
II. Retinyl acetate	CH_3 CH_3 O —CH=CHC=CHCH=CHC=CHCH$_2$OCCH$_3$
III. Retinoic acid	CH_3 CH_3 O —CH=CH—C=CHCH=CHC=CHCOH
IV. β-Carotene	CH_3 CH_3 CH_3 CH_3 —CH=CHC=CHCH=CHC=CHCH=CHCH=CCH= CHCH=CCH=CH—
V. Lycopene	CH_3 CH_3 CH_3 CH_3 —CH=CHC=CHCH=CHC=CHCH=CHCH=CCH= CHCH=CCH=CH—
VI. β-Ionone	CH_3 —CH=CHCO
VII. α-Ionone	CH_3 —CH=CHCO

opposite sides of the wound at a distance 0.5 cm. from the wound. The longer piece of fishing line was placed on the pulley, and the position of the board was adjusted so that the polyethylene bottle was freely hanging in the air. Water was added to the polyethylene bottle at a rapid but constant rate from a large reservoir (20-l. bottle) until the wound began to open. The amount of water in the polyethylene bottle was weighed and considered as the tensile strength of the wound. Two to three determinations were made on each wound. The mean of the determinations made on wounds on both sides of the animal was taken as the tensile strength of the wound.

RESULTS AND DISCUSSION

The structural formulas for the compounds are shown in Table I. The fact that retinol, retinyl acetate, and retinoic acid are all active in promoting wound healing indicates that the primary alcohol group is not essential (1). Beta-carotene, the natural precursor of retinol, has essentially the same trimethylcyclohexene ring and conjugated double-bond hydrocarbon side chain as retinol (Compound IV, Table I). The results of the effect of β-carotene on skin wound healing in rats are summarized in Table II. The mean tensile strength of the NIB control is 451 ± 9 g. (Group I). The mean tensile strength of Group II animals to which β-carotene in NIB was applied is 514 ± 7 g. The increase in tensile strength as compared with the control is 14%. The mean tensile strength of Group III animals receiving β-carotene in isopropyl myristate (IPM) is 515 ± 9 g., which is 14% higher than that of the control and is essentially the same as that of Group II animals. This also indicates that neither NIB nor IPM affects healing. The results from Group V and Group VII animals have definitely shown that topical application of β-carotene, like retinoic acid, reverses the healing-inhibitory action caused by oral administration of sodium salicylate or prednisone, as shown in Group IV and Group VI (1).

The effects of lycopene and ionones on wound healing are shown in Table III. Lycopene has essentially the same structure as β-carotene, except that it does not have the closed ring structure at either end of the molecule (Compound V, Table I). The mean tensile strength of Group II animals receiving 1% lycopene in NIB topically is 418 ± 5 g. It is clear that lycopene does not promote healing and, instead, it has a mild inhibitory effect.

Beta-ionone has essentially the same trimethyl cyclohexene ring structure as retinol or β-carotene, except that it does not have the same side chain (Compound VI, Table I). The mean tensile strength of the healing wound of animals receiving topical application of 1% β-ionone in NIB is 497 ± 11 g., which is 110% of the control (Group III). Beta-ionone is less active than β-carotene.

On the other hand, α-ionone, the isomer of β-ionone (the only difference being the position of the double bond in the trimethyl cyclohexene ring) does not have any activity in promoting healing. The mean tensile strength of the rats receiving 1% of α-ionone in NIB is 453 ± 6 g. (Group IV). It is interesting to point out the well-known fact that the α-isomer of retinol also does not have any vitamin A activity. The results from the foregoing structural relationship and

Table II—Effect of Topical Application of β-Carotene on Wound Healing

Group	No. of Animals	Drugs Given: Orally	Drugs Given: Topically	Mean Tensile Strength, g.	Percent Control
I	14	—	NIB	451 ± 9	100
II	16	—	1% β-Carotene in NIB	514 ± 7	114
III	10	—	1% β-Carotene in IPM[a]	515 ± 9	114
IV	8	NaSA	—	358 ± 10	79
V	8	NaSA	1% β-Carotene in IPM	478 ± 15	106
VI	7	Prednisone	—	346 ± 11	77
VII	9	Prednisone	1% β-Carotene in IPM	445 ± 11	99

[a] Isopropyl myristate.

Table III—Effect of Topical Application of Lycopene and Ionones on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g. ± *SE*
I	14	NIB Control	451 ± 9
II	8	1% Lycopene in NIB	418 ± 5
III	9	1% β-Ionone in NIB	497 ± 11
IV	8	1% α-Ionone in NIB	453 ± 6

wound-healing promotion activity studies indicate that β-ionone ring and conjugated double-bond hydrocarbon side chain and terminal carboxylic group are responsible for the full healing-promotion activity.

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Tetrameric Structure and Conformation of Heat-Microaggregated Human Serum Albumin

JOEL KIRSCHBAUM

Abstract □ ^{131}I-labeled heat-microaggregated human serum albumin is a colloid used in a method that measures liver blood flow through a determination of the rate of removal of microaggregates from the circulation. The heat-microaggregated material, either labeled or unlabeled, has a molecular weight (mol. wt.) of 273,000 daltons and a sedimentation coefficient of 8.6 *S*. Since the precursor, human serum albumin, has a molecular weight of 67,000 daltons and a sedimentation coefficient of 4.6 *S*, the microaggregate is a tetramer. The tetramer may be converted to subunits with a molecular weight of 72,000–76,000 daltons and a sedimentation coefficient of 4.7 *S* by the addition of buffered urea, guanidinium chloride, or formamide or by decreasing the pH to 2.2. Immunological studies indicate that this subunit has a different conformation from that of native human serum albumin. Hydrodynamic calculations indicate that the colloid has a particle size between 50 × 50 × 200 Å and 50 × 100 × 100 Å.

Keyphrases □ Albumin, human serum, heat-microaggregated—tetrameric structure, conformation, physical constants □ Human serum albumin, heat-microaggregated—structure, physical constants □ Ultracentrifugation—sedimentation coefficients □ Conformational studies—heat-microaggregated human serum albumin

Heating human serum albumin results, under certain conditions, in the formation of two protein aggregates (1, 2), made clinically useful by subsequent labeling with ^{131}I. One preparation, particulate in nature (2), is known as heat-macroaggregated human serum albumin. The other preparation is known as heat-microaggregated human serum albumin (3). The rate of disappearance of injected colloidal microaggregated particles from the circulation of mammals permits an estimation of the phagocytic capacity of the reticuloendothelial system, because this removal involves principally the Kupffer cells of the liver. Knowledge of phagocytic capacity appears to be useful in determining the extent of such diseases as pneumococcal pneumonia, typhoid fever, and Hodgkin's disease. This paper describes some physical properties of the metabolizable microaggregate, either unlabeled or labeled with ^{131}I.

EXPERIMENTAL

Source of Heat-Microaggregated Human Serum Albumin—The aggregation of human serum albumin (HSA) was performed according to the method of Iio and Wagner (3), as modified from the method of Benacerraf *et al.* (1). HSA at a concentration of 3% protein in 0.9% NaCl adjusted to pH 10 with NaOH was shaken vigorously for 20 min. at 70° and then for 15 min. at 79°. After rapid cooling, the precipitate that formed from the solution was resuspended in 0.1 *M* $NaHCO_3$. After storage, the precipitate dissolved and the solution of heat-microaggregated HSA was diluted to 10 mg./ml.

Sedimentation Coefficients—By the use of a Spinco model E analytical ultracentrifuge, the sedimentation coefficients of ^{131}I-labeled and unlabeled heat-microaggregated HSA at 20° and the viscosity and density of water, $s^0_{20,w}$ (4), were calculated at 29,500, 42,040, and 50,740 r.p.m. with 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85) as diluent. The viscosity was measured with the aid of either capillary or rotating (5) viscometers. A 10-ml. pycnometer at 20 ± 0.002° (Fisher Isotemp water bath) was used for density measurements. Viscosity and density corrections for aqueous solutions of urea and guanidinium chloride were also obtained from the data of Kawahara and Tanford (6).

Molecular Weights—The molecular weight was determined by two methods. The Archibald approach-to-sedimentation equilibrium method (7) was used with the modification of Engelberg (8) to evaluate the integral of the concentration gradient. The Yphantis meniscus-depletion (9) analyses were performed in a capillary-type double-sector cell at 20° at a speed of 20,410 r.p.m. The cell bottom was layered with FC-43 fluorochemical oil (Beckman Instruments), and 0.03 ml. of 0.1% dialyzed solution was layered over the oil. The solvent was the last dialysate.

Immunological studies utilized rabbit and horse antihuman serum albumin (Hyland Laboratories, Los Angeles, Calif.).

Polyacrylamide gel electrophoresis was performed with the Canalco model 6 apparatus (Canal Industrial Corp., Rockville, Md.).