[CONTRIBUTION FROM THE RADIOCARBON LABORATORY AND DIVISION OF ANIMAL NUTRITION, UNIVERSITY OF ILLINOIS]

Metabolism Studies with Radioactive Vitamin A[1]

BY GEORGE WOLF, SAMUEL G. KAHN AND B. CONNOR JOHNSON

RECEIVED JULY 9, 1956

Vitamin A was synthesized with C^{14} in carbon number 2.[2] Upon injection into rats, the radioactivity distribution in expired CO_2, organs and excreta was determined. The distribution of activity between vitamin A and its esters in liver was ascertained; no vitamin A aldehyde could be detected. A considerable fraction of radioactivity was found to be excreted as water-soluble compounds in urine. The principal one was characterized as an unconjugated ketoester.

Though one of the earliest vitamins discovered, very little is known about the biochemical function of vitamin A, apart from the process of vision. In the hope of obtaining some clue to its function, it appeared important to obtain information on its distribution, breakdown and transformation products in the intact animal. To that end, it was thought desirable to synthesize vitamin A labeled with radioactive carbon. Although fluorescence and spectrophotometric methods for investigating vitamin A distribution are exceedingly sensitive, labeled vitamin A extends the margin of detectability by several orders of magnitude, at least with the material here described. Using the same method of synthesis, more highly radioactive vitamin A could be made. Apart from distribution, transformation and breakdown of the molecule can be investigated best with the labeled compound.

Results and Discussion

Synthesis.—The method was adapted from that of Wendler, *et al.*,[3] and of Van Dorp and Arens.[4] Though the method of Isler, *et al.*,[5] appears to be more efficient, it involves purification of intermediates by fractional distillation, a process which is impracticable when dealing with the small quantities used in the radioactive synthesis.

β-Ionylidenecrotonic acid (obtained through courtesy of Dr. S. Rothchild, Tracerlab Inc., and of Dr. A. J. Aeschlimann, Hoffmann–La Roche Inc.) was converted to the C_{18}-ketone with methyllithium,[6] and made to react with methyl bromoacetate-2-C^{14} (obtained from Tracerlab, Inc.). The C_{20}-hydroxy ester so obtained was dehydrated with iodine and the resulting vitamin A acid ester saponified for further purification. Vitamin A acid was crystallized and assayed for per cent. carbon and radioactivity. The yield, based on methyl bromoacetate, was 8.7%. The crystalline acid was stored as such. Whenever a portion of radioactive vitamin A alcohol was required, the acid was converted to the ester, and reduced with lithium aluminum hydride.

The vitamin A so obtained was assumed to be in the all-*trans* form, on the basis of the observation of Robeson, *et al.*,[7] that the vitamin A acid prepared by the method of Van Dorp and Arens,[4] which is essentially the same as used in the present synthesis, was of that configuration.

Radioactivity Determinations.—Radioactivity was determined by combustion of samples to carbon dioxide in a Pregl microcombustion apparatus. The gas was collected in traps cooled with liquid nitrogen, the amount measured manometrically, thus giving a measure of the carbon content of the compound analyzed to within an accuracy of $\pm 0.3\%$, and then transferred to an ionization chamber. The radioactivity of the gas in the chamber was measured with a vibrating reed electrometer.[8] Rat tissues and excreta were converted to carbon dioxide by a wet combustion method using the Van Slyke–Folch combustion fluid.[9] For fractions eluted from column or paper chromatograms, an internal gas flow counter was used, with samples spread on planchets. A conversion factor from one to the other instrument was obtained by the use of a planchet containing a standard amount of radioactivity.

Distribution.—Radioactivity in expired carbon dioxide, after an intraperitoneal injection of 2.80 mg. of vitamin A-C^{14} in emulsion, is shown in Table I. The total activity expired was 5.2% of the injected dose in 24 hours. This is evidence of a small though significant breakdown of the vitamin. At least the two end carbon atoms of the side chain are split off, possibly as a two-carbon fragment in a manner similar to the breakdown of branched-chain fatty acids.[10] These fragments could, as methyl-labeled acetate, contribute to the carbon dioxide labeling, and the relatively high activity in fatty acids (Table II) (fatty acid activity of liver and intestine, 5.97% of dose). The rate of liberation of radioactive carbon dioxide was almost constant, in contrast to the large early burst of radioactivity usually obtained with water-soluble metabolites. This result suggests that either the transport of the compound to the metabolizing sites, or the actual breakdown reactions, or both, are slow and continuous over a prolonged period.

Table II shows the distribution of activity in different organs. As expected, a high percentage of activity resided in liver and carcass non-saponifiable fraction, shown to be due mainly to storage of

(1) This work was supported by the National Vitamin Foundation and the U. S. Atomic Energy Commission, Contract No. AT(11-1)-67 with the University of Illinois. Parts of it were presented at the Radioisotope Conference 1954, Oxford, England; and at the Thirty-ninth annual meeting of the Federation of American Societies for Experimental Biology, San Francisco, 1955.

(2) The numbering system used in this paper corresponds to that in "Chemical Abstracts, 1953 Subject Index."

(3) N. L. Wendler, H. L. Slates, N. R. Trenner and M. Tishler, THIS JOURNAL, **73**, 719 (1951).

(4) D. A. Van Dorp and J. F. Arens, *Rec. trav. chim.*, **65**, 338 (1946).

(5) O. Isler, A. Ronco, W. Guex, N. C. Hindley, W. Huber, K. Dialer and M. Kofler, *Helv. Chim. Acta*, **32**, 489 (1949).

(6) C. Tegner, *Acta Chem. Scand.*, **6**, 782 (1952).

(7) C. D. Robeson, J. D. Cawley, L. Weisler, M. H. Stern, C. C. Eddinger and A. J. Chechak, THIS JOURNAL, **77**, 4111 (1955).

(8) H. Palevsky, R. K. Swank and R. Grenchick, *Rev. Sci. Instr.*, **19**, 298 (1947).

(9) J. G. Burr, *Anal. Chem.*, **26**, 1395 (1954).

(10) B. K. Bachhawat, W. G. Robinson and M. J. Coon, *J. Biol. Chem.*, **219**, 539 (1956).

TABLE I

RADIOACTIVITY IN EXPIRED CARBON DIOXIDE

Dose of vitamin A-2-C^{14} injected intraperitoneally into a 205-g. male rat, 2.90 μc.; specific activity, 296 μc. per mmole

Time of collection, hr.	Activity, μc. $\times 10^{-2}$	Dose, %	Specific activity, μc./mmole $\times 10^{-4}$	Activity cumulative, μc. $\times 10^{-2}$	Dose cumulative, %
2	2.81	0.97	16.90	...	..
4	2.35	.81	12.70	5.16	1.78
6	1.98	.68	11.20	7.14	2.46
8	1.64	.57	8.81	8.78	3.03
10	1.22	.42	6.51	10.00	3.45
12	0.95	.33	5.37	10.95	3.78
16	1.58	.55	4.30	12.53	4.33
20	1.29	.45	3.50	13.82	4.78
24	1.22	.42	2.89	15.04	5.20

vitamin A and its esters. Kidney, on the other hand, had little radioactivity. The relatively large amount of radioactivity in the non-saponifiable fraction of skin was proved to be due to vitamin A. Since it could not be removed by washing with ligroin prior to saponification, it was assumed to be present in a bound form. Using classical methods, previous workers have not been able to detect the vitamin in epidermis.[11] It is known,[12] however, that local application of vitamin A to skin causes changes which suggest a reversal of epidermal keratinization, the latter being a condition of vitamin A deficiency. It seems therefore possible that skin requires a certain level of the vitamin bound to it. A very small though definite amount of activity could be detected in the eyes.

TABLE II

RADIOACTIVITY DISTRIBUTION

Duration of experiment: rat I, 24 hours; rat II, 14 hours. Weights: rat I, 205 g., rat II, 315 g. Vitamin A-C^{14} injected intraperitoneally: rat I, 2.80 mg. (2.90 μc.); rat II 4.14 mg. (4.28 μc).

	Rat I		Rat II	
	Activity, μc. $\times 10^{-2}$	Dose, %	Activity, μc. $\times 10^{-2}$	Dose, %
Urine, ligroin extract	0.058	0.02	3.895	0.91
water-soluble residue	33.943	11.67	20.373	4.76
Feces, non-saponifiable	0.667	0.23	0.000	0.00
saponifiable and water-soluble residue	64.235	22.15	0.000	0.00
Carcass, non-saponifiable	28.710	9.90	28.820	6.74
saponifiable and water-soluble residue	19.314	6.66	52.201 40.770	12.19 9.54
Liver, non-saponifiable	27.028	9.32	3.100	0.71
saponifiable	9.628	4.64		
water-soluble residue	3.335	1.61		
Intestine, non-saponifiable	6.206	2.14		
saponifiable	3.857	1.33	....	...
water-soluble residue	10.643	3.67		
Blood, non-saponifiable	0.145	0.05	....	...
saponifiable and water-soluble residue	0.435	0.15		
Kidney, non-saponifiable	1.102	0.38	0.813	0.19
Skin, non-saponifiable	5.626	1.94	1.284	0.30
Eyes, non-saponifiable	0.008	0.009	....	...

(11) H. Jeghers, *New England J. Med.*, **228**, 678 (1943).

(12) J. D. Sabella, H. A. Bern and R. H. Kahn, *Proc. Soc. Exp. Biol. Med.*, **76**, 499 (1951).

Earlier workers[13] had been unable to detect vitamin A excretion in rat urine. This fact is confirmed by the present investigation (Table II). The high level of radioactivity in the water-soluble breakdown products of vitamin A in urine was unexpected, although Prelog and co-workers[14] had found ionone derivatives excreted in pregnant mares' urine, which were presumed to be derived from vitamin A or carotene. Properties and characterization of the present urinary compounds will be described below. The rate of excretion of activity into the urine, and hence the amount of vitamin A metabolized to the urinary products, appears to be constant, at least for the two rats used in the present experiments, which were run for different times. From the activities in the urines (Table II), it is possible to calculate the amount of vitamin A-C^{14} converted per hour (rat I, 6.8 μg. per 100 g. body weight per hour; rat II, 5.4 μg./100 g./hour).

An unexplained discrepancy between the two rats was noted regarding their fecal excretion: rat II had no activity in feces, whereas rat I showed a high level of activity in the water-soluble feces fraction. This may have been caused by either a slow excretion rate of vitamin A or its metabolites into the gut (rat II was run for a shorter time), or by an inadvertent entry of a fraction of the vitamin into the gut of rat I during injection. The low activity in feces non-saponifiable fraction shows that vitamin A does not follow the metabolic pathway, from liver through bile to feces, that many fat soluble organic compounds take.

Distribution in Liver.—That liver acts as a storage organ for vitamin A, and that the larger fraction of it is found there as esters, has been amply proven. Since the active form of vitamin A in vision is vitamin A aldehyde,[15] it was of some interest to discover whether this compound is present in the liver of a rat dosed with labeled vitamin A. Table III shows the results obtained by chromatography of the liver non-saponifiable extract, using deactivated alumina. The eluted fractions were mixed with non-radioactive carrier compounds, and derivatives were prepared and assayed. No significant activity was found in vitamin A aldehyde, thus confirming results by Ames, *et al.*,[16] indicating absence of vitamin A aldehyde in the liver. Activity was almost equally distributed between vitamin A and vitamin A esters. The main bulk of activity, however (64.9%), was held to the column and could be eluted only with alcohol. It was therefore concluded to be due to metabolites of vitamin A of a more polar character than vitamin A or its esters, or to artifacts produced by air-oxidation during isolation, or both.

Metabolites in Urine.—Urine was fractionated by lyophilization and extraction into butanol. Most of the activity (85 to 93%) was extractable. This activity was only very slightly soluble in ligroin

(13) N. R. Lawrie, T. Moore and K. R. Rajagopal, *Biochem. J.*, **35**, 825 (1941).

(14) V. Prelog, J. Fuhrer, J. Hagenback and R. Schneider, *Helv. Chim. Acta*, **31**, 1799 (1949); V. Prelog and M. Osgau, *ibid.*, **35**, 981, 986 (1952).

(15) G. Wald, *Science*, **113**, 287 (1951).

(16) S. R. Ames, W. J. Swanson, H. A. Risley and P. L. Harris, *Federation Proc.*, **13**, 174 (1954).

TABLE III

RADIOACTIVITY DISTRIBUTION IN LIVER

Activity injected intraperitoneally, 4.28 μc.; dose, 4.14 mg.; duration of experiment, 14 hours; total activity of liver non-saponifiable, 0.408 μc.; activity of liver fractionated, 0.190 μc.

	Fraction in which contained no.	Amount in fractions, μg.	Activity in fractions, μc. $\times 10^{-2}$	Specific activity, μc./mM	Liver activity, %
Vitamin A	7 to 8	300	3.14	28.85	16.5
Vitamin A aldehyde	6	Not detectable	0.045		
Vitamin A esters	2 to 5	417	3.54	24.25	18.6

(4.3%) or toluene (5.7%), even after heating with acid (9.7%) or alkali (0%), thus showing the absence of vitamin A or vitamin A combined with solubilizing groups (*e.g.*, glucuronide), or vitamin A solubilized by emulsifier. The butanol-soluble fraction was redissolved in water and then divided into an ether-soluble ("water-soluble, ether-soluble," WES; 33%) and an ether-insoluble fraction ("water-soluble," WS; 67%). These fractions were subjected to paper chromatography and radioautography, by application of an aliquot to the base line of untreated sheets of Whatman No. 1 filter paper, and subsequent descending development with the solvents indicated in Table IV. After drying of the sheets, the spots were visualized, either by exposure to X-ray film, resulting in blackening by radioactivity, or by the indicator sprays described in Table V. This procedure confirmed the presence of two urinary compounds, one in the ether extract, the other in the aqueous residue of butanol-extract of urine.

Sufficient amounts of these compounds were present in urine to give a series of color reactions on paper after chromatography (Table V). The colored spots obtained with the reagents coincided exactly with the radioautographically located radioactive spots. From these reactions it was concluded that WES contained unsaturation, a hydroxyl group and an aldehydic carbonyl group; WS showed evidence only of a keto-group. However, upon acetylation of WS, from 40 to 54% of activity could be made ether soluble. The acetylated compound showed a slightly different R_f value from the original WS. It was therefore concluded that it contained one or more hydroxyl groups. Dinitrophenylhydrazone derivatives were prepared of both WES and WS. Radioactivity in both cases thereupon became toluene extractable. The R_f values of the dinitrophenylhydrazones of both WES and WS were shown to differ greatly from those of the original compounds.

TABLE IV

PAPER CHROMATOGRAPHY

Material chromatogramed	R_f in solvent systems: Butanol	Butanol satd. with water	Water satd. with butanol	93% toluene–7% butanol
Urine water-soluble (WS)	0.75–0.80			0.00
Urine water-soluble (WS) acetylated derivative	.68			.00
Urine water-soluble (WS) DNP derivative				.85
Urine ether-soluble (WES)	.87–92	0.92	0.82	.86–89
Urine ether-soluble (WES) acetylated derivative	.88			.90
Urine ether-soluble (WES) DNP derivative	.87	.92	.00	.86

TABLE V

FUNCTIONAL GROUP TESTS ON PAPER CHROMATOGRAMS

Reagents	Groups tested for	Urine water-soluble (WS)	Urine ether-soluble (WES)
$KMnO_4$	OH; —C=C—	(−)	(+)
$KMnO_4/NaIO_4$	HO-C-C-OH	(−)	(+)
$C(NO_2)_4$	—C=C—	(−)	(+)
Dinitrophenylhydrazine	C=O	(+)	(+)
$AgNO_3/NH_3$	HC=O	(−)	(+)

In order to obtain sufficient quantity for further characterization of these compounds several rats were injected with non-radioactive vitamin A and the metabolites extracted as described in the Experimental Section. The dinitrophenylhydrazone of WS was isolated and recrystallized to constant specific activity with an aliquot of radioactive dinitrophenylhydrazone of WS, as a check on its purification. Elementary analysis showed it to be a dinitrophenylhydrazone of a ketone $C_{11}H_{14}O_4$. Infrared spectrum analysis indicated an unconjugated ester, the acidic portion of which had a carbon chain of from 4 to 7 carbon atoms. Analysis of the ultraviolet absorption spectrum of WS dinitrophenylhydrazone according to the method of Jones, *et al.*,[17] revealed an absorption peak at 365 mμ, characteristic of unconjugated, aliphatic ketones. On treatment with alkali, the peak shifted to a broad band, 420 to 460 mμ, which confirms the foregoing conclusion. The disappearance rate of the absorption peak in alkali (18% of optical density in 90 minutes), according to the above authors, is also indicative of an unconjugated aliphatic ketone. Saponification of the WS dinitrophenylhydrazone yielded a dark-brown amorphous powder, soluble in alkali and insoluble in acid, which retained the radioactivity. It was therefore concluded that it is the acidic portion of the ester which is derived from vitamin A, and contains the keto-group.

Analysis of the ultraviolet absorption spectrum of WES dinitrophenylhydrazone according to the method of Jones,[17] showed a sharp peak at 375 mμ, characteristic of a conjugated aldehyde. On treatment with alkali, the peak shifted to 448 mμ, and was stable with time, confirming the structure as being an aliphatic aldehyde with a conjugated double bond.

An effort was made to determine whether radioactive acetate would be incorporated into vitamin A in the rat. Potassium acetate-1-C^{14} (9.6 mg., 900 μc.) was injected into a rat in three portions in 10 hour intervals for 25 hours. The vitamin A was extracted from the liver and, together with carrier vitamin A, chromatographed on alumina and con-

(17) L. A. Jones, J. C. Holmes and R. B. Seligman, *Anal. Chem.*, **28**, 191 (1956).

verted to the phenylazobenzoyl derivative.[18] No activity could be detected in the crystalline derivative. The urinary compounds WES and WS were found to contain no radioactivity.

Experimental

Synthesis. **Vitamin A Acid.**—C_{18}-Ketone, prepared from β-ionylidenecrotonic acid and methyllithium,[6] was purified by chromatography on acid-washed alumina (Merck), from which it was eluted with benzene. Activated granular zinc (20 mesh) (197 mg.) was weighed into a 50-ml. pear-shaped flask provided with a magnetic stirrer, reflux condenser and a capillary through which a slow stream of inert gas (dry, pure helium) swept continuously. The C_{18}-ketone was dissolved in dry benzene to make 3 ml. of solution and 0.3 ml. of it was placed in the reaction flask. Twice-distilled methyl bromoacetate (non-radioactive) (252 mg.) was dissolved in dry benzene to make 2 ml. of solution, and 0.37 ml. of it was placed in the reaction flask. The flask was then heated to 100° on an oil-bath with stirring and without condenser. As the solvent evaporated, the reaction began to take place, as indicated by the appearance of a deep-red color in the mixture. At that moment the flask was removed from the oil-bath, the rest of the C_{18}-ketone and non-radioactive methyl bromoacetate were added rapidly, followed immediately by methyl bromoacetate-2-C^{14} (207 mg.; 880 μc.; made up to 4 ml. with dry benzene). The mixture was then refluxed in a slow stream of helium for 75 minutes. Isolation of the hydroxy-ester and dehydration followed the procedure of Wendler, *et al.*[3] The ester so obtained was dissolved in ligroin (b.p. 30–60°; used throughout this purification) (10 ml.), and placed on a column of alumina (2.6 × 35 cm.), acid-washed according to the method of Garbers[19] and made up in ligroin. Non-polar impurities were first eluted by ligroin (100 ml.). Next, ligroin–benzene mixtures of the following proportions, 150 ml. each, were applied to the column in this order: 80:20; 70:30; 60:40; 50:50; 40:60. The yellow ester band was eluted completely by mixtures: 50:50 (200 ml.) and 40:60 (200 ml.). The eluted fractions from mixtures 60:40 through 40:60 were pooled and solvents were removed under reduced pressure. The residue was dissolved in ethanol (3 ml.) and saponified.[3] The vitamin A acid was extracted into ether and crystallized from that solvent at −20°. The mother liquor was withdrawn after centrifugation at 4°, using a narrow-mouthed pipet, and the crystals washed with a mixture of ligroin (b.p. 90–100°):ether, 3:1, previously chilled to −80°. The acid was recrystallized from ether in the same manner, m.p. 174–176°; ultraviolet absorption λ_{max}^{EtOH} 347 mμ, $E_{1\,cm.}^{1\%}$ 1390; yield 78 mg. or 8.7%; radioactivity, 314 μc. per millimole.

Anal. Calcd. for $C_{20}H_{28}O_2$: C, 79.96. Found: C, 80.50.

Vitamin A.—Vitamin A acid (5.87 mg.) was esterified with diazomethane and reduced with lithium aluminum hydride.[3] The vitamin A alcohol was liberated by addition of 30% aqueous potassium hydroxide solution (2 ml.) to the hydride solution, added with extremely vigorous stirring with a magnetic stirrer. The resulting emulsion was broken by centrifugation of the tube, the aqueous layer was removed by a pipet and washed with a little ether. To the pooled ether solution and washings was added α-tocopherol (5 mg.). The ether solution was then washed with water until neutral, evaporated to dryness in a rapid stream of helium, and further dried by removing traces of water under reduced pressure. The remaining vitamin A alcohol was analyzed for yield and purity by its ultraviolet spectrum λ_{max}^{EtOH} 326 mμ, $E_{1\,cm.}^{1\%}$ 1640; yield 4.7 mg. or 84%; radioactivity, 296 μc. per millimole.

Purity was checked by reversed phase paper chromatography on formamide-impregnated paper, with ligroin (b.p. 60–90°) saturated with methanol (75 ml.) and formamide (25 ml.) as solvents. The yellow spot of vitamin A alcohol (R_f = 0.7) coincided with the radioactive spot as revealed by radioautography.

For a further check, a sample of non-radioactive vitamin A synthesized by the method described, was found to have activity equal to that of natural vitamin A in a bioassay using vitamin A deficient rats.

Distribution.—Vitamin A-2-C^{14} (2.8 mg.) was emulsified together with α-tocopherol (3 mg.) in a mixture (2 ml.) consisting of ethanol (10%), "Tween 80" (16.2%) (emulsifying agent) and water (73.8%). This was injected intraperitoneally into a male albino rat (205 g.), which was then placed in a metabolism cage. Urine, feces and expired carbon dioxide were collected and assayed for radioactivity, the last-named by precipitation as barium carbonate of the sodium carbonate formed in the passage of the expired air through sodium hydroxide. Urine was collected in a vessel cooled to −80°. At the end of the experiment, the animal was killed by decapitation, bled, the organs dissected out, frozen in liquid nitrogen and stored at −20°.

Organ tissue and feces samples were saponified under nitrogen with 0.1 *N* ethanolic potassium hydroxide (15 ml. per gram of tissue), the ethanol was replaced by water and the mixture exhaustively extracted with ligroin (b.p. 60–90°), to obtain the non-saponifiable fraction. In several instances, the residual aqueous solution was acidified and extracted with ligroin to determine activity in fatty acids. The remaining aqueous solution was termed "water-soluble residue" (Table II). Liver and intestine samples were homogenized in a "Waring" blender, the homogenate extracted with ligroin and ethanol, and the organic layer saponified as described above, after removal of the solvents. Urine was directly extracted with ligroin.

To ensure the absence of extraneous radioactive compounds, possibly through leakage from the peritoneum, skin samples were taken from the back; hair, fat, connective and muscle tissue were removed, and the skin surface washed on both sides with ligroin. The samples were then saponified as described. The unsaponifiable fraction was then chromatographed on a column of alumina together with carrier vitamin A, as described in the next section. All the radioactivity was eluted with vitamin A.

Distribution in Liver.—Liver tissue of rat II was extracted by the method of Ames.[20] The tissue, while still in the frozen state, was ground with anhydrous sodium sulfate and then extracted with ether. This solvent was replaced by ligroin (b.p. 60–90°), and the solution placed on a column of alumina previously deactivated and calibrated. The deactivation was carried out by mixing thoroughly "Merck" chromatographic alumina (174 g.) with ligroin (b.p. 60–90°) (500 ml.) and water (5 ml.). The column was made up in ligroin (b.p. 60–90°), and calibrated with a synthetic mixture of vitamin A, vitamin A acetate (obtained commercially) and vitamin A aldehyde.[21] The ester was eluted with ligroin (b.p. 60–90°), the aldehyde with a mixture of ligroin:benzene, 70:30, vitamin A with ligroin:benzene, 20:80. With the liver extract, separate 5-ml. fractions were taken (Table III) and assayed by ultraviolet absorption spectrophotometry at 328 mμ for vitamin A alcohol or ester concentration, and at 369 mμ for vitamin A aldehyde concentration, and for radioactivity. Peaks obtained by plotting absorption against fraction number coincided with peaks of radioactivity against fraction number, in case of vitamin A and esters. Vitamin A was converted to the phenylazobenzoate derivative,[18] with carrier added, crystallized to constant specific activity and counted. Vitamin A ester fractions were saponified to free vitamin A in the presence of carrier, converted to the derivative, crystallized and counted.

Metabolites in Urine.—Extractions into butanol were carried out on lyophilized urine as described and paper chromatography carried out as shown in Table IV. Dinitrophenylhydrazones were prepared, extracted into toluene and used for paper chromatography and radioactivity assay. Acetylation was carried out by addition of acetic anhydride (1 ml.) and anhydrous pyridine (1 ml.) to a lyophilized aliquot of the radioactive metabolite. The mixture was allowed to stand at room temperature for two days. The acetic anhydride was then decomposed with water, the solution extracted with ether, the ether solution washed with dilute hydrochloric acid, dilute sodium carbonate solution and

(18) C. D. Robeson and J. G. Baxter, THIS JOURNAL, **69**, 136 (1947).

(19) C. F. Garbers, C. H. Eugster and P. Karrer, *Helv. Chim. Acta*, **36**, 562 (1953).

(20) S. R. Ames, H. A. Risley and P. L. Harris, *Anal. Chem.*, **26**, 1378 (1954).

(21) G. Wald, *J. Gen. Physiol.*, **31**, 489 (1947–1948): active manganese dioxide was prepared according to J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Hansen and T. Walker, *J. Chem. Soc.*, 1094 (1952).

water, and used for paper chromatography and radioactivity assay.

For the large-scale production of the urinary metabolites (in non-radioactive form), three rats were placed in metabolism cages and injected at 48-hour intervals with 1 ml. of vitamin A acetate (2.5 mg.) emulsion. Urine was collected under toluene and frozen daily. After 3 weeks, the collected urine was lyophilized and extracted with anhydrous butanol. The extract was reduced in volume and applied to the base-line of a 3-MM Whatman chromatographic paper. The bands were developed with butanol (Table IV). The marginal portion of the sheets was cut off and sprayed with dinitrophenylhydrazine reagent to locate the band of the metabolite (Table V). This band was then cut out, the metabolite eluted with water, ether-soluble material removed by ether extraction and the dinitrophenylhydrazone derivative prepared. After extraction into toluene, it was crystallized from ethanol. An aliquot of a solution of the *radioactive* dinitrophenylhydrazone of WS was added (15,868 counts per minute) prior to the first crystallization and recrystallization was carried out to constant specific activity. About one-half of the activity added (8,625 c./min.) remained in the mother liquor. The rest was crystallized, yielding a dark red solid, m.p. 182–186° (specific activity, 55 c./min./mg.); yield 108.8 mg.

Anal. Calcd. for $C_{17}H_{18}O_7N_4$: C, 52.4; H, 4.6; N, 14.4. Found: C, 53.0; H, 4.8; N, 14.5.

Upon saponification of this dinitrophenylhydrazone derivative, an acidic compound was obtained, soluble in alkali, insoluble in water, acid, ether and alcohol.

URBANA, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MARYLAND]

Reactions of Naphthoquinones with Malonic Ester and its Analogs. III. 1-Substituted Phthaloyl- and Phthaloylbenzopyrrocolines[1]

BY ERNEST F. PRATT, RIP G. RICE AND RAYMOND W. LUCKENBAUGH[2]

RECEIVED AUGUST 20, 1956

A variety of 1-substituted 2,3-phthaloylpyrrocolines (II to VI) have been prepared by the extension to other active methylene compounds of the previously reported condensation of acetoacetic ester with pyridine and 2,3-dichloro-1,4-naphthoquinone. The carbethoxy-, acetyl- and cyanopyrrocolines have been interrelated by conversion to the same acid or amide. Similarly substituted 2,3-phthaloyl-7,8-benzopyrrocolines (XIII to XXI) have been smoothly prepared by using isoquinoline in place of pyridine. The pyrrocoline structures have been confirmed by practical new syntheses of 1-acetyl- and 1-benzoyl-2,3-phthaloylpyrrocolines (II and III) from 2-acetonyl- or 2-phenacylpyridine and 2,3-dichloro-1,4-naphthoquinone. In the same way 1-acetyl-2,3-phthaloyl-5,6-benzopyrrocoline (XXIII) has been obtained from 2-acetonylquinoline.

In the preceding study of this series[3] it was found that acetoacetic ester reacted with 2,3-dichloro-1,4-naphthoquinone and pyridine to give a product for which the structure 1-carbethoxy-2,3-phthaloylpyrrocoline (I) was proposed. This reaction has now been extended to a number of analogs of acetoacetic ester. The active methylene compounds employed, the formulas of the products and the yields were as follows: acetylacetone, II, 58%; benzoylacetone, III, 10%; ethyl cyanoacetate, IV, 53%; methyl cyanoacetate, IV, 50%; phenylacetone, V, 17%; benzyl ethyl ketone, V, 1%; and nitroethane, VI, 23%. Compounds II,[4] IV[5] and V[5] were also obtained, in somewhat lower yields, from unsubstituted 1,4-naphthoquinone.

A 74% yield of the above cyanopyrrocoline (IV) resulted upon the treatment of ethyl (2-chloro-1,4-naphthoquinonyl-3)-cyanoacetate[6] with pyridine.[7] A by-product formulated as X on the basis of the results of ultimate analysis and the formation of a precipitate with aqueous silver nitrate was also isolated. Both this synthesis of the pyrrocoline and the formation of the by-product are consistent with the route of reaction previously proposed.[3] The same by-product was isolated in the synthesis of the cyanopyrrocoline from ethyl cyanoacetate and the dichloroquinone.

The hydroquinone diacetates of the cyano- and phenylpyrrocolines (IV and V) were readily prepared. By the Rast method, using camphor, the molecular weights of the acetylpyrrocoline (II) and the hydroquinone diacetate of IV were found to be 296 and 365 as compared to the calculated values of 289 and 358. The infrared absorption spectrum of the cyanopyrrocoline (IV) showed a peak at 2227 cm.$^{-1}$ indicating a cyano group conjugated with a carbon–carbon double bond.[8,9]

Upon treatment of this nitrile IV with sulfuric acid the amide VII was obtained. This crystallized as an allotropic form of the amide previously prepared[3] from the carbethoxypyrrocoline (I) *via* the acid (VIII), and the same acid was obtained from the acetyl analog II *via* the pyridinium iodide (IX).[10]

Compounds which on the basis of their melting points, elementary composition and color are identical with the carbethoxy- and acetylpyrrocolines (I and II) have been prepared in another laboratory from the dichloroquinone by essentially the same method, but the structures proposed in the initial independent reports were those of the ionic compounds Ia and IIa.[11] The results of quantitative analysis for hydrogen furnish significant support for the pyrrocoline structure since they contain two

(1) From the Ph.D. thesis of R. W. Luckenbaugh, May, 1952, and Rip G. Rice to be submitted in May, 1957.

(2) Research Corporation Fellow 1950–1951 and National Institutes of Health Fellow 1951–1952.

(3) E. F. Pratt, R. W. Luckenbaugh and R. L. Erickson, *J. Org. Chem.*, **19**, 176 (1954).

(4) It is a pleasure to acknowledge our indebtedness to Mr. Raymond L. Erickson, who carried out this experiment.

(5) It is a pleasure to acknowledge our indebtedness to Mr. Robert W. Storherr who carried out this experiment.

(6) Prepared by the method of C. Liebermann, *Ber.*, **32**, 916 (1899).

(7) Subsequent to the completion of this synthesis (ref. 12) closely related reactions were reported in ref. 11.

(8) R. E. Kitson and N. E. Griffith, *Anal. Chem.*, **24**, 334 (1952).

(9) We wish to thank Dr. Robert Spurr and his staff for the determination of the infrared absorption spectra.

(10) L. King, THIS JOURNAL, **66**, 894 (1944).

(11) B. Suryanarayana and B. D. Tilak, *Current Sci. (India)*, **22**, 171 (1953); *C. A.*, **48**, 14212 (1954); *Proc. Indian Acad. Sci.*, **39A**, 185 (1954); *C. A.*, **49**, 12411 (1955).