TABLE I,
SYNTHETIC CONDITIONS, PHYSICAL PROPERTIES, AND ANALYTICAL DATA

Compd	1(2H)-Phthalazinone	Medium	Time, hr	% yield	Solvent[a]	Mp, °C	Formula[d]
4a	4-Methyl-8-nitro-	H_2O	10	83	A–W	280–287	$C_9H_7N_3O$
4b	4-Methyl-8-nitro-2-phenyl-	AcOH	2	60	M	146–148	$C_{15}H_{11}N_3O_3$
4c	2 Benzyl-4-methyl-8-nitro-	$C_6H_5CH_3$[b]	2	71.2	B	163–165	$C_{16}H_{13}N_3O_3$
4d	8-Amino-4-methyl-[c]	H_2O	1	57.1	AcOH	277–280	$C_9H_9N_3$
4e	8-Amino-4-methyl-2-phenyl-	AcOH	2	71.1	E	129–131	$C_{15}H_{13}N_3O$
4f	8-Amino-2-benzyl-4-methyl-	AcOH	2	66	E	124–125	$C_{16}H_{15}N_3O$
4g	8-Hydroxy-4-methyl-	H_2O	1	45.7	E	228–230	$C_9H_8N_2O_2$
4h	8-Hydroxy-4-methyl-2-phenyl-	AcOH	2	76.7	E	158–160	$C_{15}H_{12}N_2O_2$
4i	2-Benzyl-8-hydroxy-4-methyl-	AcOH	35	67.6	B	170–172	$C_{16}H_{14}N_2O_2$

[a] A-W, Me_2CO–H_2O; M, MeOH; B, C_6H_6; E, EtOH. [b] Refluxed with a Dean–Stark trap until no more water separated. [c] Also obtained by reducing **4a** in ethyl acetate under 3 atm pressure of H_2 (Raney Ni). [d] All compounds showed a correct analysis for C, H, N.

We also demonstrated that 4-methyl-8-nitro-1(2H)-phthalazinone (**4a**) could be reduced in the presence of Raney nickel to 8-amino-4-methyl-1(2H)-phthalazinone (**4d**), identical in all respects with the compound obtained from the reaction of **2a** with **3a**.

Pharmacology.—The compounds were completely atoxic at levels of 2000 mg/kg *po* and ip in mice and showed no pharmacological activity in screening tests for cardiovascular, renal, and central nervous system effects. The relative lack of solubility may account for the absence of biological activity.

Experimental Section[5]

Preparation of 1(2H)-Phthalazinones.—Compounds 1, **2a**,[1] and **2b**[1] were allowed to react at reflux with 1 mole equiv of the indicated hydrazine (**3**). The reaction conditions and other pertinent data are reported in Table I.

Acknowledgment.—We are indebted to Dr. Al Steyermark and his staff for the microanalyses, to Dr. T. Williams for nmr spectra, to Dr. V. Toome for the ultraviolet spectra, and to Mr. S. Traiman for the infrared spectra as well as the fruitful discussions with them. The pharmacological data were obtained under the direction of Dr. L. O. Randall, Director of the Pharmacological Laboratories.

(5) All melting points are corrected and were taken on a Uni-Melt Thomas–Hoover capillary melting point apparatus. Ir were taken on a Beckman IR5 double beam spectrophotometer with NaCl optics as KBr pellets. The uv spectra were taken in *i*-PrOH on a Cary spectrophotometer (Model 14M). Nmr spectra (10–15% w/w DMSO-d_6 solutions, TMS) were obtained on a Varian A-60 spectrometer. Accuracy limits are about ±0.025 for chemical shifts. Absorption bands (or peaks) of spectra (uv, ir, nmr) were as expected.

Stability of Pyruvaldehyde Bis(thiosemicarbazone) and Its Copper Chelate[1]

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The tumor-inhibitory properties of pyruvaldehyde bis(thiosemicarbazone) (PTS) are increased by the concurrent administration of copper.[2] The copper chelate is still more active.[2] Studies of the fate of ^{35}S-labeled PTS showed that the distribution of the compound is altered at least quantitatively by copper,[3] and those studies showed that PTS is not eliminated in the urine as either PTS or the chelate. The antitumor activity of the chelate was variable and suggested a possible instability. The possible significance of decomposition of the chelate is the subject of this study.

Experimental Section

The PTS and the ^{35}S-labeled PTS were generously supplied by Dr. Phyllis D. Oja of the Dow Chemical Co., Walnut Creek, Calif. The 2-mercaptoethanol (MCE) was obtained from the Eastman Organic Chemical Co., Rochester, N. Y.

Preparation of Chelate.—All material and glassware used in the preparation of, and work with, the copper–PTS chelate $Cu^{II}PTS$ were washed meticulously in deionized glass-distilled H_2O in order to minimize trace metal contamination. The chelate was prepared by dissolving the PTS in 1 *N* NaOH (12 mg/0.3 ml) and diluted with H_2O to 20 ml. A saturated aqueous solution of $CuSO_4 \cdot 5H_2O$ was added dropwise. The mixture was stirred constantly. Chelate formation was considered complete when no further precipitation occurred following the addition of saturated $CuSO_4$ to an aliquot of the supernatant solution. The addition of concentrated NH_4OH to another aliquot of the supernatant produced a deep blue color indicating the presence of excess copper ions. The reddish brow precipitate was washed repeatedly with H_2O (ten times) and EtOH and Et_2O (five times) and air dried. When radioactive chelate was required, the above procedure was followed except that ^{35}S-labeled PTS was diluted with unlabeled PTS.

Heating Procedures.—Both PTS and $Cu^{II}PTS$ were studied in air and *in vacuo*. They were dissolved in DMF and placed in screw-cap tubes (2 ml/10-ml tube) for examination in air and evacuated and sealed in ampoules (1 ml/5-ml ampoule) for study in the absence of air. Samples were maintained at 4, 25, 37, and 65°.

Paper Chromatography and ^{35}S Assay.—Ascending paper chromatograms were run on Whatman No. 1 paper. The solvent system, 70% DMF and 30% *n*-BuOH, was freshly prepared each time. The chromatograms were examined under uv light. When radioactive materials were examined, the strips were cut into 20 equal parts and measured in 15 ml of toluene–PPO–POPOP scintillant in a Packard Tri-Carb liquid scintillation spectrophotometer at 9.5% gain and a window setting of 40–1000. The uv spectra of PTS and $Cu^{II}PTS$ were determined in H_2O and DMF. PTS showed $\lambda_{max}^{H_2O}$ 325 mμ (*A* 47,000) and $\lambda_{min}^{H_2O}$ 270 mμ (*A* 27,000) and λ_{max}^{DMF} 345 mμ (*A* 47,000) and λ_{min}^{DMF} 290 mμ (*A* 27,000). The values for $Cu^{II}PTS$ were $\lambda_{max}^{H_2O}$ 300 mμ (*A*

(1) This investigation was supported in part by funds from National Cancer Institute, National Institutes of Health, U. S. Public Health Service (Grant No. Ca-08748).

(2) J. G. Cappuccino, S. Banks, G. Brown, M. George, and G. S. Tarnowski, *Cancer Res.*, **27**, 968 (1967).

(3) J. G. Cappuccino and M. E. Balis, unpublished data.

24,000) and λ_{min}^{PTS} 265 mμ (A 11,000) and λ_{max}^{DMF} 312 mμ (A 24,000) and λ_{min}^{DMF} 275 mμ (A 11,000). The analysis of $Cu^{II}PTS$ was done by Galbraith Laboratories, Inc., Knoxville, Tenn., and the Karl-Fisher water analysis by the Crobaugh Laboratories, Cleveland, Ohio.

Anal. Calcd for $C_5H_8N_6S_2Cu_2(OH)_2 \cdot 1.5H_2O$: C, 14.85; H, 3.22; N, 20.79; S, 15.85; Cu, 31.43; H_2O, 6.68. Found: C, 15.31; H, 2.89; N, 20.43; S, 15.31; Cu, 31.80; H_2O, 6.09.

In view of the instability of $Cu^{II}PTS$, it was not possible to dry at high temperatures, so a partially hydrated sample was analyzed. The important values were the Cu:N:C:S ratios.

In Vivo **Tumor Assay.**—The rats used were Carworth Farm (CFN) male Wistar rats 80–100 g, maintained on a diet of Purina laboratory chow and water given *ad libitum*. The tumor was the rat W256 carcinosarcoma, implanted and evaluated as previously described.[4] The compounds studied were suspended by homogenization in carboxymethylcellulose (CMC) and were administered daily in 0.5-ml ip doses for 7 days. The tumors (long and short diameters) were measured with vernier calipers and evaluated at the termination of the therapy period.

Results and Discussion

Analyses of the chelate indicate a complex with two copper atoms and 1.5 molecules of water of crystallization. Monothiosemicarbazones form copper chelates.[5] French and Freedlander have suggested that in chelates of bisthiosemicarbazones both thiosemicarbazones are bound to one metal atom.[6] 2-Keto-3-ethoxybutyraldehyde (KTS), a compound structurally analogous to PTS, has been shown by Taylor, *et al.*,[7] to form a copper chelate with one atom of copper/molecule of KTS. This is not the case with the copper PTS chelate. The spectra of PTS and the copper complex are different in both water and DMF, and the spectral shift indicates the formation of a chelate. Addition of mercaptoethanol (MCE) or dithyoethretiol removes the copper from the chelate and the spectrum returns to that of PTS.

The calculated carbon composition was 14.85% and samples analyzed for carbon gave 15.31%, but all hydrogen analyses were low and the older the samples the lower was the hydrogen content. Chromatographic analyses revealed that the chelate is stable at −17° but slowly decomposes at 4°. Dry solid preparations of chelate decomposed at about the same rate as did solutions, thus, hydrolysis is probably not a factor, nor is decomposition catalyzed by liberated copper salts. The rate of decomposition was much greater at higher temperatures. The products formed at 4° and above could be distinguished from both chelate and PTS by chromatography in DMF–butanol (7:3). PTS had R_f 0.94; that of $Cu^{II}PTS$ was 0.65. Decomposition products 1 and 2 had R_f values 0.09 and 0.83, respectively. Mixtures of chelate and MCE gave one spot at the same R_f as PTS, probably due to the greater chelating strength of MCE. The elemental composition of the product was, with the exception of the H value, the same as the chelate. There was no gross decomposition of the material and samples containing ^{35}S showed just two products which contained ^{35}S and none of the sulfur label was volatilized. The decomposition is peculiar to the chelate (Table I). The spectral change, when PTS was heated, was less than 0.01 and no chromatographic change was noted.

TABLE I

DECOMPOSITION OF PTS AND $Cu^{II}PTS$ IN AIR AND UNDER VACUUM FOLLOWING HEAT TREATMENT (65°)[a]

Time, days	ΔOD, $Cu^{II}PTS$			
	Air		Vacuum	
	−MCE	+MCE	−MCE	+MCE
0.5	0.01	<0.01	<0.01	<0.01
1	0.03	0.015	<0.01	<0.01
2	0.02	0.125	<0.01	<0.01
3	0.03	0.105	<0.01	<0.01
5	0.08	0.215	<0.01	<0.01
7	0.12	0.280	0.010	0.040

[a] ΔOD based on OD of chelate at 300 mμ. ΔOD + MCE based on OD of PTS at 325 mμ. OD of chelate at t_0 = 0.230. OD of chelate at MCE at t_0 = 0.455.

In view of the report of free-radical reactions involved in copper chelates,[8] it is possible that a molecular rearrangement occurs *via* free-radical mechanisms. The decomposition was not due to secondary reactions resulting from disintegration of the ^{35}S, since decomposition of labeled and unlabeled chelate, followed spectrally and chromatographically, showed no differences.

The possibility that the reaction involves an oxidation was investigated by measuring the decomposition in the presence and absence of air. The change in spectrum was determined both by measuring the OD of the chelate at 300 mμ, and the OD in the presence of MCE at 325 mμ. Since the spectrum in the presence of MCE returned to that of PTS the difference is greater in the presence of MCE but the per cent decomposition was approximately the same by the two methods of assay. Both the chelate and PTS are stable when heated *in vacuo* as evidence by the change in spectrum. There was no spectral change in the material heated *in vacuo* until 7 days and even then the decomposition was very small, quite possibly due to trace contamination by air. The products are not changed chromatographically or spectrally by mixture with MCE.

Chromatographically two products are found. A plot of the data indicates that each appears to be formed as a result of first-order reactions (Figure 1). The amount of unreacted material remaining after various times of heating decreases logarithmically and the rate of decomposition is a function of the temperature. There is a lag time in the formation of product 2, which then quickly reaches a maximum level and does not seem to increase with further heating. Product 1 is formed sooner; some is apparent after one-half day of heating and the amount continues to increase until the 7th day at 65° (Table II).

The instability of $Cu^{II}PTS$ due to an irreversible oxidation with increased temperature may not be

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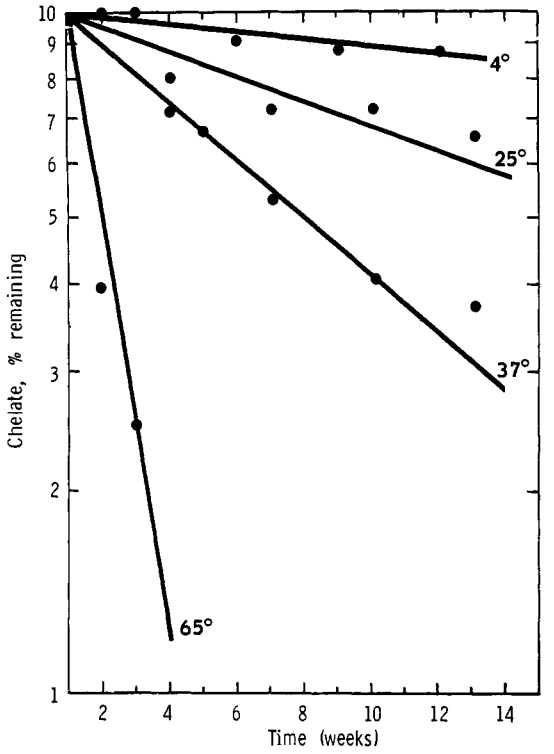

Figure 1.—Per cent chelate $Cu^{II}PTS$ remaining at various temperatures. $Cu^{II}PTS$ was heated at 4, 25, 37, and 65° in air. Samples were chromatographed and radioactivity of products and chelate was determined.

peculiar for this compound alone, but may be a phenomenon associated with all copper chelates. In view of the pharmacological activity shown by a number of chelates,[2,6,9,10] this possible reaction should be kept in mind.

TABLE II

FORMATION OF DECOMPOSITION PRODUCTS OF PTS AND $Cu^{II}PTS$ FOLLOWING HEAT TREATMENT[a]

Time, days	Air I	Air II	Air + MCE I	Air + MCE II	Vac I	Vac II	Vac + MCE I	Vac + MCE II
				$Cu^{II}PTS$				
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	4.3	0.0	5.0	0.0	0.0	0.0	0.0	0.0
1	6.0	15.0	6.5	9.6	2.5	0.0	2.9	0.0
2	10.2	21.0	12.5	18.9	1.0	0.0	3.0	0.0
3	13.0	17.6	14.1	16.4	4.0	0.0	8.0	0.0
5	26.5	15.0	20.6	14.7	6.7	0.0	2.6	0.0
7	39.1		30.5	15.5	7.6	0.0	6.5	0.0
				PTS				
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	3.3	0.0	2.2	0.0	1.8	0.0	1.6	0.0
7	4.5	0.0	4.8	0.0	1.3	0.0	0.7	0.0
10	4.0	0.0	5.2	0.0	2.2	0.0	2.3	0.0

[a] Material was heated at 65°. Values are per cent of starting materials in each product. I is material with R_f 0.09, II with R_f 0.83. The rest of the material was a chelate in sets 1 and 3 and as PTS where MCE was added in sets 2 and 4.

(9) A. C. Sartorelli, A. D. Welch, and B. A. Booth, *Federation Proc.*, **24**, 454 (1965).

(10) E. Mihich, C. L. Simpson, and A. I. Mulhern, *Cancer Res.*, **25**, 1417 (1965).

It is possible that the decomposition products are responsible for pharmacological activity of PTS. However, the products found in the urine of treated animals are chromatographically different from the breakdown products *in vitro*.[3] If the products of heating are in fact active intermediates, they are further metabolized *in vivo*. The breakdown products were tested *in vivo*. A sample heated for 9 days at 65° had about 25% of the antitumor effects and 25% as much toxicity as the chelate (Table III). Spectral analysis of the material showed that it contained 22% of unreacted chelate. Thus, the decomposition products are largely inactive.

TABLE III

THE EFFECT OF HEATED AND UNHEATED $Cu^{II}PTS$ ON THE GROWTH OF THE W256 RAT CARCINOSARCOMA[a]

Dose, mg/kg	Unheated AWC, g[b]	Unheated ATD, mm[c]	Unheated Dead	Unheated T/C	Heated AWC, g[b]	Heated ATD, mm[c]	Heated Dead	Heated T/C
0	+35	10.8	0/5	1.00	+35	10.8	0/5	1.00
1.25	+15	9.6	0/5	0.89	+32	10.5	0/5	0.97
2.50	−16	5.5	2/5	0.51	+16	9.6	0/5	0.89
5.00	...	...	5/5	NE[d]	−2	8.4	1/5	0.78
10.0	...	...	5/5	NE[d]	−29	4.5	1/5	0.42

[a] Chelate was prepared as indicated in Experimental Section. The aliquot of chelate to be heated was dissolved in DMF and incubated at 65° for 7 days. The DMF was removed and the material was dried by means of flash evaporation. The compound was suspended in CMC, homogenized, and administered intraperitoneally once daily for 7 days starting 24 hr after implantation. Tumors were evaluated at the end of the therapy period. [b] AWC, average weight change from day of implantation. [c] ATD, average tumor diameter. [d] NE, no evaluation because of animal mortality.

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New Nitrosamines[1]

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Numerous N-nitrosoamines have been prepared and studied as carcinogenic agents.[2] We prepared N-nitroso derivatives of amino compounds which had shown either carcinogenic or carcinostatic activity. The yellow, crystalline compounds listed in Table I were prepared by adding the theoretical amount of sodium nitrite to an acid alcoholic solution of the amine at 0–5°, diluting with water, and neutralizing the mixture, then recrystallizing the product from ethanol and from isopropyl ether. Several of them showed marked antitumor effects (see Table I on the following page).

(1) This investigation was supported in part by Public Health Service Research Grants CA-03717-09-10.

(2) *Cf.* H. Druckrey, R. Preussmann, S. Ivanocic, and D. Schmahl, *Z. Krebsforsch.*, **69**, 103 (1967).