[Chem. Pharm. Bull. 17(11)2340—2348(1969)]

UDC 547.472.08 : 543.426

Fluorescence Assay of α-Oxo Acids with 4′-Hydrazino-2-stibazole[1]

Saburo Mizutani, Yaeko Wakuri, Naomi Yoshida, Terumi Nakajima, and Zenzo Tamura

Faculty of Pharmaceutical Sciences, University of Tokyo[2]

(Received July 12, 1969)

4′-Hydrazino-2-stilbazole (4H2S) was discovered as a specific, selective and sensitive fluorometric reagent for α-oxo acids. Fluorescence characteristic of 4H2S-hydrazones of α-oxo acids were examined and a new fluorometric determination method of α-oxo acids was developed. By the application of this method, a simple and microdetermination procedure of total α-oxo acids in blood was established. This method was not interfered by biological materials, and the sensitivity was about 100—200 fold to that of the existing colorimetry.

α-Oxo acids exist in blood and urine, as metabolite of sugars and amino acids. Their changes in quantities or qualities indicate abnormal disturbances of biological system. Analysis of α-oxo acids in biological materials have been carried out by chemical[3,4] and biochemical[5] methods, among these a colorimetric method[3] by the formation of 2,4-dinitrophenylhydrazone has been most widely used. However, as the reaction was not specific for α-oxo acids, the method required tedious separations.[3] Moreover the colorimetry was not sensitive enough for the purpose.

The present paper deals with the discovery of a specific and sensitive fluorometric reagent for α-dioxo compounds, and its application to analysis of α-oxo acids in biological materials.

Results

Fluorescence Characteristics of 4H2S and Hydrazones

As α-oxo acids themselves did not fluoresce, carbonyl reagents with fluorophore were examined and a new fluorescence reagent, 4′-hydrazino-2-stilbazole (4H2S) (III), was developed. The whole synthetic process is listed in Chart 1.

4H2S reacted readily with α-oxo acids under mild conditions and forms a hydrazone (Table I) which showed a strong fluorescence in acid solution.

Moreover it was found that 4H2S-hydrazones were devided into two groups, concerning their fluorescence characteristics.

As shown in Fig. 1 and Table II, hydrazones of monocarbonyl compounds like acetone and benzaldehyde (group A), strongly fluoresced at higher pH, while those of α-oxo acids and α-diketones (group B) did at pH lower than 2.

1) A Part of this work was reported at "Communication to the Editor" in *Chem. Pharm. Bull.* (Tokyo), **12**, 850 (1964).
2) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo.*
3) T.E. Friedemann and G.E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).
4) E. Robins, N.R. Roberts, K.M. Eydt, O.H. Lowry, and D.E. Smith, *J. Biol. Chem.*, **218**, 897 (1956); D.J.D. Hochenhull and G.D. Floodgate, *Biochem. J.*, **52**, 38 (1952); J.E. Spikner and J.C. Towne, *Anal. Chem.*, **34**, 1468 (1962).
5) F. Kubowitz and P. Ott, *Biochem. Z.*, **314**, 94 (1943); S. Ochoa, A.H. Mehler, and A. Kornberg, *J. Biol. Chem.*, **174**, 979 (1948); P. Greengard, *Nature*, **178**, 632 (1956).

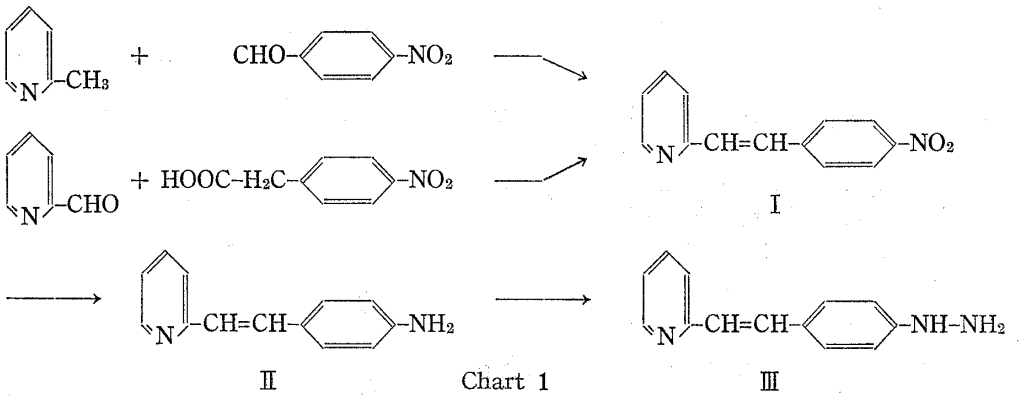
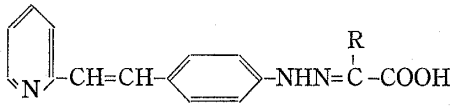
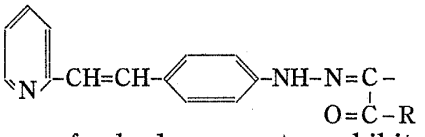


TABLE I. Hydrazones formed from α-Oxo Acids and 4′-Hydrazino-2-stilbazole



Hydrazone[a]	R	mp (°C)	Appearance[b]	Formula	Analysis (%) Calcd. C	H	N	Found C	H	N
G-Z	H	168	YO I	$C_{15}H_{13}O_2N_2$	67.31	4.90	15.72	67.31	4.96	15.61
P-Z	CH_3	203	Y II	$C_{16}H_{15}O_2N_2$	68.31	5.38	14.94	68.00	5.73	15.04
HS-P-Z	$HS-CH_2$	199	O III	$C_{16}H_{15}O_2N_3S$	61.32	4.82	13.41	61.40	5.08	13.35
KB-Z	CH_3CH_2	187	Y II	$C_{17}H_{17}O_2N_3$	69.13	5.80	14.23	69.47	5.56	13.89
MeS-KB-Z	CH_3S CH_2CH_2	176	YO II	$C_{18}H_{19}O_2N_3S$	63.30	5.61	12.31	63.41	5.83	12.64
KV-Z	$CH_3CH_2CH_2$	181	O II	$C_{18}H_{19}O_2N_3$	69.88	6.19	13.56	69.38	6.29	12.97
KIV-Z	$(CH_3)_2CH$	203	RO II	$C_{18}H_{19}O_2N_3$	69.88	6.19	13.56	69.63	6.08	13.31
KIC-Z	$(CH_3)_2CH$ CH_2	196	O II	$C_{19}H_{21}O_2N_3$	70.56	6.55	13.00	70.47	6.69	13.04
KMV-Z	CH_3CH_2CH CH_3	196	RO II	$C_{19}H_{21}O_2N_3$	70.56	6.55	13.00	70.87	6.33	12.75
OA-Z	$HOOC-CH_2$	200	YO I	$C_{17}H_{15}O_4N_3$	62.76	4.65	12.92	62.27	4.80	12.81
KG-Z	$HOOC-CH_2$ CH_2	195	YO III	$C_{18}H_{19}O_4N_3$	63.71	5.05	12.38	63.37	5.15	12.10
PP-Z	$C_6H_5CH_2$	184	Y II	$C_{22}H_{19}O_2N_3$	73.93	5.36	11.76	73.71	5.41	11.80
Cl-PP-Z	Cl-C6H4-CH2	170	Y II	$C_{22}H_{18}O_2N_3Cl$	67.43	4.63	10.72	67.41	4.91	10.30
PHPP-Z	HO-C6H4-CH2	195	Y II	$C_{22}H_{19}O_3N_3$	70.76	5.13	11.25	70.99	5.30	11.14

a) G-Z: 4H2S-hydrazone of glyoxylic acid
P-Z: 4H2S-hydrazone of pyruvic acid
HS-P-Z: 4H2S-hydrazone of mercaptopyruvic acid
KB-Z: 4H2S-hydrazone of α-oxobutyric acid
MeS-KB-Z: 4H2S-hydrazone of γ-methylmercapto-α-oxobutyric acid
KV-Z: 4H2S-hydrazone of α-oxo-*n*-valeric acid
KIV-Z: 4H2S-hydrazone of α-oxo-iso-valeric acid
KIC-Z: 4H2S-hydrazone of α-oxo-iso-caproic acid
KMV-Z: 4H2S-hydrazone of α-oxo-β-methylvaleric acid
OA-Z: 4H2S-hydrazone of oxalacetic acid
KG-Z: 4H2S-hydrazone of α-oxoglutaric acid
PP-Z: 4H2S-hydrazone of phenylpyruvic acid
Cl-PP-Z: 4H2S-hydrazone of *p*-chlorophenylpyruvic acid
PHPP-Z: 4H2S-hydrazone of *p*-hydroxyphenylpyruvic acid
b) Y: yellow, O: orange, R: red, I: prisms, II: needles, III: pillars

These results indicate that the structure of  is necessary for hydrazones to exhibit fluorescence in strong acidity (Table II). The relative fluorescence intensity was found to be stronger in the carboxyl (R:OH, OCH_3) than in carbonyl (R:H, CH_3) compounds. The reagent, 4H2S, itself showed the similar fluorescence characteristics to those of the group A.

Prerequisites to Analysis of α-Oxo Acids

As shown in Fig. 2, 4H2S fluoresced even weakly in a strong acid media. In order to make smaller the fluorescence–blank–value caused by excess of reagent, it was necessary to excite by light of 450 mμ, instead of 410 mμ which was the maximum exciting wave length.

Fluorescence spectra thus obtained were shown in Fig. 3.

The optical properties of 4H2S-

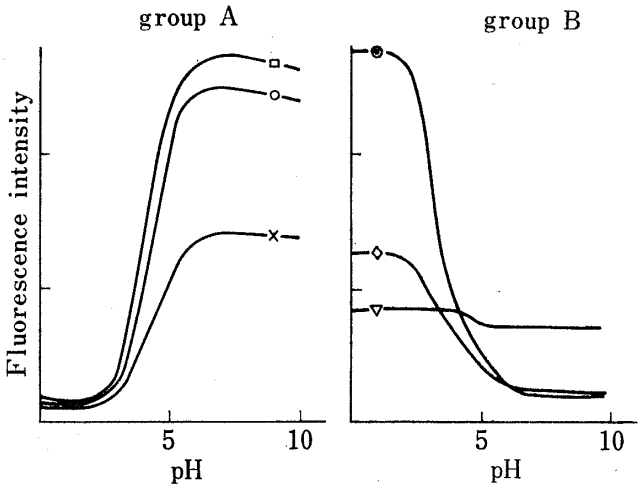
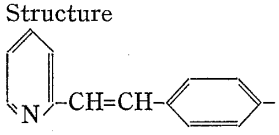


Fig. 1 Relative Fluorescence Intensity of A, B Groups at Various pH

—○—: 4H2S
—□—: 4H2S-hydrazone of acetone
—×—: 4H2S-hydrazone of benzaldehyde
—⊙—: 4H2S-hydrazone of pyruvic acid
—⊙—: 4H2S-hydrazone of methyl pyruvate
—◇—: 4H2S-hydrazone of diacetyl
—▽—: 4H2S-hydrazone of glyoxal
solution: 10^{-6}M in 50% MeOH
apparatus: turner fluorometer model 110
filter: group A primary #110—811 (Narrow pass max 360 mμ), secondary #110—821 (Narrow pass max 490 mμ)
group B #110—831 (460 mμ), #110—832 (546 mμ)

Table II. Fluorescence Characteristics and UV Absorptions

Group	Compound Name	Structure R: 	pH-range	Fl-max[a] (mμ)	Ex-max[a] (mμ)	Ab-max (mμ)
A	4′-hydrazino-2-stilbazole (4H2S)	R-$NHNH_2$	7—12	510	380	355
	hydrazone of 4H2S with acetone	R-NHN=C(-CH_3)-CH_3	7—12	510	380	345
	hydrazone of benzaldehyde	R-NHN=C(-C_6H_5)-H	7—12	510	420	385
B	hydrazone of pyruvic acid	R-NHN=C(-CH_3)-C(=O)-OH	0— 3	550	410	410
	hydrazone of methyl pyruvate	R-NHN=C(-CH_3)-C(=O)-O-CH_3	0— 3	550	430	408
	hydrazone of diacetyl	R-NHN=C(-CH_3)-C(=O)-CH_3	0— 3	550	410	405
	hydrazone of glyoxal	R-NHN=C(-H)-C(=O)-H	0— 3	550	410	408

apparatus: Hitachi Spectrophotometer EPU-2A, G-1
concentration: 2.5×10^{-5}M in MeOH
a) uncorrected

hydrazones of the other α-oxo acids were similar to that of pyruvic acid as shown in Table III.

As the most suitable conditions for condensation of 4H2S with α-oxo acids, 60 min, 20—30° and pH 4 were selected from the data shown in Table IV.

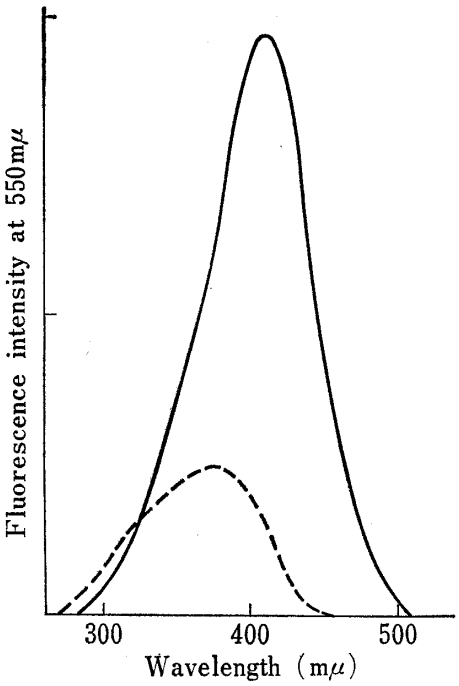


Fig. 2. Excitation Spectra of P-Z and 4H2S

------: 2×10^{-5}M 4H2S in MeOH soln.
——: 10^{-6}MP-Z in 50% MeOH soln.
apparent pH: 0.42
slit: 12(ex), 0.8(fl)mm
apparatus: Hitachi Fluorescence Spectrophotometer, MPF-2A
λ_{max}: 410mμ for P-Z, 380 mμ for 4H2S

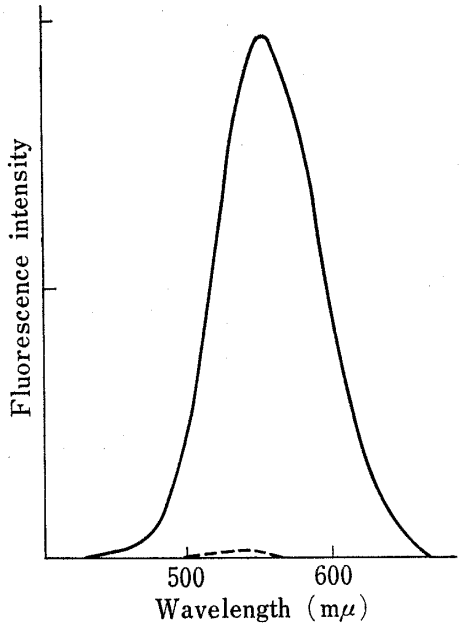


Fig. 3. Fluorescence Spectra of P-Z and 4H2S

------: 2×10^{-5}M 4H2S in MeOH soln.
——: 10^{-6}M P-Z in 50% MeOH soln.
apparent pH: 0.42
slit: 4(ex), 1.3(fl)mm
exciting wavelength: 450 mμ
apparatus: Hitachi Fluorescence Spectrophotometer, MPF-2A
λ_{max}: 550 mμ

Table III. Fluorescence Characteristics of 4H2S-hydrazones

4H2S-hydrazone	Fl-max[a] (mμ)	Ab-max[b] (mμ)	ε[b] ($\times10^4$)	RFI[c]
P-Z	550	410	4.63	100.0
HS-P-Z	550	410	3.86	91.3
KB-Z	550	407	4.76	82.5
MeS-KB-Z	545	405	3.20	78.6
KV-Z	550	407	3.78	87.4
KIV-Z	550	416	4.92	87.7
KIC-Z	550	413	5.15	103.1
KMV-Z	545	417	3.75	66.0
KG-Z	550	407	3.98	93.2
PP-Z	550	406	4.40	102.5
Cl-PP-Z	550	408	4.77	84.0
PH-PP-Z	550	410	3.72	84.0
G-Z	550	410	4.61	99.3
OA-Z	550	410	4.58	98.9

RFI: taken P-Z as 100.0 pH: 1.1
a) concentration: 2.5×10^{-5}M in MeOH, apparatus: Hitachi Spectrophotometer, EPU-2A, G-1
b) concentration: 2.5×10^{-5}M in MeOH, apparatus: Cary Spectrophotometer, Model 11 Uncorrected, equal to excitation main max.
c) concentration: 5×10^{-5}M in MeOH, apparatus: Turner Fluorometer, Model 110, filter: primary No. 110—831, secondary No. 110—832

Table IV. Effect of pH, Reaction-time and Temperature on the Relative Fluorescence Intensity produced by Reaction of 4H2S with Pyruvic Acid

pH	3			4			5		
Temp. (°C) / Time (min)	20—30	40	50	20—30	40	50	20—30	40	50
15	106	96	97	100	91	93	97	88	93
30	107	97	90	105	83	82	101	68	62
60	104	87	84	105	72	59	96	66	48
120	103	82	77	105	68	35	90	58	34

concentration of pyruvic acid: 1.6×10^{-5}M apparatus: Turner Fluorometer, Model 110

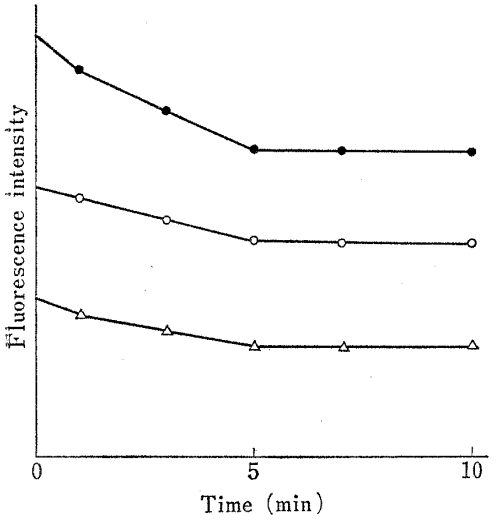


Fig. 4. Stabilization of Fluorescence Intensity of 4H2S-hydrazone by Irradiation at 405 mμ

Concentration:
—●— 2.0×10^{-4}M pyruvate
—○— 1.5×10^{-4}M pyruvate
—△— 1.0×10^{-4}M pyruvate
pH: 1.1
apparatus: hitachi fluorescence spectrophotometer MPF-2A

Durnig the measurement in strong acid media, fluorescence intensity of produced hydrazone decreased a little, and so, before the determination, it was irradiated by a light of 405 mμ for 10 min, owing which the fluorescence intensity was stabilized as shown in Fig. 4. The figure indicates the existence of isomeric equilibrium (perhaps *cis* and *trans*) caused by the irradiation.

Procedure for Determination of α-Oxo Acids

Reagents

a) Aqueous solution of specimen of α-oxo acids
b) Buffer solution, pH 4 (1N HCOONa 2 vol+0.5N HCl 1 vol)
c) 4H2S·2HCl 15 mg is disolved in water to 100 ml
d) 1N HCl

To 1.0 ml of a) each 1.0 ml of b) and c) were added, mixed throughly, and allowed to stand for 1 hour at 20—30° in a dark place. To this mixture, 2.0 ml of d) was added, mixed well, and after irradiation by a light of 405 mμ, at 10 min, the fluorescence intensity was measured at the fluorescence maximum wave length (550 mμ) by the excitation at 450 mμ. The blank solution was similarly prepared and the blank value was taken off from the fluorescence intensity.

Limit and Scope

Linear relationship between the concentration and fluorescence intensity existed with pyruvic acid below 8×10^{-5}M. The limit of determination of pyruvic acid was 2×10^{-6}M (Fig. 5) as at that concentration the fluorescence intensity became only twice of that of the blank.

Other α-oxo acids were determined under the same condition and their fluorescence intensity showed approximately the same value with pyruvic acid (Table V) excepting KIV and KMV.

But the other carbonyl compounds in biological materials did not fluoresced as shown in Table VI.

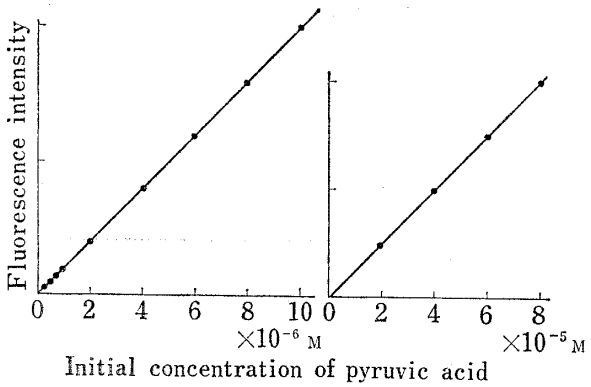


Fig. 5. Scope of Relationship between Fluorescence Intensity and Concentration of Pyruvic Acid

apparatus: hitachi fluorescence spectrophotometer MPF-2A

Table V. Relative Fluorescence Intensity of α-Oxo Acids (taken Pyruvic Acid as 100)

α-Oxo acid	G	P	HS-P	KB	MeSKB
RFI	91.8	100.0	92.2	79.8	70.5
α-Oxo acid	KV	KIV	KIC	KMV	OA
RFI	82.8	33.8	90.2	13.5	89.9
α-Oxo acid	KG	PP	Cl-PP	PHPP	
RFI	92.5	79.9	87.7	85.3	

concentration: 5×10^{-5}M
apparatus: Turner Fluorometer, Model 110
filter: primary No. 110—831, secondary No. 110—832

Table VI. Relative Fluorescence Intensity of Other Substances (taken Pyruvic Acid as 100)

Ketones		Aldehydes		Sugars		Others	
Acetone	0	formaldehyde	2.5	glucose	0	ovalbumin	0
Methyl ethyl ketone	0	acetaldehyde	0	xylose	0	heparin	0
Cyclohexanone	0	propionaldehyde	0	sodium 2-oxogluconate	0	disodium citrate	0
Acetophenone	0	caprylaldehyde	1.5	glucuronic acid	0	lactic acid	0
Diacetyl	54.2	capronaldehyde	0	ascorbic acid	0	levulinic acid	0
Glyoxal	27.0	benzaldehyde	0.7				
Acetylacetone	0	salicylaldehyde	4.6				
		p-anisaldehyde	3.1				

apparatus: Turner Fluorometer, Model 110
concentration: 2×10^{-5}M
filter: primary No. 110—831, secondary No. 110—832

Fluorometry of Total α-Oxo Acids in Human Blood

The main α-oxo acids in blood are pyruvic acid and α-oxoglutaric acid, and their fluorescence intensity of equimolar solutions were approximately equal as shown in Table V. Therefore, pyruvic acid was adopted as standard substance for the fluorometry of total α-oxo acids in blood.

To 1.0 ml of 0.33M $HClO_4$, 0.1 ml of blood was added, mixed vigorously and centrifuged, then 0.5 ml of supernatant was taken and fluorescence intensity was measured by the procedure described above.

For the estimation of total concentration of α-oxo acids, two calibration methods (I and II) were compared. In the first (I), a standard solution of pyruvic acid was used as the reference, while the second (II) was a standard addition extrapolation method (Table VII).

The recovery data indicate that the method (II) was recommended to obtain correct value, because interfere substances for fluorescence reaction, though a little, exsist in blood. The reliability of the method (II) is confirmed by a linear line obtained by plotting $(B)_1$ $(B)_2$ and $(B)_3$ (Fig. 6).

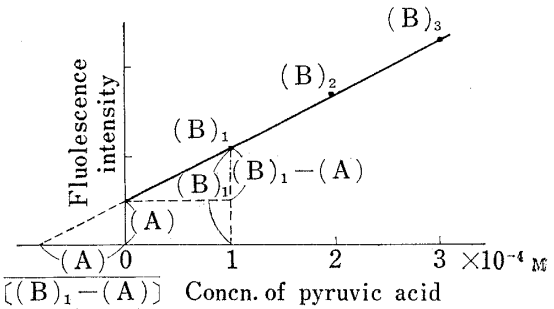


Fig. 6. Standard Addition Extrapolation Method

Table VII. Total α-Oxo Acids in Human Blood and Recovery of Standard Pyruvic Acid added to Blood

Human blood Sample No.	n[a]	Fluorescence intensity: Intrinsic (A)	Fluorescence intensity: After addition of pyruvic acid $(B)_n$	Fluorescence intensity: Pure pyruvic acid $(C)_n$	Recovery (%) (I)[b]	Recovery (%) (II)[c]	Total α-oxo acid (10^{-4}M)
1	1	12.4	27.8	16.3	94.5		0.81
	2		42.9	32.6	93.5	99.0	
	3		57.9	49.0	92.8	98.5	
2	1	32.6	47.4	16.8	88.1		2.20
	2		62.3	33.4	88.9	100.3	
	3		77.2	50.0	89.2	100.5	
3	1	15.4	29.3	15.6	89.1		1.11
	2		43.1	31.0	89.4	99.6	
	3		56.8	46.4	89.2	99.3	
4	1	15.4	31.6	18.1	89.5		0.95
	2		47.9	36.3	89.5	100.3	
	3		64.3	54.1	90.4	100.6	
5	1	14.7	31.2	17.8	92.7		0.89
	2		47.7	35.4	93.2	100.0	
	3		64.2	52.8	93.8	100.0	
average	1				90.8		
	2				90.8	98.8	
	3				91.1	99.8	

a) 0.5 ml of $n \times 10^{-4}$M pyruvic acid was added. b) $I = \frac{(B)_n - (A)}{(C)_n} \times 100$ c) $II = \frac{(B)_n - (A)}{n[(B)_1 - (A)]} \times 100$

Discussion

Variation of UV-spectra with pH were measured with typical 4H2S compounds in group A and B (see Fig. 1).

As shown in Fig. 7, there exist distinct differences between group A and B, namely in strong acid media group A scarcely shows absorbance at about 410 mμ (see Fig. 2), while group B shows strong absorbance. Such phenomena seem to be related to the selective characteristics of fluorescence.

Perchloric acid, used as deproteinizer, does not affect the fluorescence reaction of pyruvic acid, and does not decompose pyruvic acid (Table VIII).

As already described (see Table VII), deproteinized serum contains some interfering substances and the grade of interference varies serum to serum and so the standard addition

Table VIII. Stability of α-Oxo Acid kept in a Cold Room after Deproteinization with Perchloric Acid

Concn. of pyruvic acid ($\times 10^{-4}$M) / Period (days)	Aq. soln. 1.0	Aq. soln. 2.0	Aq. soln. 3.0	Blood sample 0.76	Blood sample 1.95	Blood sample 0.99	Blood sample 0.84
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	98.5	98.3	98.2	103.6	98.0	100.0
2	103.1	104.0	104.1	96.1	100.3	100.0	
3	104.1	102.6	102.4	98.7	100.1		
4	101.0	101.0	100.7				
6	101.0	101.0	101.4				

extrapolation method is recommendable. The values thus obtained for healthy ordinary human blood are almost similar to those in a report.[6]

The sensitivity of this fluorometric determination is higher than 100 fold in comparison with the existing colorimetry, therefore, only 0.1 ml of blood is quite enough for this method, while 2—5 ml of blood is required for the colorimetry.[3]

4H2S is selective reagent for α-oxo acids, moreover, as the wave lengths of excitation and measurement are unusually long, the method is not affected by other fluorescent substances in biological materials. Such specifisity can not be achieved by ordinal colorimetric methods.

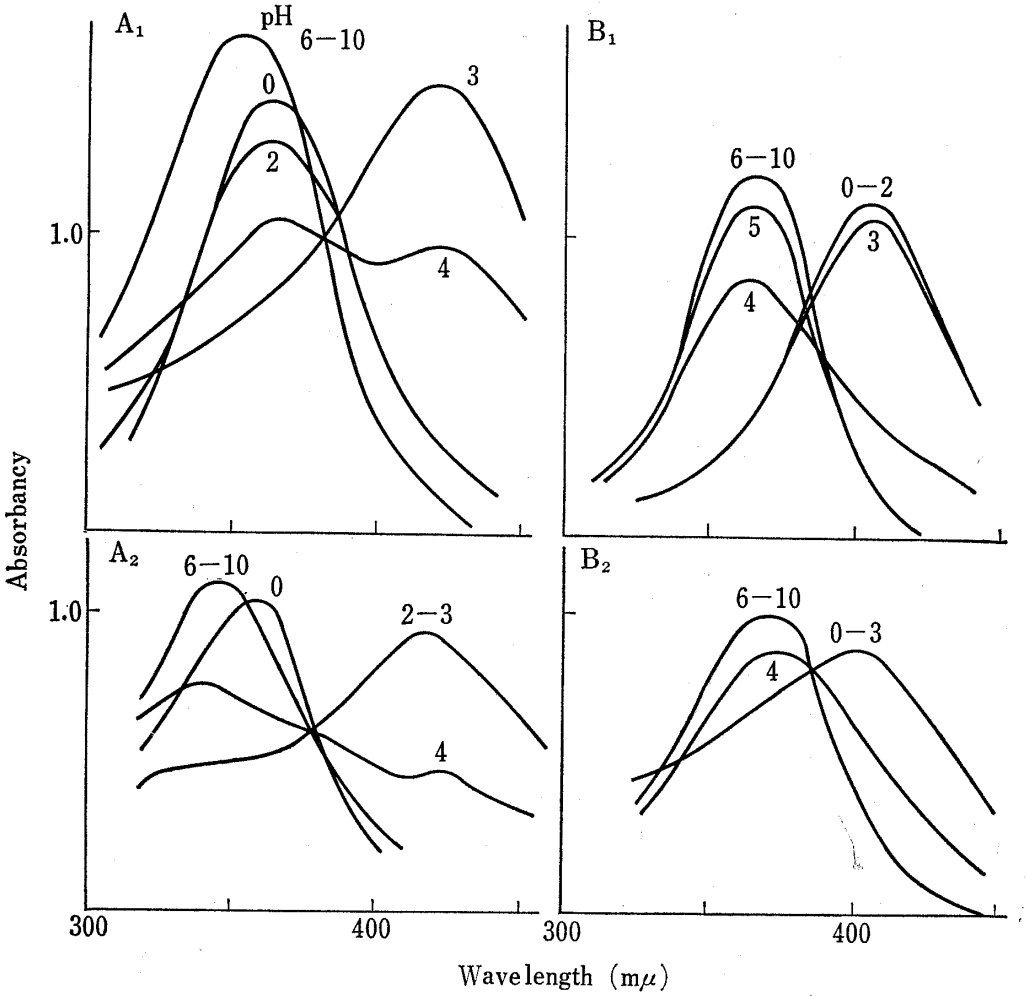


Fig. 7. Effect of pH on Absorption Spectra of Compounds in Group A and B

group A: A_1=4′-hydrazino-2-stilbazole (4H2S)
A_2=4H2S-hydrazone of acetone
group B: B_1=4H2S-hydrazone of pyruvic acid
B_2=4H2S-hydrazone of diacetyl
concentration: A_1, A_2……5.0×10^{-5}M in 50% MeOH
B_1, B_2……2.5×10^{-5}M in 50% MeOH
apparatus: cary spectrophotometer, Model 11

Experimental[7]

4′-Nitro-2-stilbazole (I)——A mixture of 5.4 g of *p*-nitrophenylacetic acid, 3.2 g of pyridine-2-aldehyde and 5 ml of piperidine was heated in an oil bath at 140°, 2 hr to finish evolution of CO_2, on cooling, ppt. was filtered and then recrystallized from EtOH to give yellow prisms, mp 126°. *Anal.* Calcd. for $C_{13}H_{10}O_2N_2$:

6) F.L. Iber, and T.C. Chalmers, *J. Clin. Invest.*, **36**, 706 (1957).
7) Melting points are uncorrected.

C, 69.01; H, 4.46; N, 12.38. Found: C, 68.96; H, 4.63; N, 12.27. On the otherhand, I was obtained from α-picoline and *p*-nitrobenzaldehyde in autoklave.[8]

4′-Amino-2-stilbazole (II)——I was reduced according to reference,[9] recrystallized from EtOH to give pale yellow needle, mp 139°. *Anal.* Calcd. for $C_{13}H_{12}N_2$: C, 79.56; H, 6.16; N, 14.28. Found: C, 79.81; H, 6.18; N, 14.14.

4′-Hydrazino-2-stilbazole (III)——A solution of 700 mg II in 7 ml of 20% HCl was diazotized by an aqueous solution of $NaNO_2$ (270 mg in 1 ml) at 0° to yellowish syrup, and then reduced by a solution of 4.2 g of $SnCl_2 \cdot 2H_2O$ in 4.2 ml of conc. HCl at 0°. To yellow precipitate 400 ml of water was added to dissolve and then 2 ml of conc. HCl was added, to which H_2S was passed. Precipitate was filtered off, the filtrate was concentrated *in vaccuo*, then neutralized to pH 8 with $NaHCO_3$. Produced precipate was filtered, washed with H_2O, then recrystallized from PrOH to give pale yellow prisms, in 75 mg (10%) yield, mp 138°. It was almost insoluble in ligroin, water, easily soluble in EtOH, MeOH, Benzene, EtOAc. *Anal.* Calcd. for $C_{13}H_{13}N_3$: C, 73.90; H, 6.20; N, 19.89. Found: C, 73.84; H, 6.11; N, 20.14.

4′-Hydrazino-2-stilbazole Dihydrochloride——One gram of III was dissolved in 9 ml of 4% HCl and filtered. The filtrate was added 10 ml of conc. HCl. Produced yellow precipitate was recrystallized from dil. HCl to give yellow prism, in 80% yield, mp 200°, soluble in water, EtOH, MeOH.

Anal. Calcd. for $C_{13}H_{15}N_3Cl_2$: C, 54.94; H, 5.32; N, 14.79. Found: C, 53.67; H, 5.61; N, 14.42.

α-Oxo Acids——Pyruvic acid was refined[10] from commercial source. Other α-oxo acids were synthesized by the method desribed: G,[11] P-Li,[10] P-Me,[12] HS-P,[13] KB,[14] KV,[14] KIV,[14] KIC,[14] KMV,[14] MeSKB,[15] OA,[16] KG,[17] PP,[18] ClPP,[14] PHPP.[19]

Hydrazones of 4H2S with α-Oxo Acids——Refer to Table I concerning each properties.

a) 4H2S was disolved in EtOH, to which a solution of equivalent α-oxo acid in EtOH was added warmed in a water bath. Separate hydrazone was recrystallized from *p*-dioxane or dil MeOH. By this method, P-Z, OA-Z, KG-Z, PP-Z, Cl-PP-Z, and PHPP-Z were synthesized.

b) To an aqueous solution of 4H2S·2HCl, an aqueous solution of equivalent α-oxo acid sodium or ammonium salt was added, warmed in a water bath and added aqueous solution of NaOAc. Separate hydrazone was recrystallized from *p*-dioxane or MeOH. By this method, G-Z, HS-P-Z, KB-Z, MeS-KB-Z, KV-Z, KIV-Z, KIC-Z, and KMV-Z were synthesized.

P-Me-Z——was synthesized instead of α-oxo acid using methyl pyruvate by method b), yellow amorphous, mp 187°. *Anal.* Calcd. for $C_{17}H_{17}O_2N_3$: C, 69.13; H, 5.80; N, 14.23. Found: C, 69.52; H, 5.98; N, 13.98.

Glyoxal 4H2S-Monohydrazone——Was prepared by method b), instead of α-oxo acid, 40% aqueous solution of glyoxal was used, red amorphous, mp 196°. *Anal.* Calcd. for $C_{15}H_{13}ON_3$: C, 71.69; H, 5.21; N, 16.72. Found: C, 71.82; H, 5.26; N, 16.41.

Diacetyl 4H2S-Monohydrazone——Was prepared by method b), orange red amorphous, mp 230°. *Anal.* Calcd. for $C_{17}H_{17}ON_3$: C, 73.09; H, 6.13; N, 15.04. Found: C, 72.91; H, 6.36; N, 14.94.

Acetone 4H2S-Hydrazone——Was prepared by method b), yellow needle, mp, 128°. *Anal.* Calcd. for $C_{16}H_{17}N_3$: C, 76.46; H, 6.82; N, 16.72. Found: C, 75.97; H, 6.61; N, 16.52.

Benzaldehyde 4H2S-Hydrazone——Was prepared by method a), using a solution of benzaldehyde in MeOH, instead of α-oxo acid, yellow prisms, mp 187°. *Anal.* Calcd. for $C_{20}H_{17}N_3$: C, 80.24; H, 5.72; N, 14.04. Found: C, 79.79; H, 5.70; N, 14.04.

Standard Substance——Lithium pyruvate prepared according to Wendel[10] was used. $CH_3C(OH)_2COOLi$ or $CH_3COCOOLi \cdot H_2O$, mol. wt. 112.012. *Anal.* Calcd. for $C_3H_5O_4Li$: C, 32.14; H, 4.46. Found: C, 32.01; H, 4.24.

Acknowledgement The authers are indebted to the members of the Central Analysis Room of this Faculty for elemental analyses.

8) B.D. Shaw and E.A. Wagstaff, *J. Chem. Soc.*, **1933**, 78; K. Feist, *Chem. Ber.*, **34**, 466 (1901); *idem*, *Arch. Pharm.*, **240**, 249 (1902).
9) R. Baumert, *Chem. Ber.*, **39**, 2972 (1906).
10) W.B. Wendel, *J. Biol. Chem.*, **94**, 717 (1932).
11) N.S. Radin and D.E. Metzler, "Biochem. Prep.," **4**, 60 (1955).
12) A. Weissberger and C.J. Kibler, "Org. Synth.," Coll. Vol. III, 610 (1955).
13) J. Ward, *J. Chem. Soc.*, **123**, 2209 (1924).
14) F. Weygand, W. Steglich, and H. Tanner, *Ann.*, **658**, 128 (1962).
15) A. Meister, *J. Biol. Chem.*, **197**, 309 (1952).
16) C. Heidelberger, "Biochem. Prep.," **3**, 59 (1953).
17) L. Friedman and E. Kosower, "Org. Synth.," Coll. Vol. III, 510 (1955).
18) R.M. Herbst and D. Shemin, "Org. Synth.," Coll. Vol. II, 519 (1943).
19) G. Billek, "Org. Synth.," **43**, 49 (1963).