

## CHROMATOGRAPHIC STUDY OF "REIGELHAUPT" CHROMOGENS IN URINE\*

ARTHUR YUWILER† and MARY H. GOOD

Schizophrenia and Psychopharmacology Joint Research Project of the  
University of Michigan and the Ypsilanti State Hospital

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CONSIDERABLE neurochemical and pharmacological evidence has accumulated suggesting a relationship between indole metabolism and mental illnesses such as pellagra, Hartnup's disease and phenylketonuria. Recently these studies have been extended to schizophrenia with encouraging but inconclusive results. Among the many findings is the discriminatory test proposed by REIGELHAUPT<sup>(1)</sup> who observed a marked incidence of "positive" Hopkins Cole reactions among urines from schizophrenics. Although others have confirmed these results we were unable to do so in a previous study,<sup>(2)</sup> when schizophrenic and non-schizophrenic subjects were identically maintained in respect to diet, medication and ward environment. Rather, a preponderance of positive reactions was obtained on subjects in both groups.

The Reigelhaupt test consists of the formation of violet chromogens at the interface between sulfuric acid and a mixture of glyoxylic acid, urine and copper sulfate. Because it is conceivable that our experimental conditions introduced "spurious" chromogens masking the "true" chromogens, the present study was undertaken to explore the nature of the contributory compounds. The relationship of chromogens to diagnosis, urinary toxicity, and "abnormal" aromatic amino acid metabolism was also examined. To this end the Reigelhaupt test was adapted for chromatographic use and applied to: whole urine concentrates; *n*-butanol extracts which reportedly concentrate Reigelhaupt chromogens<sup>(3)</sup>; extracts of charcoal treated urines producing differential toxicity to mice<sup>(4)</sup>; butenone-2-extracts reportedly containing differential quantities of 6-hydroxyskatole sulfate<sup>(5)</sup>; and urine concentrates from subjects receiving phenothiazine medication.

The results obtained will be discussed with regard to the apparent significance of 6-hydroxyskatol sulfate, Mauve factor, Depression factor, and bufotenine in urine, as well as to the nature of Reigelhaupt chromogens.

### METHOD

Subjects were drawn from the population of the Ypsilanti State Hospital upon unanimous diagnostic agreement obtained independently by three psychiatrists. The subjects were maintained on a constant repetitive diet with vitamin supplements throughout the course

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† Present address (July 20, 1962) Neurobiochemistry Laboratory, Veterans Administration Center, Sawtelle and Wilshire Boulevards, Los Angeles 25, California.

of the study. Medication was discontinued two months before initial testing and reinstated after test completion when drug studies were carried out. Urine concentrates of a total of 40 schizophrenics (24 chronic undifferentiated, 1 simple, 1 hebephrenic, 9 paranoids, and 5 type uncertain) and 17 non-schizophrenics (5 non-psychotic chronic brain syndromes, 8 sociopaths, 3 alcoholics, 1 psychotic brain syndrome) were examined for Reigelhaupt chromogens. Butanol extracts were prepared from the urines of 5 schizophrenics (chronic undifferentiated); concentrates of urinary toxic factors from 2 non-schizophrenics (sociopaths), 5 schizophrenics (2 paranoid and 3 chronic undifferentiated), and 3 subjects of uncertain diagnosis; butenone extracts, from 18 schizophrenics (9 chronic undifferentiated, 7 paranoid, 2 type uncertain) and 8 non-schizophrenics (5 sociopaths, 3 chronic brain syndrome); and concentrates from subjects on phenothiazines, from 5 schizophrenics (3 paranoid and 2 chronic undifferentiated).

#### *Specimens*

Urines were collected over a twenty-four hour period and refrigerated between collections. Aliquots of the twenty-four hour urine were either used immediately or stored at  $-15^{\circ}\text{C}$ . The Reigelhaupt test was carried out in the manner previously described, using mixtures of copper sulfate and glyoxylic acid.<sup>(2,6)</sup> In some studies, only samples giving clear positive tests at the time of preparation as well as at the time of initial collection were chromatographed, so as to minimize decomposition of abnormal chromogens.

#### *Procedures*

Samples of whole and treated urine were concentrated either by lyophilization or by reduced pressure evaporation in a flash evaporator at a bath temperature of  $40^{\circ}\text{C}$ . Both methods gave identical chromatographic results. Precipitate formed during evaporation was left in contact with the concentrated liquid and chromatogrammed, either by dabbing onto the origin or by application after solution in acetone-water. Chromatograms were spotted in quadruplicate; each spot containing the equivalent of 0.5–10 ml of initial urine, depending upon the experiment and the sample used. Comparisons between diagnostic groups were carried out using that amount of concentrate containing 0.50 g of creatinine.

Butanol extracts were prepared by shaking 100 ml of acidified whole urine with 50 ml of *n*-butanol, centrifuging and concentrating. The method of SPRINCE *et al.*<sup>(5)</sup> was used to prepare butenone-2 extracts. Concentrates of urinary factors toxic to mice were obtained through the courtesy of Dr. N. S. Ging.

Chromatographic analysis was carried out in a Chromatocab, using Whatman 1 paper as the stationary phase. The usual solvent systems employed were *n*-butanol; acetic acid: water (12:3:5) and isopropanol: ammonium hydroxide: water (8:1:1) but, in special instances other mixtures were employed as described in the text. Acid chromogens were detected with a spray of sulfuric acid (9N), copper sulfate ( $6.5 \times 10^{-3}$  M) and glyoxylic acid (1.2 M). Other location and class reagents are described in the body of the paper. Standards were run with each paper.

Glyoxylic acid was obtained either from Calbiochem or freshly prepared from oxalic acid and magnesium to give a 9.2% solution. Chlorpromazine excretion was monitored by the method of EIDUSON and WALLACE<sup>(7)</sup> and spectrophotometric analysis was carried out using a Beckman DU or a Coleman Universal.

## RESULTS

*Comparison of sulfuric acid sprays*

Various authors have carried out the Reigelhaupt test with glyoxylic acid, acetic acid or water added to a sulfuric acid-copper sulfate mixture. Although all three reagent mixtures appeared to yield very similar qualitative results<sup>(2,6)</sup> it seemed important to determine whether differences existed in their specificity or reactivity. Consequently, urine concentrates equivalent to 0.5-0.9 ml of whole urine were spotted on Whatman 1, developed with isopropyl: ammonia: water (8:1:1), and separate sheets sprayed with sulfuric acid-copper

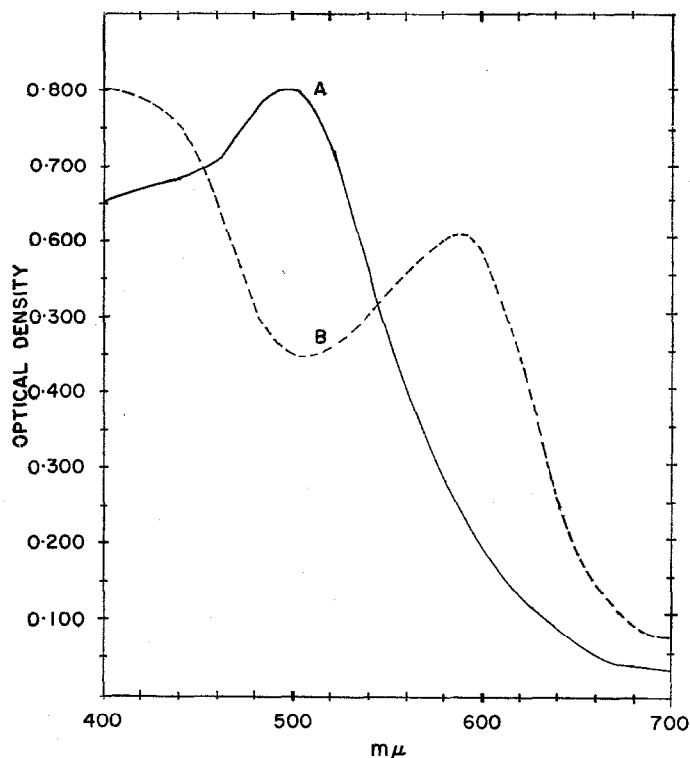


FIG. 1. Absorption spectra of indican chromogens.

Indican absorbed on Whatman 1 was treated with a spray mixture of 9N sulfuric acid and  $6 \times 10^{-8}$  M copper sulfate with (solid line) and without (dotted line) 1.2 M glyoxylic acid. The developed chromogen was eluted with acetone and the spectrum determined with a Coleman Universal.

sulfate mixtures containing glyoxylic acid (1.2 M), glyoxylic acid (1%), acetic acid (8.7 N), or water. Both urine concentrates and untreated whole urine from these subjects gave "positive" glyoxylic acid tests. Indican and 5-hydroxyindole-3-acetic acid were employed as chromatographic markers. All four reagents were found to produce identical patterns of chromogens; however, indican developed a reddish mauve upon treatment with the glyoxylic acid sprays rather than the purple wine observed after the other sprays (Fig. 1).

Despite the apparent equivalence of these mixtures, the glyoxylic acid spray was routinely employed to preclude the possibility of unique reactions.

#### *Specificity and sensitivity of spray reagent*

A variety of authentic substances was studied for chromogen production under chromatographic and Reigelhaupt test conditions. The results in Table 1 show good agreement between these procedures despite some differences. Chromogen formation on paper was considerably slower than that in solution and followed a well defined sequence of color development usually proceeding from yellow through pink to some shade of the final hue. Ambient temperature appeared related to the rate of chromogen production and concentration sometimes modified the tint of hues observed. In the series tested, indican was the first to appear (5–15 min) and tryptophan the last (30–60 min).

Differences in the shade of chromogens developed in solution and on paper were not uncommon and, in the case of serotonin, the final hue differed; green to brown-red chromogens developing in solution and pink-green, green-red or purple ones developing on paper. However, in no case was a chromogen detected by the Reigelhaupt test without the corresponding appearance of a distinctive, and usually related, chromogen on paper. The converse was not always true. For example, tryptophan slowly formed a marked red to purple chromogen on paper while only a feeble yellow was seen in the ring test.

The sequential changes in chromogens formed on paper had their parallel color variations in the ring test. Thus, indole variously formed yellow, orange, red, or violet chromogens, psilocybin green to violet ones, and indolacetamide and indoleacetic acid yellow to pink violet ones, apparently depending on the sharpness of the interface and the temperature accompanying sulfuric acid ionization.

Patterns similar to those obtained with the glyoxylic acid spray were observed using 4 N HCl containing copper sulfate (2.6%) or ferric chloride (1%), sodium nitrite in nitric acid, and ferric chloride in perchloric acid, and these mixtures were employed as ancillary sprays.

The glyoxylic acid spray routinely employed could detect less than 2.0  $\mu\text{g}$  of indole-3-acetic, indican, or bufotenine and 5  $\mu\text{g}$  of 5-hydroxyindoleacetic acid. Sensitivity towards other compounds was not determined.

#### *Chromogens in whole urine*

*Indican.* All concentrates contained varying quantities of a substance, identified as indican (Fig. 2), forming a reddish purple upon exposure to glyoxylic acid sprays. This material showed intense, pale blue fluorescence under ultraviolet; an orange-brown color after p-dimethylaminobenzaldehyde and a positive Fluorindal upon exposure to concentrated ammonium hydroxide; a faint orange changing to orange-pink with diazosulfanylic acid; a very light grey with ammonical silver; faint yellow with p-nitroanaline becoming olive after sodium carbonate; an initial pink becoming blue after ferric chloride in HCl; and no reaction with 1-nitroso-2-naphthol. The substance could be removed from neutral urine by passage through a Dow 2 (C1) or Dow 1 (C1) column but freely passed Dow 50 (H) and IRC-50 (H). Subsequent elution from Dow 2 could not be achieved. These properties were all identical with those observed for authentic indoxylsulfate run concurrently.

CHROMATOGRAPHIC STUDY OF "REIGELHAUPT" CHROMOGEMS IN URINE

The R<sub>f</sub> of this substance was higher than that of authentic indican when crude urine concentrates were developed with either acidic or basic solvent systems. Band-splitting often could be detected, with urea sandwiched between a top and bottom layer of chromogen, the bottom layer having the R<sub>f</sub> of authentic indican. Both layers produced identical chromogens to the full spectrum of location reagents, both were removed from neutral

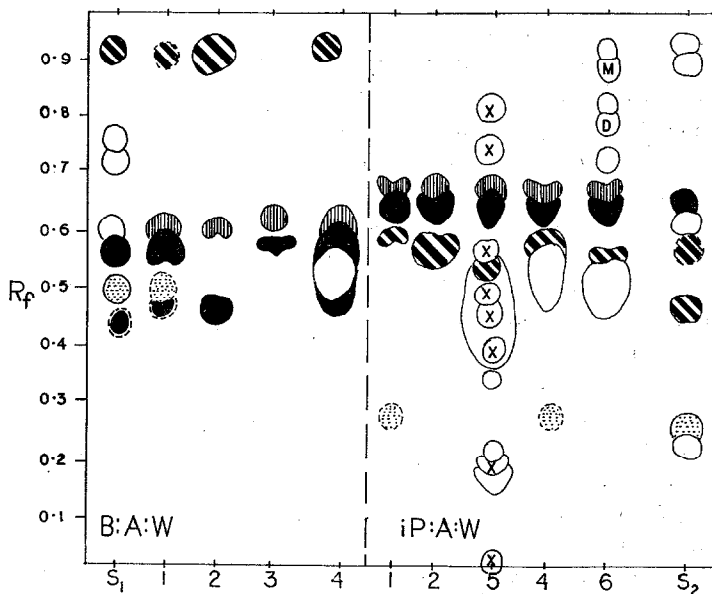


FIG. 2. Composite chromatographic patterns.

Indican designated by black areas, indole-3-acetic acid by lateral stripes, tryptophan by dotted areas and 6-hydroxyskatole sulfate by thin vertical stripes. Dotted outlines designate infrequent occurrence. S<sub>1</sub> Standards. In order of decreasing R<sub>f</sub>, indole-3-acetic, 5-hydroxy-indole-3-acetic, tryptamine, bufotenine, indican, tryptophan. Dotted blackened area shows position of indican in the absence of urea.

1. Urine concentrate developed with glyoxylic acid spray.
2. Butanol extract of urine developed with glyoxylic acid spray.
3. Urinary "toxic" factor extract developed with glyoxylic acid spray.
4. Urine concentrate developed with Ehrlich's reagent.
5. Urine concentrate of subject on chlorpromazine developed with glyoxylic acid spray. Areas marked "X" were removed by passage through Amberlite IRC-50.
6. Butenone extract developed with Ehrlich's reagent. "M" designates "Mauve Factor", "D" designates "Depression Factor".

S<sub>2</sub> Standards. In order of decreasing R<sub>f</sub>, bufotenine, tryptamine, indican, serotonin, indole-3-acetic acid (in presence of urea), indole-3-acetic acid, tryptophan and 5-hydroxyindole-3-acetic acid.

Developed chromatograms were copied and reduced in size by use of a pentagraph and R<sub>f</sub>'s were corrected on the basis of concurrently run standards.

solution by Dow 2 (Cl) and Dow 1 (Cl), and the Fluorindal reaction showed permeation, through urea, between the bands. Permeation could be enhanced by addition of authentic indican to the sample, and both R<sub>f</sub> elevation and band splitting could be replicated by addition of urea to authentic indican. Urease pretreatment of urine concentrates decreased

the  $R_f$  so that it was equal to (basic solvent systems) or only slightly greater than (acidic systems) that obtained with synthetic indican and eliminated band splitting. Both indican and the urinary substance had identical  $R_f$ 's in the neutral solvent systems *n*-butanol: ethanol: water (4:1:1), and 75% ethanol in water. Two-dimensional chromatography of a urine concentrate-indican mixture with isopropanol: ammonia: water (40:1:9), and *n*-butanol: acetic acid: water (4:1:1) revealed only one spot with the characteristic red-wine of indican.

Because of its universal appearance, no diagnostic significance could be attached to the qualitative presence of this material. Further, there appeared to be no relationship between diagnosis and a crude quantitative index, based upon a one-to-five rating of chromogen intensity divided by the urinary equivalent applied.

*6-Hydroxyskatole Sulfate.* Glyoxylic acid treatment of chromatograms often elicited a stable blue chromogen with a characteristic position just above indican in both acidic and basic solvents. Darkened trails of fast running compounds sometimes obscured its presence in the former case. This material corresponded in  $R_f$  to a bright blue-green fluorescent area most clearly seen after development with isopropanol: ammonia: water mixtures. The fluorescent characteristics of this substance are shown in Fig. 3.

Treatment with Ehrlich's dip resulted in the appearance of a bright cobalt blue which faded at a variable rate, sometimes completely disappearing in less than a minute, on other occasions changing to a relatively persistent greyish-blue-green. This variance was a characteristic of the run and did not reflect differences in the samples under examination. Exposure to ammonium hydroxide in the Fluorindal reaction led to the immediate disappearance of color without eliciting discernible fluorescence. Diazotized sulfanilic acid — ammonium sulphamate elicited the violet chromogen presumed characteristic of 6-hydroxyindoles. The material could be removed from neutral solutions by passage through Dow 1 (C1) but not Dow 50 (H) and could be partly extracted into butanol and methyl ethyl ketone.

Although authentic material was not available for comparison, the chromatographic characteristics, color reactions, and ionic properties of this substance are in conformity with those reported for 6-hydroxyskatole sulfate.<sup>(6,8)</sup> Failure to elicit the orange-red Fluorindal fluorescence, reportedly<sup>(9)</sup> characteristic of skatole indicanoids, may be due to interfering substances in the samples or, more likely, may indicate that 6-hydroxyskatole sulfate is an exception to this reaction since others<sup>(3,13)</sup> have also obtained negative reactions with their corresponding materials.

This substance also appears to be identical to that material variously called "Q. F. B.",<sup>(3,10)</sup> "Spot 2",<sup>(11)</sup> "5",<sup>(12)</sup> "U-2",<sup>(13)</sup> and "Q",<sup>(14)</sup> reported more frequently in urine from schizophrenics than from normals. In this study, Ehrlich's reagent indicated the presence of this substance in 65 per cent of the urines from schizophrenics, it appeared to be absent in 30 per cent and was equivocal in 5 per cent. On the other hand, 63 per cent of the urines from non-schizophrenics showed this material, it was absent in 27 per cent and equivocal in 10 per cent. If the blue chromogen produced with glyoxylic acid was taken as a criterion, 46 per cent of the schizophrenics showed this substance as against 43 per cent of the non-schizophrenics. A blue glyoxylic acid chromogen always had a corresponding cobalt blue Ehrlich's reactor. The converse was not always true, presumably because of the

CHROMATOGRAPHIC STUDY OF "REIGELHAUPT" CHROMOGEMS IN URINE

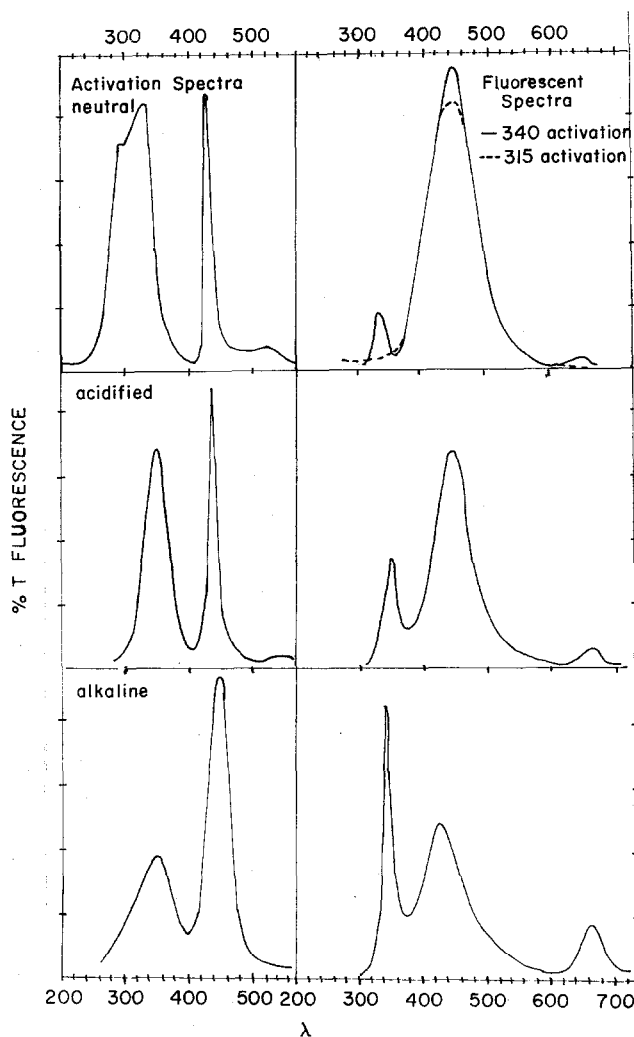


FIG. 3. Fluorescent characteristics of 6-hydroxyskatole sulfate (?)

Material thought to be 6-hydroxyskatole sulfate was located on chromatograms by characteristic placement relative to indican, blue green fluorescence, and position, of material forming a quickly-fading cobalt blue after Ehrlich's reagent and a blue grey after glyoxylic acid reagent. Corresponding regions of the untreated chromatogram were excised, homogenized in water and passed through a Millipore filter. Fluorescent characteristics of the solution were determined in neutral, acidic (2 N HCl), and basic (2 N NaOH) solution using an Aminco Bowman Spectrofluorophotometer.

greater sensitivity of the Ehrlich's reagent. The Reigelhaupt test on a butanol concentrate of this material eluted from chromatograms elicited a pale brown, violet, to red ring and a lavender solution after mixing (Table 1).

The incidence of this substance in urines from schizophrenics agrees with other findings. The high incidence among controls, however, differs from some reports, especially those

## ARTHUR YUWILER AND MARY H. GOOD

TABLE 1. COMPARISON OF ACID CHROMOGENS UNDER REIGELHAUPT AND CHROMATOGRAPHIC CONDITIONS

Compound***	Reigelhaupt Test*		Chromatographic**	
	Ring	Solution	0-2 hr	24-48 hr
<b>Indoles</b>				
Bufotenine Oxalate‡	G-GB	G	R-G	GBk
Dimethyl tryptamine§	P	R-Bn	G	G
5-Hydroxytryptophan‡	G	G	G	G
5-Hydroxytryptamine creatinine sulfate‡	RBn-G	G	R-G	GyG-P
5-Hydroxyindoleacetic acid cyclohexylamine‡	YG-P	G-GyP	Pi-G	GyG
6-Hydroxyskatole sulfate?††	Y-B-R	Pi-R	B	BGy
Indole†	Y-O-R-V	pY-R	O	R
Indole-3-carboxylic acid‡‡	fY	fY	X	—
Indole-3-acetic acid†	Y-PiV	YPi-V	Y-Pi	GyG-Pi
Indole-3-propionic acid†	GY	Y	X	—
Indole-3-butyric acid†	Y	Y	X	—
Indole-3-acetamide¶	Y-PiV	Y-V	Y-Pi	Pi-G
Indoxyl-o-sulfate‡	V-PR	V	Pi-RV	RV
Psilocybin§	G-V	G	GyG	GyG
Skatol	Y-R	YO-OR	Y-R	P-PR
Tryptamine . HCl†	YBn	RBn	Y-Pi	GyG
Tryptophan†	Y	X-Y	fPi	PB
<b>Catecholes</b>				
3,4-Dihydroxy phenylalanine‡	X-Gr	Gr	Pi(s)	Bn(s)
Epinephrine . HCl‡	Y	Y	fPi(s)	Bn(s)
Norepinephrine . HCl‡	Y	Y	fPi(s)	Bn(s)
<b>Phenothiazines</b>				
Chlorpromazine . HCl	RV	RV	RV	RV
Imipramine§§	YG	fG	X	Y
Prochlorperazine	RV	RV	RV	RV
Trifluoperazine	RV	RV	RV	RV
<b>Miscellaneous</b>				
Creatinine†	X	X	X	X
Histamine†	X	X	X	X
Kynurenine†	X	X	X	X
Kynurenic acid†	X	X	X	X
Proline†	X-fY	X	X	X
Thorzylamine . HCl	RV	RV-P	Pi	Pi
Urea†	X	X	X	fYBn
Uric acid†	X	X	X	X

\* The reaction solution consisted of the compound in 3.2 ml of 1.2 M glyoxylic acid and  $6.5 \times 10^{-3}$  M copper sulfate to which was added 0.2 ml of *n*-butanol. The chilled solution was slowly underlayered with 2 ml of 36  $\text{NH}_4\text{SO}_4$  and the color of sharp and stirred interface noted (column 1). The two phases were slowly mixed by swirling in an ice bath and the color of the solution estimated after 10 min (column 2).

\*\* Compounds were chromatogrammed in isopropanol : ammonium hydroxide : water (8:1:1) and chromogens developed with a spray consisting of sulfuric acid (9N), copper sulfate ( $6.5 \times 10^{-3}$  M) and glyoxylic acid (1.2 M). Some compounds such as tryptamine . HCl<sub>2</sub> formed "iso-smears" under these conditions and the colors reported are those for both major and minor spots.

\*\*\* †Nutritional Biochemicals; ‡Calbiochem; §Sandoz Pharmaceuticals; ¶Smith Kline and French; ¶Mann Biochemical; ††eluted from undeveloped chromatograms (see text); ‡‡courtesy Dr. F. Campbell, Parke Davis & Co.; §§Geigy.

Symbols: X = no color; B = blue; Bn = brown; Bk = black; G = green; Gy = grey; O = orange; P = purple; Pi = pink; R = red; V = violet; Y = yellow; f = faint; (s) = streaks; — color range.



using hospital personnel or normal volunteers as the comparison population. It does agree with other findings, of high incidence among all mentally ill subjects<sup>(11)</sup> and among "organic psychosis," "complicated affective disorders," and "complicated neurosis".<sup>(10)</sup>

*Indole-3-acetic acid.* Two pinkish spots also were seen occasionally in whole urine. The first was tentatively identified as indole-3-acetic acid on the basis of: co-chromatography with authentic indole-3-acetic acid; oxidation by nitrite-nitric acid to a bright pink slowly going to brown; reaction with ferric chloride-perchloric acid to a bright pink; and formation of a pink to purple color eventually turning blue with Ehrlich's reagent. Its Rf in untreated urine concentrates exceeded that of authentic material in basic solvent systems, where it appeared just above urea. Addition of urea to authentic compound resulted in a similar elevation in Rf and pretreatment with urease decreased it to that reported in the literature. The Rf in acidic solvents corresponded with literature values but its detection with the glyoxylic acid spray was often difficult because of the darkened trails of fast moving compounds. Its presence could be demonstrated with Ehrlich's or with oxidizing reagents. Glyoxylic acid spray revealed this material in about 10 per cent of both schizophrenic and non-schizophrenic subjects and its presence was apparently unrelated to clinical state. Ehrlich's reagent elicited it in 80 per cent of the urines from subjects in both diagnostic categories.

*Tryptophan.* The fourth chromogen, was observed particularly in a limited series of "Reigelhaupt positive" urines in which precipitate from the urinary concentrate was employed. The material was present in urines from 4 of 13 schizophrenics and 1 of 3 non-schizophrenics. It gave the same color reactions as tryptophan and had the same Rf in the solvent systems *n*-butanol: ethanol: water (4:1:1) and *n*-butanol: acetic acid: water (4:1:5). Further attempts at identification were not made both because it appeared unrelated to diagnosis and because of its infrequent occurrence even in "positive" urines.

*n-Butanol Extracts.* Chromatograms of butanol extracts equivalent to 6-8 ml of whole, positive-reacting, urine revealed only three chromogenic spots. Two of these were common to all urines and were identified as indole-3-acetic acid and indoxyl sulfate on the basis previously described. The third substance appeared in one of the four urines and had the properties already described for 6-hydroxyskatole sulfate. Interestingly the indole-3-acetic acid spot was considerably more intense in these extracts than in whole urine, while indican showed the reverse trend.

*Urinary toxic factors.* Five biologically potent extracts<sup>(4)</sup> were chromatographically examined individually and in an equal volume pool, and were compared to a pool of biologically weak extracts. All of these crude extracts gave a weakly positive Reigelhaupt test and contained a single glyoxylic acid chromogen, indican; one also contained 6-hydroxyskatole sulfate. Chromogens could not be observed with purified preparations of the biologically active components, and such preparations produced only a faint brown ring on the Reigelhaupt test. Whatever their nature, these substances are clearly unrelated to the active principal in the Reigelhaupt test.

*Butenone-2-Extracts.* Three glyoxylic acid chromogens were detected in volumes of butenone-2-extracts of ether-extracted urine equated to 2.5 mg of creatinine. Indican was observed in all cases and no relation was found between intensity of color and any pathological index. Indole-3-acetic acid was observed in 38 per cent of the extracts from

schizophrenics and 31 per cent of those from non-schizophrenics. The third substance, identified as 6-hydroxyskatole sulfate, was observed in urines from 46 per cent of the schizophrenics and 57 per cent of the non-schizophrenics. Somewhat higher figures were obtained with Ehrlichs' dip reagent, where "Quickly Fading Blue" was present in 63 per cent of the schizophrenics, absent in 26 per cent, and ambiguous in 11 per cent, as against 76 per cent positive, 12 per cent negative, and 12 per cent uncertain among non-schizophrenics.

Several other Ehrlich-reactors were observed on chromatograms developed in isopropanol-ammonia-water. Among these were substances apparently identical with the recently described "Mauve" (associated with psychosis) and "Depression" factors.<sup>(15)</sup> The "Mauve Factor" had an Rf of 0.89-0.92 (Bufotenine control = 0.92) forming a red-purple product after Ehrlichs' reagent, which slowly turned dusky lavender. This substance was found in 66 per cent of the schizophrenic and 38 per cent of the non-schizophrenic populations — the greatest difference encountered. Three of the 7 most psychotic subjects in this study (determined by psychological testing) were negative, however, as was the only non-schizophrenic considered possibly psychotic. The 3 non-schizophrenics excreting this material showed no evidence of psychotic derangement. Only a 43 per cent concordance was observed between this factor and quickly-fading-blue material although both are purportedly related to psychosis. The Depressive Factor had an Rf of 0.77-0.80 and formed a pink-red product with Ehrlichs' reagent. This substance was detected in 66 per cent of the schizophrenics and 71 per cent of the non-schizophrenics. Of the three subjects considered as greatly depressed, two excreted this material and one did not.

*Medication.* Many phenothiazines react with strong acid to produce chromogens and a number of analytical tests for this compound are based upon this reaction (Table 1). Urines of subjects treated with chlorpromazine often produce purple-lavender chromogens in the Reigelhaupt test. Therefore, it seemed important to examine urines of phenothiazine treated subjects for glyoxylic acid chromogens to assess the extent of phenothiazine interference with the Reigelhaupt test.

The urines of 5 schizophrenic subjects (receiving 150 mg of Thorazine Concentrate daily for three weeks) were concentrated and chromatogrammed. Between 6 and 13 glyoxylic acid chromogens were observed, 4 to 11 of which were never observed in urines of untreated subjects. These chromogens ranged in color from grey-green to red-pink. Among all the chromogens observed, 3 to 6 (including indican and 6-hydroxyskatole sulfate) were not adsorbed by Amberlite IRC-50. The nature of the remaining chromogens has not been investigated but these results clearly indicate both that phenothiazine metabolites cannot be ignored in studies involving chromogen production and that assays for phenothiazine metabolites which are dependent upon Amberlite IRC-50 adsorption may not account for all its metabolic products.

## DISCUSSION

In an earlier study on the Reigelhaupt test, a high and equal incidence of "positives" was found among both schizophrenics and non-schizophrenics. These results could have been due to an overgenerous criterion of a positive reaction or to the experimental introduction of an extraneous masking chromogen. The first explanation could account for the high incidence of positive tests but would indicate also that the procedure had little

diagnostic relevance, inasmuch as the criteria were the same for both groups. The results of the present study strongly mitigate against the second explanation as well.

A glyoxylic acid chromogen unique to schizophrenics might avoid detection by failing to form chromogens on paper while forming them in solution. No example of such behavior was observed upon testing a variety of diverse compounds. A unique chromogen might escape detection if it were unstable to the acidic, neutral and alkaline chromatographic solvents employed. Such instability is possible but unlikely. Finally, the compound could have been destroyed or lost in sample preparation. Because of the variety of preparations employed, however, this could only occur if the substance were both strongly polar in acidic solution and yet distilled from it during reduced pressure evaporation, and were either not adsorbed onto charcoal or not eluted from it by alkaline acetone water. Few compounds could fit these criteria.

The results also demonstrate the lack of specificity of this test. Three of the chromogens detected in these studies produce a positive Reigelhaupt test and at least two of them, indican and indole-3-acetic acid, are common in normal urine. A wide variety of other compounds, including some of the most common therapeutic agents in psychiatric medicine, also produce red to violet chromogens and more positive Reigelhaupt tests than otherwise observed in this laboratory. The observation that chlorpromazine elevates indican excretion and that elevated urinary chlorpromazine and indican levels may persist for significant periods after termination of chronic medication<sup>(2)</sup> makes it possible that the reported difference between diagnostic populations is due to treatment, not disease. Other workers have reached similar conclusions.<sup>(16,17)</sup>

The requisite metabolic pathways for 6-hydroxyindole formation have been demonstrated in mammalian tissues<sup>(18,19)</sup> but their relationship to endogenous psychosis is uncertain. Six-hydroxyskatole sulfate itself is probably formed exclusively by intestinal flora<sup>(8)</sup> and its equal incidence among schizophrenics and non-schizophrenics in this study strongly suggests that factors other than disease account for its apparent relationship to schizophrenia. The comparison population in this study consisted largely of non-schizophrenic sociopaths differing from hospital personnel and other "normal" controls not only in psychopathology but probably more critically, in sharing with schizophrenics the identical environment, diet, activity restrictions and the like, and so presumably their intestinal flora and any endogenous subclinical infections. The similar findings in our populations may well reflect this greater control for similarity in environment.

Parallel comments apply to the present observations on the "Mauve" and "Depression" factors. Although the preparations employed differed from those of the original worker,<sup>(15)</sup> their lack of diagnostic relevance in this study again suggests that spurious factors account for earlier findings of differences. In no case was bufotenine detected in urinary preparations although the location reagents employed were sensitive to this compound. Again these preparations differed somewhat from those employed in the original reports,<sup>(20)</sup> but this could hardly be decisive. Interestingly enough, bufotenine has a similar Rf to 6-hydroxyskatole sulfate in butanol: acetic: water (12:3:5) and could conceivably have been mistaken for it.

SUMMARY

A chromatographic analysis failed to detect any glyoxylic acid chromogen unique to schizophrenics in whole urine, butanol extract, butenone-2-extracts, or charcoal purified preparations. Compounds identified as indican, indole-3-acetic acid, 6-hydroxyskatole sulfate, and tryptophan were detected in the preparations examined. The incidence and intensity of these materials was identical for both schizophrenic and non-schizophrenic populations. The results support the view that the Reigelhaupt test has little diagnostic significance.

The incidence of the compounds designated "Quickly Fading Blue", and "Depression Factor" in preparations from both schizophrenic and comparably handled non-schizophrenic hospital populations was the same as that generally reported for schizophrenics. "Mauve Factor" had a higher incidence among the schizophrenics but none of these substances appeared specifically related to pathology.

No evidence for the occurrence of bufotenine could be found in urinary preparations from either schizophrenics or non-schizophrenics.

The results are discussed in relation to true and spurious factors, and the importance of control of many environmental factors is stressed.

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## CHROMATOGRAPHIC STUDY OF "REIGELHAUPT" CHROMOGENS IN URINE

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