THE DEGRADATION OF TRYPTOPHAN DURING PAPER CHROMATOGRAPHY WITH OBSERVATIONS ON SOME METABOLITES OF TRYPTOPHAN*

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INTRODUCTION

Partial degradation of compounds during the process of paper chromatography has been observed[1-4]. HUGGINS AND MOSES[1] noted glycine breakdown largely dependent on the first dimension solvent employed and the pH of the paper. A more detailed study[2] extended these observations to eight additional amino acids. SCHWARZ AND BITANCOURT[3], using the technique of double chromatography, reported the decomposition of indole derivatives as evidenced by the appearance of multiple spots from a single substance. OPIEŃSKA-BLAUTH *et al.*[4] have demonstrated instances of the instability of DL-tryptophan in paper and in operations preparatory to paper chromatography.

Application of paper chromatography for the isolation of tryptophan and its urinary metabolites made necessary a reexamination of the conditions for the first dimension development in conventional solvents[5] and equally important, a study of the stability of the compounds during drying and storage of the chromatograms prior to second development with another solvent. The existence of a serious drying and storage problem was clearly indicated by developing some known compounds of interest (tryptophan, anthranilic acid, 3-hydroxyanthranilic acid) with the same solvent in both dimensions[3,6]. Even under conditions yielding adequate stability (one spot from one compound) after the first development, multiple spots resulted from the second development. The problem has been studied in detail for the case of tryptophan. Variables found to determine the extent of degradation of tryptophan in the chromatogram after the first development are temperature during the first dimension development, drying, and storage, irradiation by visible or ultraviolet light, and duration of storage prior to second development. Conditions that minimize or eliminate the degradation reactions of the compounds in the chromatograms have been found.

MATERIALS AND METHODS

Whatman 3 MM paper (46 × 57 cm) was used for chromatography. In each

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case 50 μg of DL-tryptophan* to which had been added 0.1 μC DL-tryptophan 1'-^{14}C* (sp. activity 7.65 mC/mM) were applied to the paper. Fluorescent compounds in developed chromatograms were located with a Mineralight model V-41 ultraviolet light (254 mμ), and ultraviolet fluoroscopes[6] aided in the visualization of compounds that absorb light of 254 mμ. A flow gas hand-scanner having a 5 cm aluminized mylar end-window** and attached to a decade scaler was used to measure radioactivity[7]. Radioautographs were made with Eastman-Kodak No-Screen X-Ray film.

The degradation was clearly elucidated by two developments in the organic phase of freshly prepared *n*-butanol–acetic acid–water (4:1:5)[5]. The standard procedure was to carry out the first development in the lengthwise direction of the paper (referred to as +Y) and the second development with the paper rotated 90° in a clockwise direction (+X)[6]. In this system, with the point of application designated the origin, the location of points in such papergrams may be expressed by conventions of analytic geometry. This simplifies not only the description of direction of solvent flow but also the position of one compound relative to another in the developed chromatogram. Location of a compound on a line drawn diagonally across the chromatogram from the origin to the intersection of the +Y and +X solvent fronts qualifies it as a constituent of the mixture originally applied to the paper. Fig. 1 illustrates an application of this principle to tryptophan and some metabolites processed under non-degradative conditions. Note that all spots lie on the diagonal line.

Completed two-dimensional papergrams were dried in a current of air at room temperature, usually 24–25°, and examined under ultraviolet light with the lamp held at a distance of 3 to 6 in. from the paper***. To locate non-fluorescent, ultraviolet-absorbing deposits, the chromatograms were laid briefly directly on the lamp with the fluoroscope superimposed on the area of the chromatogram being studied.

The radioactive areas were counted directly in the chromatogram, with measurements made on both sides of the paper. These data confirm recent work demonstrating that an accurate estimate of ^{14}C in paper requires averaging of counts on both sides of the chromatogram[8]. Tryptophan was identified by its characteristic R_F in this solvent, its ultraviolet absorption, and its high radioactivity (usually 10,000 to 13,000 counts/min). Because of this high radioactivity, counts of less than 85 counts/min above background were disregarded except when they occurred in areas occupied by visible fluorescent or ultraviolet-absorbing compounds.

Radioautographs of representative chromatograms from each category studied were prepared to delineate location of closely adjacent radioactive areas.

The effect of the following conditions on the degradation of tryptophan was studied.

Temperature during first dimensional chromatography

(*1*) *27–37°*. Temperature within the chromatography cabinet was recorded at frequent intervals throughout the development. These studies were made during the months of warm weather; experiments at 37° were carried out in a constant temperature incubator.

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*** The lamp used produced 45 milliwatts/sq. ft. of U.V. radiation at a distance of 2 ft. from the source.

(2) *24–25°*. Chromatography was carried out in an air-conditioned room.

(3) *12–22°*. Temperature within the chromatography cabinet was recorded at frequent intervals throughout the development. These experiments were carried out during the months of cool weather with the heat in the room shut off and the windows open.

(4) *4°*. Chromatography was carried out in a constant temperature cold room.

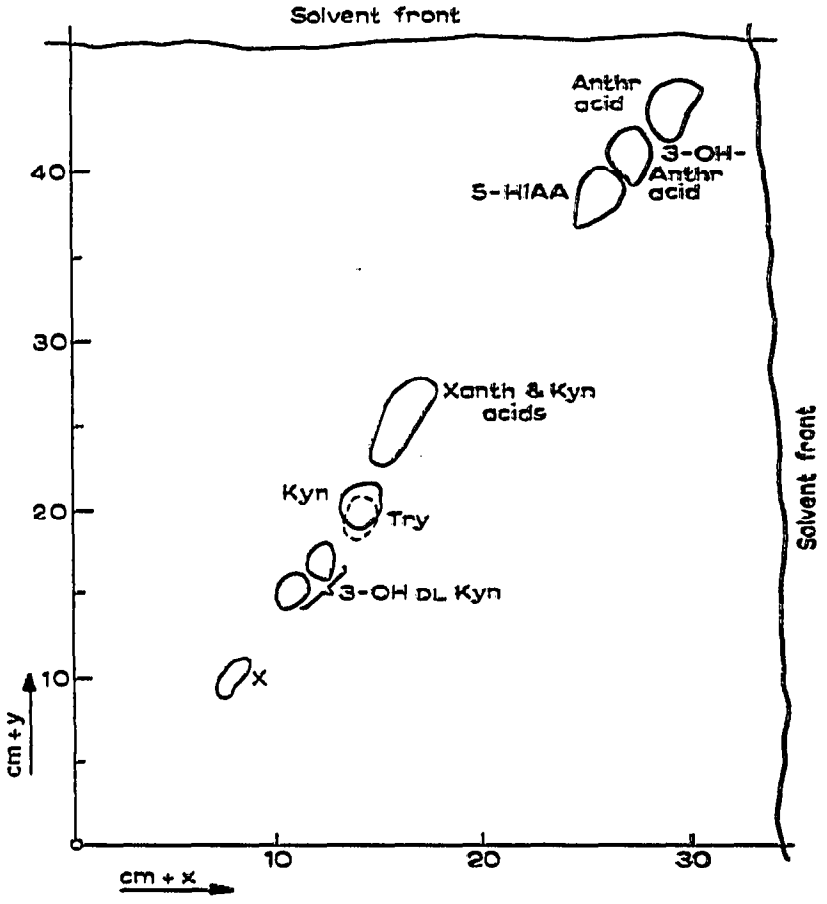

Fig. 1. Illustrating principle of investigative approach: chromatogram of mixture of tryptophan and metabolites prepared under non-degradative conditions. Mixture contained 30 μg of each compound; developed in +X and +Y directions in *n*-butanol–acetic acid–water (4:1:5) by descending technique. Temperature maintained at 12–22° during and between developments. Second development followed first by 2.5 h. Abbreviations as follows: Try = tryptophan; 3-OH DL Kyn = 3-hydroxy-DL-kynurenine; Kyn = kynurenine; Kyn acid = kynurenic acid; Xanth acid = xanthurenic acid; 5-HIAA = 5-hydroxyindoleacetic acid; Anthr acid = anthranilic acid; 3-OH-Anthr acid = 3-hydroxyanthranilic acid. The tryptophan appeared as a strong U.V.-absorbing deposit (dashed outline) overlapping the fluorescent L-kynurenine. The D- and L-isomers of 3-hydroxykynurenine separate in this solvent[5]. The spot marked X was an impurity originally present in the kynurenine.

Storage of chromatcgrams between first and second development

(1) *3–5 days storage in room light*. The chromatograms were exposed during the daylight hours to light ranging from 2 to 25 foot-candles.

(2) *3–5 days storage in total darkness*. The chromatograms were stored in black plastic envelopes in a dark cabinet for 3 to 5 days.

(3) *Less than 12 hours storage.* The papergrams were shielded from room light by sheets of black plastic between first and second development.

These conditions of storage were studied at all the temperatures listed above.

Pretreatment of paper

(1) Unwashed.

(2) Washed in 0.1 *N* acetic acid prior to use*.

Exposure to U.V. light (254 mμ) prior to second development

(1) Exposed.

(2) Unexposed.

Comparisons were made only on chromatograms developed in pairs where all procedures used corresponded in every detail except that one of each pair was not exposed to ultraviolet radiation between developments.

The effects of storage and temperature were studied on the following non-labeled metabolites of tryptophan: kynurenine, 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid, kynurenic acid, xanthurenic acid, and 5-hydroxyindoleacetic acid. Unwashed paper was used in these studies.

RESULTS

Effect of temperature

Table I shows the effect of temperature under the various conditions of storage studied. The greatest degradation, as measured by the amount of ^{14}C in non-trypto-

TABLE I

EFFECT OF TEMPERATURE AND STORAGE ON DEGRADATION

Storage of less than 12 h in the dark in all cases; all observations at 24–25° made on unwashed paper; all chromatograms studied at 4° developed by descending technique.

Temperature	*Condition of storage*	*Number of observations*	*% Degradation**	
			Range	*Average*
27–37°	3–5 days light	13	7.6–33.5	24.5
	3–5 days dark	10	1.2–24.5	7.3
	Less than 12 h	17	0.5–25.8	7.2
24–25°	3–5 days light	4	3.0–18.8	11.4
	3–5 days dark	4	1.2–10.0	5.7
	Less than 12 h	5	0 – 6.6	2.7
12–22°	3–5 days light	11	2.0–25.2	6.8
	3–5 days dark	11	0 – 8.0	1.7
	Less than 12 h	38	0 – 2.2	0.2
4°	3–5 days light	5	2.7– 8.2	5.1
	3–5 days dark	6	0–1.5	0.4
	Less than 12 h	4	No degradation	

* Measured as % ^{14}C not in tryptophan after development.

* No observations at 24–25°.

phan areas, was found on chromatograms developed between 27° and 37°. Within this range, papergrams developed by the ascending technique and stored in room light showed the most extensive breakdown with 26.7 % to 33.5 % of the total radioactivity off the diagonal line. Fig. 2 is a radioautograph of a chromatogram developed under these extreme conditions of temperature and storage. Here radioactive degradation products are seen across the chromatogram from X = 0 to the solvent front.

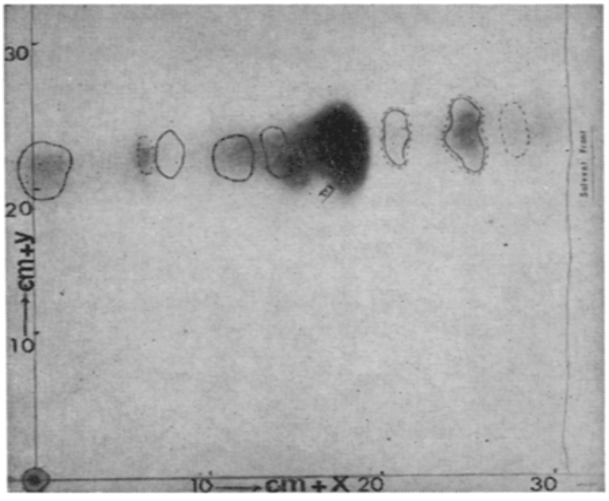

Fig. 2. Radioautograph of chromatogram prepared under degradative conditions. ≡≡≡ = fluorescent and absorbing area; —— = fluorescent deposit; - - - - = non-visible radioactive area, location verified by radioautography. Chromatographic procedure: developed 70 h + Y by ascending technique in *n*-butanol–acetic acid–water (4:1:5); temperature 27–30°; exposed to room light for 3 days; developed 24 h + X ascending in same solvent.

Although few observations were made under each of the storage conditions at 24–25° it was obvious that while development at this temperature represented an improvement over development at the more elevated temperatures, there was still a serious degradation problem.

At temperatures between 12 and 22° entirely satisfactory results were achieved where storage was limited to less than 12 hours or carried out in total darkness. With development at 4°, storage in total darkness resulted in negligible degradation and with less than 12 hours storage, no breakdown could be detected.

Effects of storage of dry chromatograms in light and dark

Of 33 chromatograms observed after exposure to room light for 3 to 5 days (Table I), every one showed degradation following second development. Storage in total darkness sharply reduced breakdown in chromatograms developed at temperatures above 27° and nearly eliminated it below 22°. When the period of storage was confined to 12 hours or less, papergrams developed at temperatures under 22° exhibited virtually no degradation and only a minimal breakdown was seen at 24 to 25°.

Effect of pretreatment of the paper

Table II indicates that use of acid-washed paper did not itself eliminate breakdown during chromatography. However some decrease in degradation was observed under all conditions of storage when this measure was employed.

Visible degradation products

All chromatograms developed at temperatures above 27° had one or more fluorescent or ultraviolet-absorbing areas outside of the deposit identified as tryptophan (Table III). In general, as the temperature during first development decreased, the number of such spots and the amount of radioactivity in these same areas decreased also. Of 62 chromatograms stored for less than 12 hours preceding second development, 20 contained 1 to 3 visible areas in addition to tryptophan and, of these 20, 17 had been developed at temperatures above 27°.

Exposure to light also contributed to the appearance of the visible extra-tryptophan deposits. Table III shows that the number of these extraneous visible areas was five times as great in chromatograms stored in room light for periods up

TABLE II

EFFECT OF PRETREATMENT OF PAPER ON DEGRADATION

Condition of storage	*Washed paper*			*Unwashed paper*		
	Number of observations	*% Degradation*		*Number of observations*	*% Degradation*	
		Range	*Average*		*Range*	*Average*
3–5 days light	12	2.0–33.5	13.9	17	2.3–33.0	13.9
3–5 days dark	10	0 – 6.5	1.0	17	0–24.5	4.7
Less than 12 h	22	0 –25.8	0.6	40	0–13.0	2.3

TABLE III

EFFECT OF DEGRADATIVE CONDITIONS ON THE NUMBER AND % RADIOACTIVITY OF VISIBLE NON-TRYPTOPHAN DEPOSITS

Condition	*Total number of observations*	*Number with visible breakdown products*	*Number of visible breakdown products per chromatogram*		*% Activity*	
			Range	*Average*	*Range*	*Average*
Temperature						
27–37°	39	39	1–5	2.6	0.5–36.3	10.4
24–25°	13	9	0–3	1.2	0.5– 6.2	1.8
12–22°	60	14	0–2	0.3	0.6– 5.5	0.3
4°	15	5	0–2	0.5	0.4– 4.9	0.5
Storage						
3–5 days light	33	32	0–5	2.5	0.9–36.3	9.4
3–5 days dark	31	15	0–3	0.8	1.4–20.2	1.9
Less than 12 h	62	20	0–3	0.5	0.4–10.3	1.3
Pretreatment of paper						
Washed	44	21	0–4	1.1	0.5–27.1	3.8
Unwashed	72	41	0–5	1.2	0.6–36.3	3.7
Exposure to U.V. light						
Exposed	53	32	0–5	1.2	0.5–27.4	3.6
Unexposed	53	26	0–5	1.1	0.5–27.1	3.6

to five days as in those where light had been eliminated, and the percentage radioactivity in these areas was more than seven times as great in the chromatograms stored in room light as in the papergrams not so exposed.

The appearance of these visible non-tryptophan areas was not a random occurrence. Careful observation of the chromatograms revealed that under identical conditions of development and storage, spots with the same fluorescence and/or U.V.-absorbance properties were always found at the same R_F in the + X dimension. This was true even in chromatograms developed several months apart and indicated that under given conditions degradation products of a specific chemical composition were formed. No attempt was made to characterize the degradation products.

Radioactivity in non-visible areas and at X = o

Radioactivity in areas not visible with ultraviolet illumination (Table IV) follows the same pattern as that found in visible areas, the highest concentrations being found in papergrams developed at elevated temperatures and/or stored in room light between developments. However the amount of radioactivity in these non-visible areas was almost never as great as in the visible deposits. This also held true for radioactivity which did not move in the + X direction as shown in Table V. Where no storage intervened between the first and second development, only one chromato-

TABLE IV

DEGRADATION IN NON-VISIBLE RADIOACTIVE AREAS

Condition	*Total number of observations*	*Number with non-visible radioactive areas*	*% with non-visible radioactive areas*	*% Radioactivity*	
				Range	*Average*
Storage					
3–5 days light	37	31	86.5	o–12.4	3.3
3–5 days dark	33	15	45.5	o– 5.8	1.1
Less than 12 h	60	20	33.3	o– 8.2	0.9
Temperature					
27–37°	38	31	81.6	o–12.4	2.3
4–25°	92	41	44.6	o–11.1	1.3

TABLE V

RADIOACTIVITY WHICH DID NOT MOVE IN THE + X DIRECTION

Condition	*Total number of observations*	*Number with radioactivity at X = o*	*% with radioactivity at X = o*	*% Radioactivity*	
				Range	*Average*
Storage					
3–5 days light	37	32	83.8	o–16.5	2.2
3–5 days dark	33	8	24.3	o– 4.0	0.4
Less than 12 h	60	1	0.06	0.9% on one paper	
Temperature					
27–37°	38	17	44.8	o– 7.4	1.1
4–25°	92	23	25.0	o–16.5	0.56

gram of the 60 studied contained radioactivity at X = 0 and in this instance the temperature during the first development exceeded 27°. Of the 32 chromatograms stored in room light which contained radioactive degradation products at X = 0, ten had visible fluorescent deposits in this region. These were not included in Table III.

Effect of exposure to ultraviolet light between developments

Exposure to ultraviolet light between developments resulted in only a minimal amount of breakdown. For instance in 16 chromatograms where exposure to ultraviolet light after first development preceded storage in room light there was an average degradation of 13.2 %, whereas in 16 corresponding chromatograms where exposure to U.V. was omitted, the average degradation was 11.6 %. This effect was consistently present, however, raising the question as to whether a more prolonged exposure to ultraviolet light would result in further degradation. In this study a location of compounds after the first development of the single compound applied required an exposure to ultraviolet light of approximately 30 sec. However, when examining a chromatogram in which an unknown mixture has been partitioned with the resultant presence of multiple deposits, a more prolonged examination is necessary. Therefore the problem was of more than academic interest.

Clarification of this subject was attained in the following way. Five one-dimensional chromatograms were prepared as described under Methods. Descending development in the *n*-butanol–acetic acid–water solvent was carried out at a temperature under 22°. The dried chromatograms were treated as shown in Table VI. After exposure the papergrams were immediately placed for development in the +X direction in freshly prepared solvent at a temperature under 22°. The results obtained after this development are shown in Table VI. It is apparent that prolonged exposure of tryptophan in a paper chromatogram to ultraviolet light is destructive to the compound and should be avoided.

TABLE VI

RESULTS OF PROLONGED EXPOSURE TO ULTRAVIOLET LIGHT BETWEEN DEVELOPMENTS

The exposure was carried out in a dark room with the ultraviolet lamp as the only source of illumination. During the prolonged exposures the lamp was fixed at a point six inches from the previously marked tryptophan spot. The temperature was constant throughout at 22°. Chromatography on unwashed paper; development by descending technique.

Chromatogram	*Treatment*	*% Degradation*
UEX	No exposure to ultraviolet light	0.0
EX	Exposed to U.V. light for location of tryptophan	0.0
EX + 5	Exposed to U.V. light for location of tryptophan + 5 additional minutes	0.0
EX + 15	Exposed to U.V. light for location of tryptophan + 15 additional minutes	4.0*
EX + 30	Exposed to U.V. light for location of tryptophan + 30 additional minutes	7.9**

* 1.6 % radioactivity at X = 0; 2.4 % radioactivity in non-visible radioactive areas.

** 2.0 % radioactivity in 3 extra-tryptophan fluorescent deposits; 2.3 % radioactivity at X = 0; 3.6 % activity in non-visible radioactive areas.

Studies on metabolites of tryptophan

At temperatures below 22° the metabolites of tryptophan investigated underwent no appreciable breakdown in the *n*-butanol–acetic acid–water solvent when storage between developments was omitted. Table VII shows the effect of storage and elevated temperatures on these compounds. Fig. 3 is a chromatogram of a mixture of tryptophan and some of its metabolites showing the effect of storage in room light subsequent to first development at temperatures between 12° and 22°. Extensive degradation may be observed to occur in many of the compounds studied.

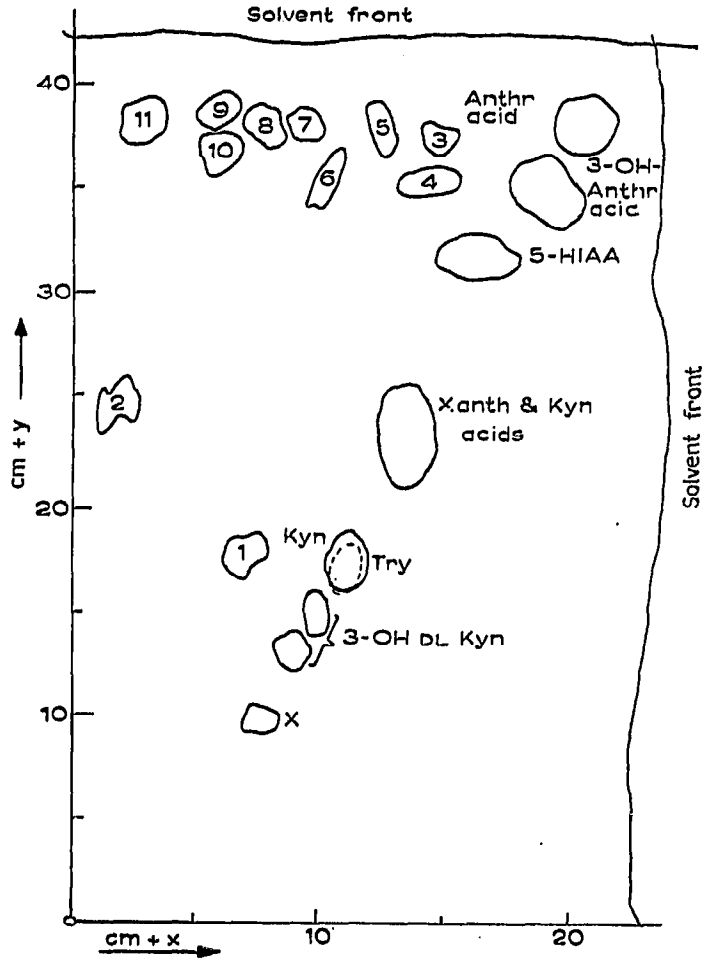

Fig. 3. Effect of exposure of chromatogram of tryptophan and metabolites to room light. + Y development as in Fig. 1; + X development in same solvent preceded by 3 day exposure to room light. Numbered areas designate degradation products. Abbreviations as in Fig. 1.

DISCUSSION

The special methods employed facilitated the recognition and study of the degradation described. Double development in the same solvent verified degradation in instances where the use of two different solvents would have raised doubt as to whether multiple spots from a single applied compound resulted from degradation or from impurities originally present in the material applied to the paper. The use of

labeled tryptophan allowed detection of non-visible degradation products and permitted measurement of all the degradation products derived from the 1-carbon of the tryptophan side-chain. Radioautography confirmed data obtained by counting and provided a permanent record of exact locations of the radioactive degradation products. The availability of ultraviolet fluoroscopes made it possible to visualize areas which did not fluoresce at 254 mμ without subjecting the chromatograms to procedures[4,9] which might damage the compound being studied. This was especially important when observing the papergrams after first development where exposing them to temperatures above 100° in order to render the tryptophan spot fluorescent would have resulted in breakdown of the tryptophan.

While exposure of the chromatograms to light between first and second development resulted in extensive degradation, there were a very few instances where, when the first development had been carried out at a temperature under 22°, exposure of the papergrams to light resulted in breakdown of 3 % or less. This at first appeared to minimize the importance of light as a factor in the degradation phenomenon. Closer inspection of the data, however, disclosed that in these cases $^2/_5$ to $^3/_4$ of the storage was carried out over a week-end or holiday period either late in December or early in January when during the daylight hours the window-light reaching the chromatogram ranged from 2 to 5 foot-candles as contrasted with 5 to 25 foot-candles to which the papergrams were exposed in the customary laboratory illumination during the regulation working day. The effect of light as a factor contributing to degradation is emphasized by observations on the total percentage breakdown following development at elevated temperatures. Table I shows that the chromatograms stored in total darkness for 3 to 5 days exhibited no more decomposition than where storage in the dark was limited to less than 12 hours. Storage in room light, however, caused a three-fold increase in degradation. Observations on extra-tryptophan visible deposits are also pertinent. As noted previously, Table III shows the marked increase in both the number of visible decomposition products and in the radioactivity present within these deposits resulting from exposure to light.

The fact that no breakdown products were located until after the second development indicated that the actual degradation occurred only after the termination of the first development during the drying of the paper and during subsequent storage. In view of this a possible explanation for the extensive degradation found after first development at elevated temperatures is that at warm temperatures the highly volatile butanol may have evaporated at a far more rapid rate (despite the use of relatively air-tight chromatography cabinets) than would have been the case at more normal temperatures. This would have left in the chromatogram a high concentration of the acetic acid resulting in the degradation of tryptophan during the drying process. The data in Tables I and III appear to support this theory.

In the case of glycine[1] the pH of the paper has been implicated as a major factor in breakdown and this view was given added credence[2] and extended to 8 additional amino acids when it was observed that the destructive effect of phenol as a solvent* was mitigated to a large extent by employing a more highly purified grade of phenol. However, degradation as high as 9 % was found when glycine was chromatographed on oxalic acid-washed paper using a butanol–propionic acid–water

* Reported elsewhere[4] to cause tryptophan degradation when used as the solvent at the same concentration.

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TABLE VII

EFFECT OF TEMPERATURE AND STORAGE ON THE CHROMATOGRAPHY OF TRYPTOPHAN METABOLITES

Compound	*Number of deposits not on the diagonal line*					
	Light		*Dark*		*Less than 12 h*	
	12–22°	*27–30°*	*12–22°*	*27–30°*	*12–22°*	*27–30°*
Kynurenine	0	1 (at X = 0)	0	1 (at X = 0)	0	0
3-Hydroxykynurenine	0	0	0	0	0	0
Kynurenic acid	2	3 (1 at X = 0)	0	0	0	0
Xanthurenic acid	0	0	0	0	0	0
Anthranilic acid	3 (1 at X = 0)	4 (1 at X = 0)	2 (1 at X = 0)	3 (1 at X = 0)	0	1
3-Hydroxyanthranilic acid	2 (1 at X = 0)	4 (1 at X = 0)	2 (1 at X = 0)	3 (1 at X = 0)	0	1
5-Hydroxyindoleacetic acid	0	1	0	0	0	0

solvent in the first dimension and redistilled phenol in the second. The present study showed no degradation of tryptophan or the tryptophan metabolites investigated, when developed on unwashed paper, when optimum conditions of temperature and exclusion of light were maintained. Under extreme degradative conditions, use of washed paper ameliorated degradation only moderately.

It can be concluded that tryptophan and its investigated metabolites can be successfully separated by means of paper chromatography if the following conditions are observed:

(1) Maintaining the temperature below 22° during the solvent development and drying of the chromatogram.

(2) Either eliminating substantial periods of storage between first and second dimension development or carrying out any necessary storage in total darkness.

(3) Using acid-washed paper for chromatography where possible.

(4) Limiting exposure to ultraviolet light between first and second development.

Failure to adhere to these conditions may result in serious misinterpretation of chromatograms prepared from biological material and of corresponding radioautographs.

SUMMARY

The possible degradation of tryptophan and seven of its metabolites during and after paper chromatography with *n*-butanol–acetic acid–water has been investigated. It has been found that extensive degradation results from developing and drying the chromatograms at temperatures over 27° and storing them in room light for extended periods before the second development. Lesser amounts of degradation were found after limited exposure of compounds to ultraviolet light between developments and occasionally with the use of unwashed paper. Procedures to eliminate degradation have been described.

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