CONDENSATION OF GLYCINE WITH PHENOL DURING PAPER CHROMATOGRAPHY*

K. E. RICHARDSON AND N. E. TOLBERT

Department of Physiological Chemistry, Ohio State University, Columbus, Ohio (USA)
and
Department of Biochemistry, Michigan State University, East Lansing, Mich. (USA)

(Received November 1st, 1963)

The identification of metabolic intermediates by two-dimensional paper chromatography has been employed extensively in biochemical investigations. When this method is used to determine the quantitative distribution of intermediates, errors due to the presence of non-enzymically produced products become significant. This problem is considerably enhanced when the precursor or its metabolic products complex with the chromatographic solvent. Apparent concentrations may be significantly lowered with respect to the other intermediates or precursors present. Multiple or irregular spots, variability in R_F values, and poor quantitative recovery are often encountered in paper chromatography, particularly when phenol solvents are employed with amino acids[1–5]. Such formation of multiple spots has been attributed to interaction between amino acids[3,6], to excessive concentrations of the amino acids applied[7], to oxidation of the amino acid[4], and to different ionic forms as related to the pH of the solvent system employed[8]. This report concerns the formation of stable glycine condensation products during paper chromatography with water saturated phenol. The effects of pH, distillation of phenol, and the presence of formaldehyde are considered.

MATERIALS AND METHODS

Whatman No. 1 filter paper (22 × 17 in.) was used either untreated or treated with oxalic acid by immersion for 2 h in a 1% solution and then washed exhaustively with distilled water. Designated amounts of radioactive glycine-2-^{14}C (1.08 mC/m*M*) from New England Nuclear or Volk were applied to the origin point. The two-dimensional chromatograms were developed first in water-saturated phenol (prepared from Mallinckrodt 88% phenol, liquified, preservative free) and secondly in *n*-butanol–propionic acid–water[9]. Between solvents, the chromatograms were dried overnight in a hood at room temperature in a slow moving current of air. Timed studies were initiated from the instant of solvent addition to the chromatographic system. Radioactive areas were located on the chromatograms by radioautography employing Dupont Xtra Fast No. 508 X-ray Film. The relative distribution of radio-

* Aided in part by grant no. GM-07865 from the National Institutes of Health to K.E.R. and in part by grant no. G-20436 from the National Science Foundation to N.E.T.

activity in the various areas was determined by counting with an end window Geiger tube[10]. Identification of labeled unknowns was made by co-chromatography with unlabeled compounds and comparison of radioautographs with ninhydrin sensitive areas. Exact coincidence of areas was accepted as indicating identical compounds. Distillation of phenol was performed either in the absence of any additives or by the method of DRAPER AND POLLARD[11]. The water-saturated phenol solvent was prepared by adding the distillate or residue to a limited amount of water until a single phase was obtained. Acid and alkaline hydrolysis of the glycine condensation products was performed in a sealed tube at 20 lb. pressure and 250° F for 2 hours.

DISCUSSION AND RESULTS

A two-dimensional chromatogram of glycine-2-^{14}C on Whatman No. 1 filter paper with water-saturated phenol as the initial solvent and butanol–propionic acid–water as the second solvent is shown in Fig. 1. While a number of active areas were present in addition to glycine, only three gave evidence of being formed during the chromatographic process. These areas, labeled I, II and III, consistently streak in the water-saturated phenol solvent with their tailing end coinciding with the glycine area. This type of streaking suggested that the unknowns were being formed during the development of the chromatogram in phenol. Their R_F values in the second solvent system were considerably larger than that of glycine, thus providing excellent re-

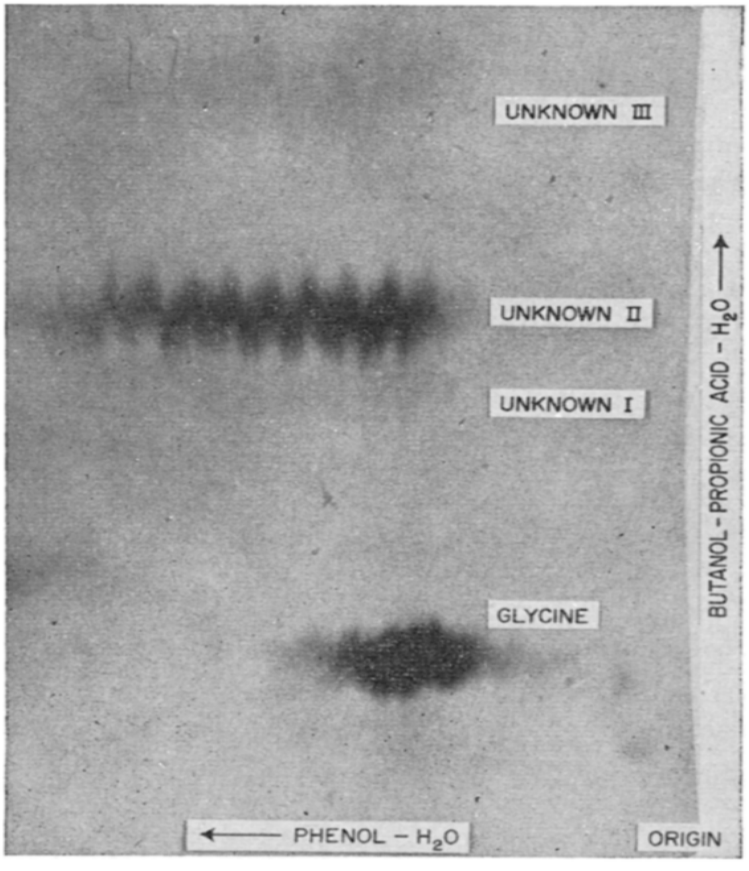

Fig. 1. Radioautogram of chromatogram of glycine-2-^{14}C. Whatman No. 1 filter paper. Solvents: phenol–water (first dimension); *n*-butanol–propionic acid–water (second dimension).

solution. No significant streaking occurred in the second solvent, indicating that no complex formation was taking place. The other areas of activity on the chromatogram were well defined spots with no evidence of streaking and appear to be contaminants of the original glycine preparation or, at least, were formed prior to chromatography.

At adequate levels of material all three unknowns could be detected with standard ninhydrin sprays. Since the ^{14}C-labeled areas and the ninhydrin-sensitive areas exactly coincided, the ninhydrin-sensitive compounds and the ^{14}C-labeled products were the same. Unknown I exhibited the typical purple color of glycine with ninhydrin sprays as soon as sprayed. Unknowns II and III exhibited a yellow color during the five minute heating process following spraying, but two to five days later the color turned to the typical purple color of ninhydrin with glycine. This type of color development (first yellow and then purple) was characteristic of such compounds as glycylglycine and glycylglycylglycine, except that the glycine peptides showed ninhydrin color development from yellow to purple within two to three hours.

Failure by other workers[4] to detect ninhydrin color reaction with these unknown substances from glycine may be due to the low concentration of material used. With commercial liquid phenol and untreated Whatman No. 1 filter paper unknowns I and II were easily detectable with 25 μg applications of glycine at the origin. Unknown III could not be detected at this level. Even applications as low as 10 μg of glycine yielded a detectable ninhydrin sensitive area for unknowns I and II. Chromatography of unlabeled glycine from four different commercial sources with the two-dimensional solvent system yielded, in each case, unknown I and II which could be detected readily with ninhydrin.

Unknowns I and II were stable and could be eluted from the filter paper readily with water. Rechromatography in the same solvent system gave a reproducible R_F with a discrete spot and no evidence of tailing. The approximate R_F values in butanol–propionic acid–water were 0.6 for I, 0.7 for II and 0.9 for III. In phenol–water the R_F values for all three unknowns were nearly the same at about 0.8 to 0.9.

Upon hydrolysis of ^{14}C-labeled unknown II with ammonium hydroxide all the ^{14}C cochromatographed identically with unlabeled glycine (Fig. 2). Partial hydrolysis was obtained with HCl, formic acid, and acetic acid. In each case glycine-^{14}C was released. These results supported the hypothesis that unknown II was a stable glycine condensation product rather than a product of glycine oxidation as has been suggested[4]. However, unknown II did not cochromatograph with α-phenyl-glycine, N-phenylglycine, glycylglycine, glycylglycylglycine, diketopiperazine and phenylglycinate and thus did not appear to be one of these types of possible condensation products.

The glycine condensation was directly related to the phenol employed and to the length of time that the glycine was exposed to the solvent (Table I). When redistilled phenol (redistilled without preservatives present) was used, a four fold increase in condensation products formed during an initial five hour period from glycine-2-^{14}C occurred as compared to the use of nonredistilled commercial phenol or the distillation residue.

Phenol redistilled in the presence of aluminum turnings and sodium bicarbonate yielded only 1.6 % of the total activity as condensation products after twenty hours of chromatography. This suggested that formation of glycine unknowns required the

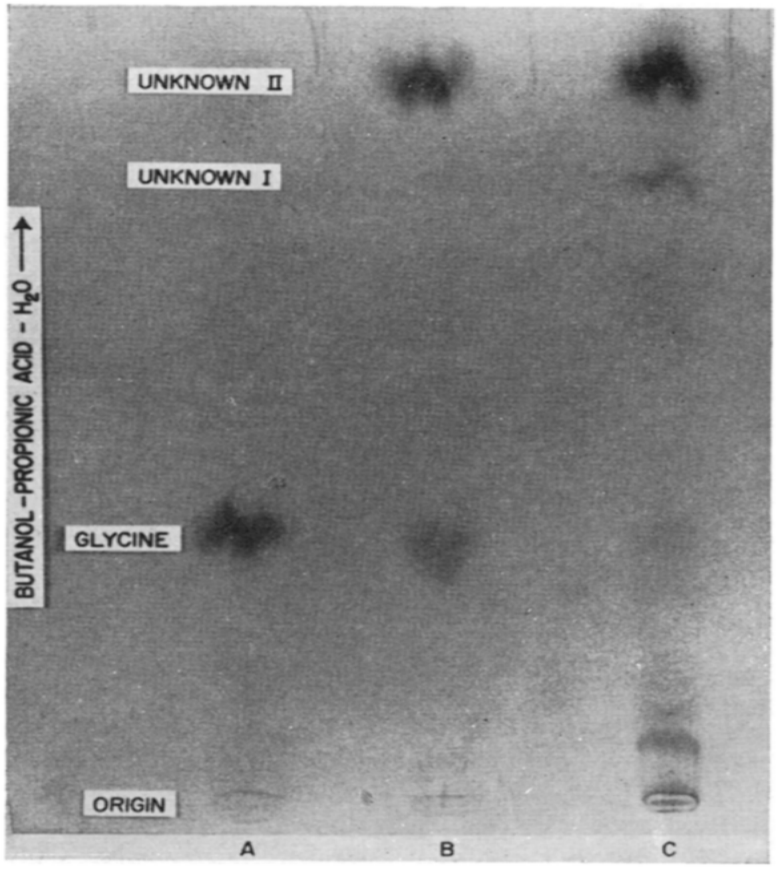

Fig. 2. Radioautogram of the hydrolysis products of unknown II. Whatman No. 1 filter paper. Solvent: *n*-butanol–propionic acid–water. A. Hydrolysis with ammonium hydroxide. B. Hydrolysis with HCl. C. No hydrolysis.

presence of a contaminant which was essentially removed or destroyed by distillation in the presence of aluminum turnings and sodium bicarbonate.

The increase in amount of the glycine condensation products with length of time of chromatography confirmed that their synthesis occurred during the chromatographic process. Direct contact between the glycine and the phenol solvent was required. Suspensions of glycine aliquots spotted on Whatman No. 1 filter paper in a phenol saturated atmosphere for two weeks produced no detectable condensation products.

Adjustment of the pH of the phenol solvent from 3.0 to 9.0 caused no significant decrease in condensation products. However, prewashing Whatman No. 1 filter paper with oxalic acid decreased glycine condensation by about one third. Saturation of the phenol solvent system with 20 % saponin, which has been suggested as a means of preventing amino acid interaction[12], failed to curtail glycine condensation.

Chromatography of glycine in phenol solvent containing 1 % (v/v) formaldehyde for twenty hours resulted in complete conversion of glycine to unknown III. Addition of 1 % (v/v) formaldehyde to the *n*-butanol–propionic acid–water solvent had no effect on glycine chromatography.

One hour incubation of solutions containing glycine-2-^{14}C, formaldehyde, and phenol produced condensation products II and III, while incubation for 24 h caused complete conversion of glycine-2-^{14}C to unknown III (Table II). The formation of

TABLE I

FORMATION OF GLYCINE-2-^{14}C CONDENSATION PRODUCTS IN REDISTILLED PHENOL

Hours in phenol	*Per cent of total radioactivity found in condensation products*		
	Commercial phenol	*Redistilled phenol**	*Phenol residue**
0	0	0	0
5	3.0	13.4	3.5
10	7.4	21.1	6.4
15	10.8	25.5	9.2
20	20.0	36.4	12.5
25	21.4	43.3	19.3

* Distillation of the phenol was performed in the absence of any additives.

TABLE II

EFFECT OF FORMALDEHYDE ON THE FORMATION OF GLYCINE-2-^{14}C CONDENSATION PRODUCTS

Compound	*Phenol* c.p.m.*	*Phenol* + HCHO c.p.m.*
Glycine	16,488	234
Unknown I	170	0
Unknown II	94	3,803
Unknown III	0	17,719

* Phenol was redistilled from aluminum turnings and sodium bicarbonate. Mixtures were incubated at room temperature for 1 h.

unknown II predominated at low formaldehyde concentrations and short incubation times, while unknown III was formed with longer incubation times and high formaldehyde concentrations. Thus unknown II may have been the precursor of unknown III.

By cochromatography it was shown that unknown II was the same material whether formed in commercial nonredistilled phenol or in the presence of added formaldehyde. Radioactive ^{14}C was found in condensation products II and III irrespective of whether formaldehyde-^{14}C or glycine-2-^{14}C were employed (Fig. 3). The differences in distribution of radioactivity between II and III on the chromatogram resulted from differences in formaldehyde concentration. The formaldehyde-^{14}C mixtures contained much less formaldehyde than the glycine-2-^{14}C plus added unlabeled formaldehyde. On chromatograms of biological experiments which utilized formaldehyde-^{14}C, unknown III was a common product. In such experiments unused formaldehyde-^{14}C and a reservoir of glycine from the biological material were usually present.

The structures of unknowns I, II and III have not been established. Our evidence indicates that they are condensation products of glycine, phenol and formaldehyde. Such condensation products have been reported[13]. While such reports have been concerned with formation of glycine–phenol–formaldehyde polymers, polymerization at the low concentrations employed in paper chromatography has not been considered. *Ortho*, *meta* and *para*-hydroxybenzyl alcohols, the postulated initial products of formaldehyde condensation with phenol[14], were considered as intermedi-

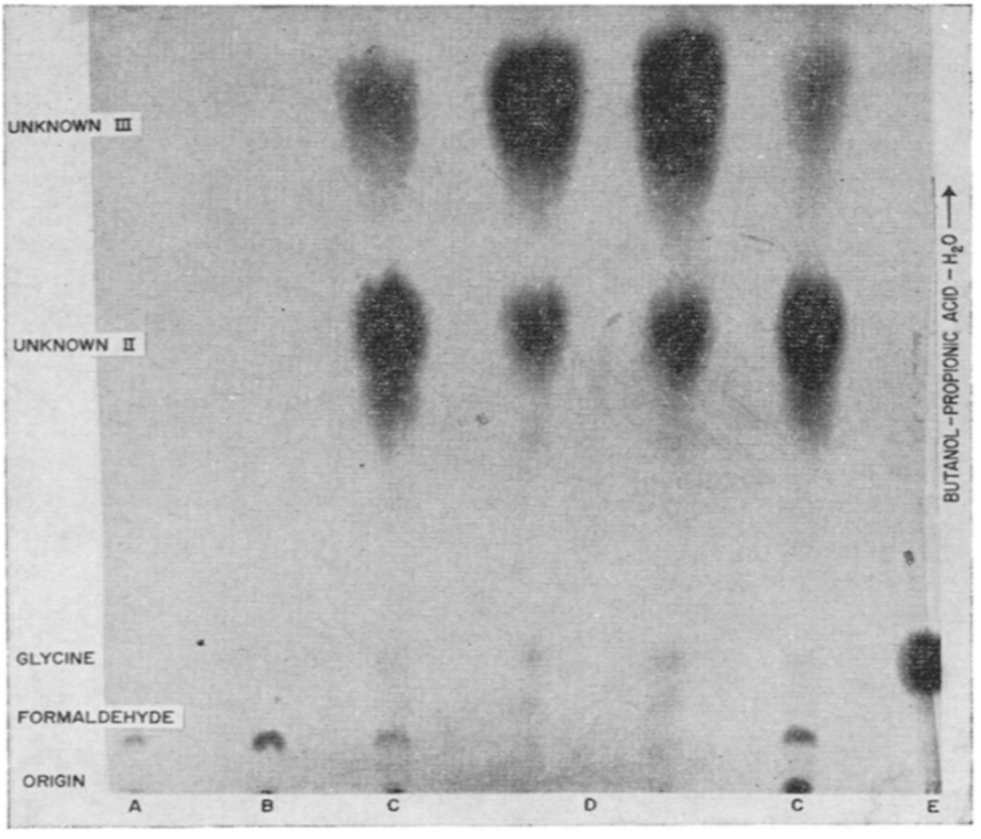

Fig. 3. Radioautogram of a chromatogram of the following mixtures incubated for 1 h prior to spotting on the filter paper. A. Formaldehyde-^{14}C in water-saturated phenol. B. Formaldehyde-^{14}C in water-saturated phenol. C. Formaldehyde-^{14}C and glycine in water-saturated phenol. D. Glycine-2-^{14}C and formaldehyde in water-saturated phenol. E. Glycine-2-^{14}C in water. Solvent for chromatography was *n*-butanol–propionic acid–water.

ates in formation of unknowns I, II and III. However, no increase in the ^{14}C condensation products from glycine-^{14}C was observed when the hydroxybenzyl alcohols were incubated with glycine and phenol mixtures.

Experimental results suggest that commercial phenol contains trace amounts of formaldehyde or formaldehyde condensation products which condense with glycine during paper chromatography. In addition, it appears that ethanolamine forms similar condensation unknowns during paper chromatography with phenol solvents. In chromatography where such condensation should be eliminated, redistillation of the phenol from aluminum turnings and sodium bicarbonate[11] and pretreatment of Whatman No. 1 filter paper with oxalic acid minimize the problem.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the technical assistance of Mrs. LYNN ZELLER and Mrs. EMILY BADERTSCHER.

SUMMARY

Three products formed during the paper chromatography of glycine with water-saturated phenol as a solvent have been investigated. These unknowns were stable

condensation products whose formation was dependent upon the presence of glycine and formaldehyde. They were ninhydrin sensitive. Alkaline hydrolysis yielded glycine. For chromatographic practices, the amount of glycine or formaldehyde which may be converted into the condensation products will depend upon the amount of the other component in the mixture. Addition of 1 % formaldehyde normally complexes all of the glycine. In usual chromatographic experiments part of the glycine may be complexed by residual formaldehyde present in chromatographic phenol. Glycine condensation may be minimized by redistilling the phenol solvent with aluminum turnings and sodium bicarbonate and by washing the filter paper with 1 % oxalic acid.

REFERENCES

1 V. MOSES, *J. Chromatog.*, 9 (1962) 241.
2 S. ARONOFF, *Science*, 110 (1949) 590.
3 M. T. BECK AND P. EBREY, *Acta Chim. Acad. Sci. Hung.*, 4 (1954) 231.
4 A. K. HUGGINS AND V. MOSES, *Nature*, 191 (1961) 668.
5 L. FOWDEN, *Biochem. J.*, 48 (1951) 327.
6 M. T. BECK AND P. EBREY, *Biochim. Biophys. Acta*, 20 (1950) 393.
7 R. H. HACKMAN AND M. LAZARUS, *Biochim. Biophys. Acta*, 17 (1955) 147.
8 R. FUERST AND J. AWPARA, *Anal. Chem.*, 23 (1951) 162.
9 A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710.
10 R. C. FULLER, *Science*, 124 (1956) 1253.
11 O. J. DRAPER AND A. L. POLLARD, *Science*, 109 (1949) 448.
12 P. EBREY, *Chemist-Analyst*, 48 (1959) 36.
13 L. L. MCKINNEY, E. A. SETZKORN AND E. H. UHING, *U.S. Pat.*, 2,717,263, Sept. 6, 1955.
14 S. R. FINN AND J. W. JAMES, *J. Appl. Chem.*, 6 (1956) 466.