Reaction of Gossypol With Amino Acids and Other Amino Compounds

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Abstract

The reactions of gossypol with certain amino acids and other amino compounds have been studied spectroscopically with respect to the effect of time and pH in the range from 5.7 to 7.5 at 37 C. The rate of reaction of gossypol with amino acids increases with increase in pH and has been shown to be related to the distance of the amino group from the carboxyl group within the molecule. Reaction products of gossypol with amino acids and other amino compounds were subjected to various purification procedures and analysis to determine combination ratios. In addition to the expected gossypol-to-amino compound ratio of 1:2, dictated by the formation of Schiff base-type bonds with the two aldehyde groups of gossypol, compounds with ratios of 1:3 and 1:4 were isolated. These results indicate that each of the two aldehyde groups of gossypol can react with two amino groups under the conditions studied.

Introduction

Gossypol, a yellow polyphenolic pigment of cottonseed, has long been known to form complexes with protein, with the binding site thought to consist principally of the ε-amino group of lysine. The nature of the reaction products of gossypol with cottonseed protein and their detrimental effect on its digestion and utilization have been subjects of active research by chemists and biochemists for 40 years. The chemistry of cottonseed pigments is not only a problem of intriguing scientific interest, but is also of major economic importance to the cotton industry. Because of their toxicity to monogastric animals, cottonseed pigments are responsible for loss of revenue to the cottonseed processing industry in terms of restricted markets and increased processing costs necessary to detoxify them.

Clark (1) proposed that the detoxication of gossypol achieved by heating moist cottonseed was the result of chemical reaction between gossypol and the free amino groups of cottonseed protein. Baliga and Lyman (2,3) found that lysine accounted for most of the free amino groups of cottonseed protein and that reaction of gossypol with cottonseed protein decreased lysine availability from 82.9% to 48.7%. Further investigation of gossypol-protein complexes by Lyman et al. (4) identified the free ε-amino groups as the principal site of binding gossypol with the molar ratio of lysine to gossypol varying from 2:1 to 1:1 as the concentration of gossypol was increased. Sedimentation velocity studies of these gossypol-protein complexes indicated the presence of from one to four different components, thus indicating that gossypol probably reacts to form a cross link between two or more protein molecules.

Several investigators (4–6) have demonstrated that the presence of bound gossypol in proteins lowers their digestibility. The extent of this lowered digestibility is greater than would be expected from the binding of gossypol of the ε-amino groups of lysine alone, thus raising the possibility that subsequent reactions occur between gossypol and free amino acids or peptides or both as the degradation of the protein progresses in digestion and more amino groups become available.

A wide variety of derivatives of gossypol with various aliphatic and aromatic compounds has been produced by workers interested in the compound (7–9,10). These derivatives have usually been formed, however, under conditions more rigorous than those found in physiological systems. Witkop and Beiler (11) demonstrated the greater reactivity of the ε-amino group of lysine as compared to the alpha-amino group and Alley and Shirley (12) utilized this property to produce the Schiff base derivative of gossypol with lysine.

In the present study, the amino acids arginine, ornithine, glutamine, asparagine, glycine and lysine were selected as ones which would be likely to react with gossypol. Aspartic and glutamic acid were also used in order to compare the reaction with α-amino groups and amide nitrogen. This communication reports the results of an investigation of the reaction of gossypol with these amino acids and other amino compounds under physiological conditions of temperature and pH.

Experimental Procedures

Analytical Methods

Standard Methods for Free and Total Gossypol. Free gossypol was routinely determined by the method of Pons and Guthrie (13) with the exception that 80% 2-propanol (2-PrOH:H_2O, 80:20, v/v) was used as the diluting solvent instead of 95% ethanol. In samples whose gossypol content was lower than the range of the usual determination (less than 0.02 mg in the aliquot) or in samples whose size precluded the use of the standard procedure, the following modifications were used. The initial volume of the aqueous 70% acetone was reduced from 50 to 25 ml, a 2 ml aliquot was taken, and the volume of the para-anisidine and acetic acid reagents was reduced from 3 to 1 ml. However, if the amount of gossypol in the aliquot is greater than 0.02 mg, the standard volumes must be used to prevent para-anisidine from being the limiting factor.

Total gossypol determinations were made by the method of Pons et al. (14). For small samples or ones containing low levels of gossypol, the modifications mentioned above were used in addition to reduction of the initial volume for oxalic acid hydrolysis from 100 to 25 ml. A standard curve, which demonstrated the linear relationship of aliquots of a standard solution containing 0.001 to 0.02 mg. of gossypol, was prepared using these modifications.

Amino Acid Analysis. Quantitative determination of amino acid composition was performed on Model 120A and Model 120C Beckman Amino Acid An-

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alyzers using columns of Beckman Custom Research Resin Types AA-15, PA-28 and PA-35, by the procedure of Spackman et al. (15). A modification of Spackman's 30 C–50 C system for physiological fluids was used to separate ornithine and γ-amino butyric acid from lysine and 3-aminopropanol from ammonia. A 0.9 × 23 cm column of Beckman Type PA-35 ion-exchange resin was eluted with pH 4.25 sodium citrate buffer containing 1% 1-propanol and pH 5.36 sodium citrate buffer and temperatures of 32.5 C and 62.5 C. β-Alanine was determined by increasing the run time on the regular long column (PA-28) run for acidic and neutral amino acids from 190 min to 220 min. β-Alanine is eluted after phenylalanine at about 190 min.

The samples were hydrolyzed by placing in heavy wall glass tubes with 6 N hydrochloric acid, freezing in acetone-dry ice solution, degassing and sealing under vacuum. After heating in an autoclave at 110 C for 22 hr, the ampules were opened and the samples transferred to round bottom flasks with deionized water. The samples were reduced to dryness on a rotary evaporator, washed once with deionized water, and diluted to volume with pH 2.2 sodium citrate sample-dilution buffer (Beckman). No correction has been made in calculating amino acid concentration for the destruction of amino acids during acid hydrolysis.

Reaction of Gossypol With Amino Acids

Gossypol-amino acid complexes were formed by adding gossypol to solutions of various amino acids and incubating in test tubes in a water bath at 37 C. Gossypol was dissolved in 95% ethanol and added to the amino acid which was dissolved in 0.03 M sodium phosphate buffer. Aliquots were taken at intervals and the reaction was monitored by successive scans from wavelengths of 320 to 500 mμ with a Beckman Model DB spectrophotometer. The reaction mixtures were shaken before and after sampling. A gossypol control was run to ascertain whether the spectral changes were due to changes in gossypol itself or to the formation of complexes with the amino acids.

Each of the amino acids arginine, asparagine, glutamine and glycine were incubated with gossypol at pH 7.5 in a reaction mixture composed of 1 ml (2 μmoles) of gossypol and 1 ml (50 μmoles) of amino acid. For scanning, 0.05 ml of the reaction mixture was diluted with 3 ml of 95% ethanol: 0.03 M sodium phosphate buffer (1:1 v/v). Lysine was incubated with gossypol at pH 5.7, 6.1, 6.5, 7.1 and 7.5 to determine the effect of pH on the rate of reaction.

To further compare the effect of pH under the conditions utilized for enzymic digestion of gossypol-protein complexes, gossypol was incubated with ornithine, glutamine, asparagine, glutamic acid and aspartic acid at pH 5.7 and 7.5. This also permitted a comparison of the rate of reaction of gossypol with glutamic and aspartic acid and their amides. The possibility of oxidation was minimized by running these reactions in an atmosphere of nitrogen during incubation.

Gossypol-γ-Amino Butyric Acid. γ-Amino butyric acid (Calbiochem) was dissolved in 0.03 M sodium phosphate buffer pH 7.4 and combined with gossypol-acetic acid dissolved in methanol in a 10:1 molar ratio. The container was flushed with nitrogen, stoppered, and shaken in the dark at 37 C for 12 hr. The reaction mixture was lyophilized and extracted with ether. The ether-extracted residue was analyzed for gossypol and micro-Kjeldahl determinations were made for nitrogen content. This residue was dissolved in the butanol upper phase of a 2 phase system composed of 1-butanol and water containing 0.01 M NaCl (1:2 v/v) and washed three times with the aqueous lower phase to remove water-soluble free γ-amino butyric acid. The butanol-soluble material was chromatographed on thin layer plates (0.375 mm layer) of Adsorbosil-5 (Applied Science Laboratories, Inc., State College, Pa.) which had been activated at 110 C for 1 hr. A solvent system composed of ethyl acetate/2-propanol/water (65:24:11, v/v/v) produced the best separation of the reaction products.

Gossypol-β-Alanine. β-Alanine was combined with gossypol by dissolving 173 μmoles of gossypol-acetic acid in 25 ml of methanol and mixing with 25 ml of 0.03 M sodium phosphate buffer, pH 7.4, containing 122 μmoles of β-alanine. The flask was flushed with a stream of nitrogen, stoppered, and shaken in the dark at 37 C for 12 hr. The methanol was removed from the reaction mixture on a rotary evaporator and the remaining aqueous solution was extracted 5 times with 25 ml of ether. The ether extract was reduced to dryness on a rotary evaporator and suspended in methanol. This methanol solution was fractionated by a series of preparative thin layer plates coated with a 0.5 mm layer of Adsorbosil-5. The plates were developed in ethyl acetate/2-propanol/water (65:24:11 v/v/v) and the corresponding bands from each plate were scraped off and pooled. The pooled bands were placed in funnels on Whatman No. 1 filter paper and eluted with 95% ethanol-water (70:30 v/v). The eluates were reduced to dryness on a rotary evaporator and transferred to test tubes with methanol, leaving in the round bottom flask the small amount of adsorbent which had been dissolved in the ethanol-water elution step. Concentration of these solutions and gossypol and amino acid content were determined.

Preparation and Purification of Gossypol Complexes with Other Amino Compounds

Hippuryl-L-Lysine-Gossypol. The complex of gossypol and hippuryl-L-lysine (benzoyl-glycyl-L-lysine, Mann Research Laboratories, Inc.) was prepared by dissolving 100 μmoles of hippuryl-L-lysine in a 2:1 (v/v) mixture of 95% ethanol and 0.03 M sodium phosphate buffer pH 7.4 and adding to it 50 μmoles of gossypol dissolved in 95% ethanol. This mixture was heated in a capped test tube, cooled, filtered, and stored in a refrigerator at 4 C. A sample of the reaction mixture was chromatographed on a 2.5 × 44 cm column of LH-20 Sephadex (Pharmacia Fine Chemicals, Inc.) which had been equilibrated with a 1:1 (v/v) mixture of 95% ethanol and 0.03 M sodium phosphate buffer pH 7.4. The sample was pumped onto the column with a Holter Model RL155 Peristaltic Pump using upward flow with a rate of 41 ml/hr. The column effluent was monitored at 380 mμ by means of a flow cell in a Beckman Model DB recording spectrophotometer and fractions were collected as they were eluted from the column. Absorption spectra and gossypol and amino acid content were determined.

Gossypol-3-Aminopropanol. One hundred seventy-three micromoles of gossypol-acetic acid was dissolved in 25 ml of methanol and combined with 25 ml of 0.03 M sodium phosphate buffer, pH 7.4, con-

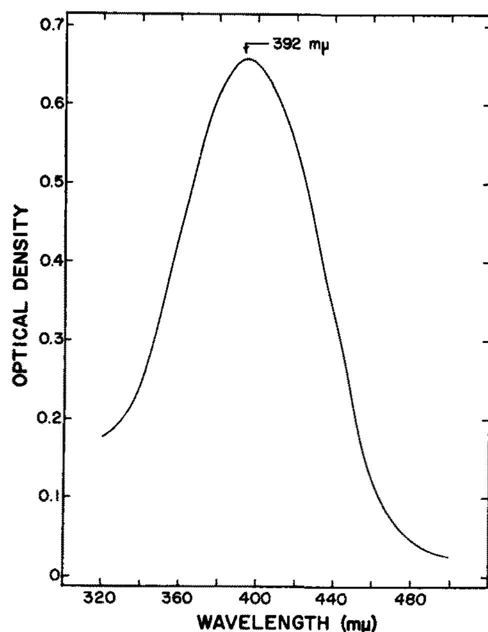

FIG. 1. Absorption spectrum of gossypol in 95% ethanol-0.03 M sodium phosphate buffer (1:1 v/v) pH 7.5 at 37 C.

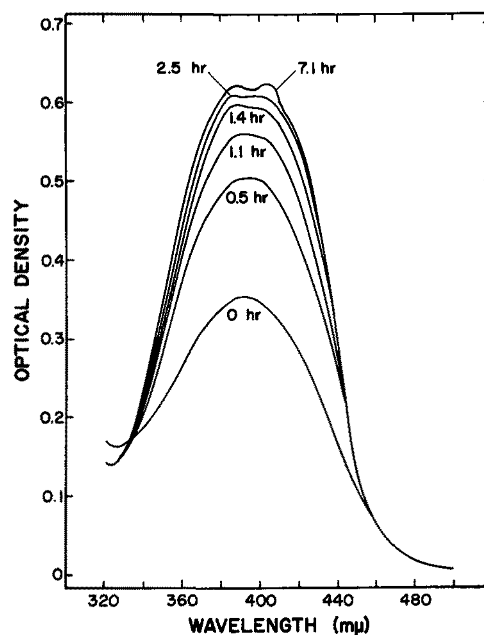

FIG. 2. Change in the absorption spectrum (320–500 mμ) of gossypol on incubation with lysine in 95% ethanol-0.03 M sodium phosphate buffer (1:1 v/v) pH 7.5 at 37 C.

taining 3.88 mmoles of 3-aminopropanol (Matheson, Coleman and Bell). The flask was flushed with nitrogen, stoppered, and shaken in the dark at 37 C for 12 hr. A yellow precipitate was present at the end of the reaction time and was removed by filtration through Whatman No. 5 filter paper. The precipitate was washed with methanol and ether and dried in a vacuum desiccator.

The precipitate was fractionated by streaking it on a series of thin layer plates coated with a 0.5 mm layer of Adsorbosil-5 and developing with ethyl acetate/2-propanol/water (65:24:11 v/v/v). The bands were scraped off and pooled and the material was eluted from the pooled adsorbent with the ethyl acetate solvent system. Aliquots were taken for the determination of concentration, gossypol and amino acids.

Results and Discussion

The absorption spectrum of gossypol in the solvent system used is shown in Figure 1 exhibiting maximum absorbance at 392 mμ. Gossypol was incubated in this system for 24 hr with no change in the spectrum other than a slow decrease in overall absorbance toward the end of the period. Figure 2 illustrates the changes in the absorption spectrum of gossypol during incubation with lysine. The incubation of gossypol with a number of other amino acids, peptides and amino compounds produced the same spectral changes, specifically the formation of a peak in the 406–408 mμ area and a shoulder at 432 mμ. The spectrum becomes stable after a period of time, depending on the compound involved in the reaction, and then begins to exhibit the same overall decrease in absorbance as was noted with gossypol alone. Stabilization of the absorbance spectrum may indicate the completion of the reaction or attainment of equilibrium. In monitoring the reaction of gossypol with amino compounds, the difference in absorbance at 406 mμ and 392 mμ was used as a measure of the extent of complex formation, unreacted gossypol having maximum absorbance in this system at 392 mμ and the gossypol-amino complex at 406 mμ.

In the experiments described in this paper, lysine was incubated with gossypol at pH values from 5.7 to 7.5 to determine the effect of pH on the formation of the gossypol-lysine complex at body temperature (37 C). Figure 3 depicts the effect of pH on the rate of reaction, revealing that, although reaction occurs at all levels, it proceeds more rapidly as the pH increases from 5.7 to 7.5. Higher pH values were not investigated because of the instability of gossypol in this region.

The rate of reaction of the various amino acids with gossypol is illustrated in Figure 4 in which the ranking, in order of reactivity with gossypol, appears to be as follows: arginine > lysine > ornithine > glutamine > asparagine > glycine > aspartic acid > glutamic acid. This is in general accord with the order expected on the basis of structure and charge on the molecules at pH 7.4. Gossypol is an acidic compound intermediate in acidity between phenols (pKa about 10) and carboxylic acids (pKa about 5). This greater acid strength relative to other phenols has been explained in terms of the delocalization of the negative charges in the divalent anion formed by loss of protons from the hydroxyl groups ortho to the aldehyde groups (16) as shown in Figure 5. Titration of gossypol shows pK_1 to

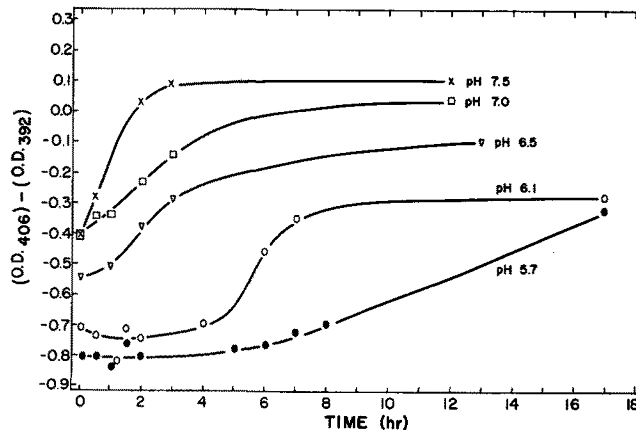

FIG. 3. Effect of pH on rate of reaction of gossypol with lysine.

TABLE I

Analysis of Gossypol Complexes With β-Alanine and 3-Aminopropanol Separated by Thin Layer Chromatography[a]

Sample	R_f value	Gossypol µM/mg	Amino acid µM/mg	Molar ratio Goss./AA[b]	
Gossypol-β-alanine (TLC Band 1)	0.06	0.6943	1.8735	1:2.70	(1:3)
Gossypol-β-alanine (TLC Band 2)	0.29	0.3079	1.1614	1:3.77	(1:4)
Gossypol-β-alanine (TLC Band 3)	0.47	0.7739	1.2862	1:1.66	(1:2)
Gossypol-β-alanine (TLC Band 4)	0.72	0.1792	0.2496	1:1.39	(1:1)
Gossypol-β-alanine (TLC Band 5)	0.87	0.2070	0.3906	1:1.89	(1:2)
Gossypol-3-aminopropanol (TLC Band A) Ppt.[c]	0.84	0.5204	1.4354	1:2.76	(1:3)
Gossypol-3-aminopropanol (TLC Band A) Soln.[c]		0.3857	1.4083	1:3.65	(1:4)
Gossypol-3-aminopropanol (TLC Band B)	0.76	0.1266	0.4127	1:3.26	(1:3)

[a] Adsorbent layer, Adsorbosil-5; solvent system, Ethyl acetate/2-propanol/water (65:24:11 v/v).
[b] Gossypol/amino acid.
[c] After isolation, this material separated and the two fractions were treated separately.

be in the vicinity of pH 6 (17), therefore, at pH 7.4, both gossypol and the carboxyl groups of the amino acids involved in this study would bear a negative charge and would be mutually repelling. An α-amino group near to the carboxyl group would, consequently, be expected to react more slowly with gossypol than would an amino group in a position more distant from the carboxyl group and a dicarboxylic amino acid should react more slowly than a monocarboxylic amino acid. Figure 4 supports this hypothesis as can be seen by the fact that arginine and lysine react more rapidly than glycine which reacts more rapidly than aspartic or glutamic acid.

Gel filtration of hippuryl-L-lysine-gossypol on Sephadex LH-20 produced three major absorption peaks. The absorption spectra (Fig. 6) of fractions within each of these peaks and the order of their elution suggested that the first peak was di-(hippuryl-L-lysine)-gossypol, the second, mono-(hippuryl-L-lysine)-gossypol, and the third, free gossypol. The molar ratios between gossypol and lysine, obtained by amino acid analysis and gossypol determination on the fractions, did not, however, clearly confirm this hypothesis. The ratio of 1 mole of gossypol to 2.8 moles of lysine found in Peak 1 is somewhat higher than would be expected on the basis of the reaction of a single ε-amino group of lysine with each of the two aldehyde groups of a gossypol molecule, or a ratio of 1 to 2. This ratio of 1:2.8 (or 1:3) may be obtained if each of the aldehyde groups of gossypol can combine with more than one amino group.

The composition of the fractions of complexes of gossypol with β-alanine and 3-aminopropanol isolated on thin layer plates is presented in Table I with Band No. 1 being nearest the origin and Band No. 5 being nearest the solvent front for the gossypol-β-alanine complex and Band A being nearest the solvent front for the gossypol-3-aminopropanol complex. Since, in this solvent system, β-alanine and 3-aminopropanol barely move from the origin, while gossypol moves almost with the solvent front, it would be expected that various combinations of the two compounds with gossypol would have R_f values ranging between 0 and 1, with the more highly substituted complexes having the smaller R_f values. This is generally true for the β-alanine complexes, although the R_f value of the 1:4 complex (Band No. 2) exceeds that of the 1:3 (Band No. 1), and that of one of the 1:2 complexes (Band No. 5) exceeds both the other 1:2 complex (Band No. 3) and the 1:1 complex (Band No. 4). An explanation for the greater R_f value of Band No. 5 may be that the two β-alanine molecules are both attached to a single aldehyde group on the gossypol molecule, leaving the other aldehyde group free and thus allowing greater affinity for the mobile phase as compared with Band No. 3 in which one β-alanine molecule may be attached to each aldehyde group.

In the case of the gossypol-3-aminopropanol com-

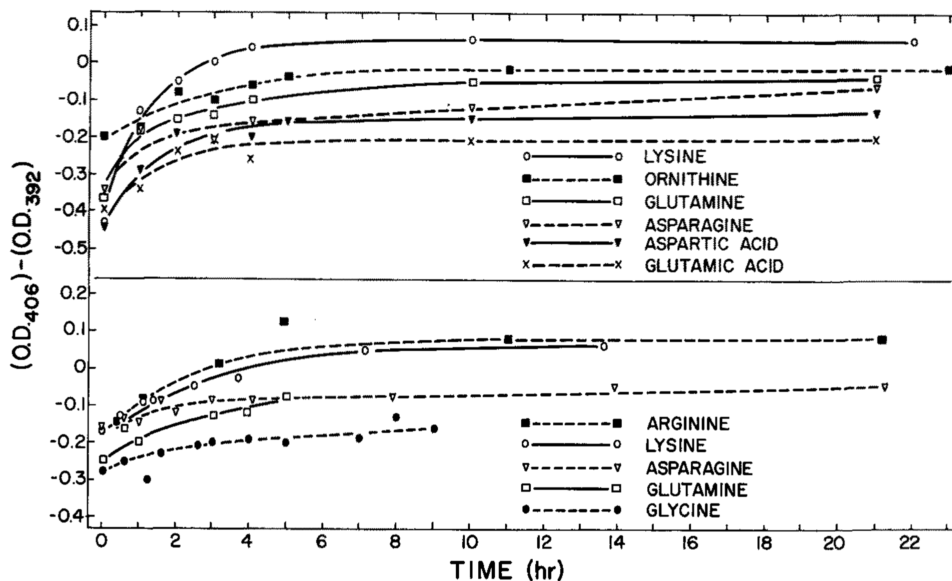

FIG. 4. Rate of reaction of amino acids with gossypol.

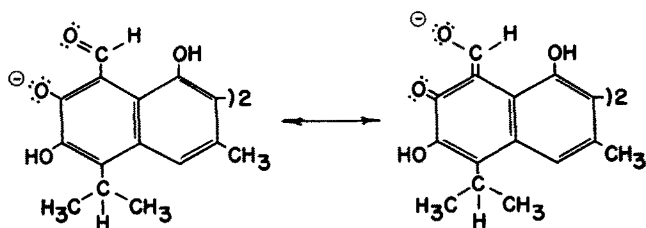

FIG. 5. Resonance stabilized anion of gossypol.

plex, Band A contained a mixture of 1:3 and 1:4 while Band B contained only 1:3. Apparently, the free hydroxyl groups of the 3-aminopropanol impart a greater affinity for the mobile phase, thus resulting in higher R_f values than for the comparable complexes with β-alanine. The ratios of gossypol to amino compounds in these various complexes of from 1:1 to 1:4 can be explained in terms of the tetraanilino-gossypol complex of Murty and Seshadri (18). In this compound, two amino groups are bound to each aldehyde carbon giving a gossypol to aniline ratio of 1:4. Obviously, ratios of 1:1, 1:2 and 1:3 could also be produced. The tetraanilino product was isolated at ambient temperatures and could be converted to the dianilino form by heating for 4 hr at 110 C. Since the complexes under discussion were not subjected to temperatures in excess of 60 C, it is possible for molar ratios of 1:4 to have been produced.

In the case of γ-amino butyric acid, gossypol to γ-amino butyric acid ratios were found to be as high as 1:18 in some products. The fact that γ-amino butyric acid forms polymers rather easily under certain conditions (19) probably accounts for these ratios.

The reaction of gossypol with amino groups other than the ε-amino group of lysine at physiological pH and temperature offers a possible explanation for the unaccountably large effect of bound gossypol on the digestibility of proteins containing this material. As the protein is broken down by the enzymes in the digestive tract and more amino groups become available, they may react with gossypol, with the resulting complex being resistant to further enzymic degradation because of steric hindrance by the bulky gossypol molecule. Evidence for this mechanism has been found in other studies on the effect of bound gossypol on amino acid availability. (Cater and Lyman, manuscript in preparation). It also presents a possible explanation of the mode of toxicity of free gossypol within the body in the binding of gossypol to the surface of enzymes, in particular in the area of the active center. Reaction with the ε-amino group of lysine may result in a more stable bond than reaction with amino groups in other amino acids which are closer to the carboxyl group which would account for the identification of the ε-amino group as the site of binding for bound gossypol. Samples containing bound gossypol have usually been subjected to temperatures in excess of 110 C

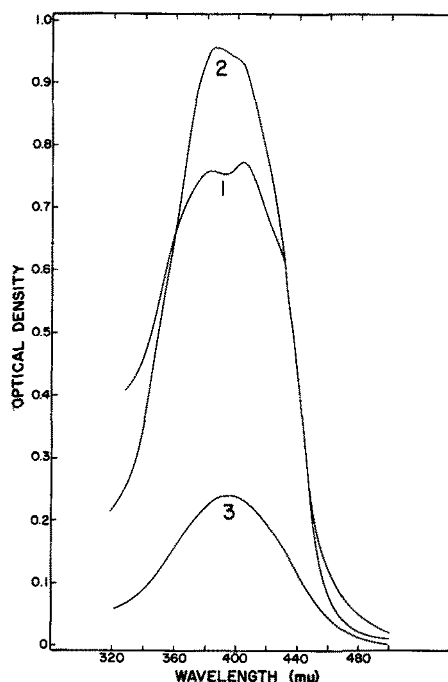

FIG. 6. Absorption spectra of fractions of hippuryl-L-lysine; gossypol reaction mixture eluted from LH-20 Sephadex.

either in processing or drying. The reaction of gossypol with amide nitrogen, as indicated by the differences in the rates of reaction between aspartic and glutamic acids and asparagine and glutamine with gossypol, suggests these groups as additional binding sites for gossypol on protein molecules.

ACKNOWLEDGMENTS

Supported in part by research grants from the National Cottonseed Products Association; amino acid analysis by Vicky Boyd and W. V. Stewart, chromatographic analysis and technical assistance by Dorothea Hedges and Brenda Oglesby.

REFERENCES

1. Clark, E. P., J. Biol. Chem. *76*, 229 (1928).
2. Baliga, B. P., Ph.D. dissertation, A&M College of Texas, College Station, Texas, 1956.
3. Baliga, B. P., and C. M. Lyman, JAOCS *37*, 21 (1957).
4. Lyman, C. M., B. P. Baliga and M. W. Slay, Arch. Biochem. Biophys. *84*, 486 (1959).
5. Jones, D. B., and H. C. Waterman, J. Biol. Chem. *56*, 501 (1923).
6. Tillman, A. D., and K. Druse, J. Animal Sci. *21*, 290 (1962).
7. Shirley, D. A., and W. C. Sheehan, J. Org. Chem. *21*, 251 (1956).
8. Dechary, J. M., and L. E. Brown, JAOCS *33*, 76 (1956).
9. Dechary, J. M., G. Wakaboyashi and C. R. Grau, Ibid. *34*, 548 (1957).
10. Gonzales, C. O., H. M. Cappi, M. Salem and C. Staffa, Ibid. *43*, 678 (1966).
11. Witkop, B., and T. W. Beiler, J. Am. Chem. Soc. *76*, 5589 (1954).
12. Alley, P. W., and D. A. Shirley, J. Org. Chem. *24*, 1534 (1959).
13. Pons, W. A., Jr., and J. D. Guthrie, JAOCS *26*, 671 (1949).
14. Pons, W. A., Jr., C. L. Hoffpauir and R. T. O'Connor, Ibid. *27*, 390 (1950).
15. Spackman, D. H., W. H. Stein and S. Moore, Anal. Chem. *30*, 1190 (1958).
16. Shirley, D. A., Proceedings of Conference on Inactivation of Gossypol With Mineral Salts, New Orleans, April 1966, p. 12.
17. Tanksley, T. D., Ph.D. Dissertation, Texas A&M University, College Station, Texas, 1968.
18. Murty, K. S., and T. R. Seshadri, Proc. Indian Acad. Sci. *A16*, 141 (1942).
19. Gutfreund, H., Biochem. J. *42*, 544 (1948).

[Received April 21, 1969]