amounts of 2000 μg of each sample of white and red variety and 750 μg in the case of Madras variety have been found to be suitable for spotting on the TLC plate. With these concentrations all the extracts give rise to identical chromatograms consisting of several pink colored spots of varying intensities detected by glycine–formaldehyde reagent. Among these the major three components of each sample having the same R_f values with respect to each other were estimated. From Table II it appears that the amount of individual lachrymators in both white and red varieties of onion is nearly the same, whereas in the Madras variety these individual lachrymators are present in higher amounts. The small Madras onion is believed to be a "strong" variety in terms of pungency and lachrymatory factor. The present data show that the total lachrymator content in this variety is considerably higher than the other two varieties studied.

The results of the incubation study described previously could give an overall estimation of total lachrymators in onion. This method is, however, not suitable when quantitative information on the relative abundance of individual lachrymator components of onion is required.

The present TLC method of separation of lachrymators followed by estimation of the individual component gives a better understanding of various onion lachrymators, which in turn reflects the quality of onions in respect to their flavor strength.

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Effect of Free and Bound Gossypol on the Absorption of L-[^{14}C]Lysine, L-[^{14}C]Methionine, and L-[^{14}C]Valine from the Rat Small Intestine

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Studies were conducted to determine the relationship of any alteration in amino acid transport and an observed decrease in nitrogen uptake from the gut due to gossypol. The amino acids used were ^{14}C-labeled L-lysine, L-methionine, and L-valine. The effects of free and bound gossypol on the in vivo removal of these amino acids from surgically produced sacs were studied. In vitro determinations of the effect of two forms of gossypol on the kinetic values K_t and V_{max} for these amino acids using everted intestinal sacs were used to evaluate transport. Transport of gossypol alone was studied. In vivo removal of lysine was not significantly altered; methionine was reduced by free gossypol and showed an increase in the sac wall; bound gossypol increased the removal of valine, lessened the amount in the sac wall, and increased deposition in the liver. In the in vitro studies, K_t and V_{max} were increased above the control for lysine-bound gossypol and with free gossypol were increased over the controls for methionine. K_t was unchanged from the control for valine-bound gossypol, while the V_{max} was increased. Gossypol alone was not actively transported.

The nutritive quality of cottonseed meal is affected by a toxic polyphenol, gossypol (Withers and Carruth, 1915), which is largely detoxified during processing by its combination with the free amino groups of the protein with a concomitant reduction in nutritive quality (Smith et al., 1958). Research on cottonseed meal as a nutrient has been directed toward determining the level of the residual free gossypol that may be safely fed to production animals (Heywang and Bird, 1955; Hollon et al., 1958; Sharma et al., 1966), and on the mode of gossypol toxicity in the animal body (Smith, 1957; Albrecht et al., 1968; Skutches et al., 1973).

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Little attention has been given to the interaction of gossypol with the absorptive surface of the gut which is the true interface of nutrients with the body. Fecal nitrogen from rats fed diets with and without bound gossypol was higher for the former as were the individual amino acids in the small intestinal chyme of rats an hour after the diet was consumed (unpublished data). An increased rate of passage of digesta, inhibition of proteolytic enzymes (Lyman et al., 1959; Wong et al., 1972; Finlay et al., 1973; Tanksley et al., 1970), and the action of gossypol on amino acid absorption are all possible factors involved in such observations. Possibly, gossypol in cottonseed protein could affect all amino acid transport systems to effectively reduce the protein level fed, or it could affect one system to effect an amino acid imbalance (Delhumeau et al., 1962).

To determine if gossypol alters the intestinal absorption of amino acids, two experiments were conducted, one in vivo and the other in vitro, using, in both experiments, lys-

ine, methionine, or valine with either free or bound gossypol.

MATERIALS AND METHODS

In Vivo Study of the Effect of Two Forms of Gossypol on Amino Acid Removal from Surgically Produced Sacs Made in Situ in White Rats. Two experiments were conducted, experiment 1 with gossypol at 0.5% of the amino acid and experiment 2 with gossypol at 0.9% of the amino acid by weight respectively. In both experiments, the amino acids L-[^{14}C]lysine, L-[^{14}C]valine, or L-[^{14}C]methionine (purity determined by thin-layer chromatography) were present at a concentration of 10 m*M*. Each experiment tested the removal of L-[^{14}C]lysine, L-[^{14}C]methionine, or L-[^{14}C]valine with either no gossypol, free gossypol, or bound gossypol from surgically produced sacs in the live rat. Each of the nine individual amino acid × gossypol treatments employed four rats in each of which two sacs were made. The two experiments involved a total of 72 white rats of the Wistar strain weighing between 220 and 250 g, which had been fed a commercial rat chow from weaning.

All solutions introduced into the gut sacs in the in vivo experiments were based on a modified saline solution as follows (m*M*): NaCl, 70; KCl, 2.8; $CaCl_2$, 1.5; KH_2PO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.7. The pH was adjusted to 7.4 with 0.05 *N* NaOH. This concentration was chosen in preference to distilled water to approximate the conditions present in the lower small gut. Solutions used in gossypol-free control treatments were 10 m*M* including about 2μCi of ^{14}C-labeled L-lysine monohydrochloride, L-methionine, or L-valine, respectively. Solutions used in the free gossypol treatments were made up similar to the control solutions except that the sodium salt of gossypol (Skutches et al., 1973) was added in an aqueous solution in the proper amount to provide 0.5 or 0.9% of gossypol in experiments 1 and 2, respectively.

A complex of each of the amino acids, lysine, methionine, and valine, was formed with gossypol by a modification of the method of Cater and Lyman (1969). Each of the amino acids was dissolved in the desired amount of distilled water, and after adding sufficient ^{14}C-labeled amino acid, the pH of the resulting solutions was adjusted to 7.60, 7.45, or left unadjusted for lysine, methionine, and valine, respectively. A fivefold excess of gossypol dissolved in ethanol was added to the solution which was then incubated in a shaking water bath at 60° for 6 hr. Any remaining gossypol not bound to the amino acid in the procedure was removed by washing with ether and the sample was diluted to the volume desired. Spectral curves of the resultant solutions indicated a peak absorbance shift from the 392 mμ expected with unreacted gossypol to approximately 405 mμ, a value characteristic of a gossypol–amino acid complex (Cater and Lyman, 1969). The products were analyzed for free and total gossypol by the procedures of Smith (1968 and 1958, respectively), with suitable modifications made for aqueous samples. Bound gossypol was taken as the difference between total and free gossypol. Solutions of amino acids with bound gossypol were made up for treatments by using the proper amounts of both cold amino acid and the amino acid–gossypol complex to produce a 10 m*M* amino acid solution with either a 0.5 or 0.9% gossypol content, depending on the experiment.

Prior to the surgical procedure involved in making the in situ sacs, the rats were starved 18 hr. Two sacs were made in the intestine of each rat. Locations for sacs were chosen at mid-jejunum and in the lower ileum approximately 5 to 10 cm proximal to the ileocecal junction. The order of production of the two sacs in each of the four rats per treatment was alternated to remove any time bias in the mean of each sac. Each of the two sacs was 7 cm long and they were filled with 1 ml of the treatment solution which contained a 10-μmol amino acid load. After completion of the sacs the incision was closed and the rats were allowed to regain consciousness. After a 1-hr period had elapsed from the time the first sac was filled, the rat was exsanguinated by decapitation, the sacs were removed, and the contents quantitatively emptied and rinsed into a 10-ml volumetric flask which was then brought to volume with rinse fluid and water. The sac tissue was frozen for later assay. An aliquot of the 10-ml sample was counted in a liquid scintillation spectrometer using a scintillator described by Patterson and Greene (1965). The total length and exact locations of the sacs in the small gut were measured. The liver was also removed, weighed, and frozen for later assay.

The dry weight of each sac was determined after lyophilizing the tissue for 24 hr. The sac and liver tissue were oxidized in a Packard Model 305 Tri-Carb sample oxidizer according to the instruction manual (1972). The radioactivity of the oxidized samples was determined in a Tri-Carb liquid scintillation spectrometer. Previous work indicated that only the liver and intestinal sac tissue need be oxidized to locate the majority of initial counts after 1 hr. Micromoles of amino acid in the two tissues and in the fluid remaining in each sac were determined by comparison against an aliquot of the original treatment solution containing the amino acid in known concentration. Statistical analyses were conducted on the micromoles of amino acid removed from the sac (determined by differences from the known original amount and the measured remaining amino acid in the sac), and the micromoles of amino acid present in the entire sac tissue, and the micromoles of amino acid found in the entire liver for both experiments 1 and 2.

In Vitro Study of the Effect of Bound and Free Gossypol on the Intestinal Transport of Amino Acids. The experimental design was similar to the in vivo study in that it involved three amino acids, lysine, valine, or methionine, tested with none, 0.5% free, or 0.5% bound gossypol. The difference in design between the in vivo and in vitro experiments was that while the former employed two gossypol levels both at the same amino acid level, the latter employed only one gossypol level (0.5%) and four levels of the amino acids. The in vitro study employed 144 rats over three gossypol forms and three amino acids at four relative concentrations of 1, 10, 50, and 100. The lowest actual concentration for methionine and valine was 0.1 m*M* and that for lysine was 10 μ*M*. The need for the tenfold reduction in actual lysine concentration was determined in preliminary experiments. The 100-fold difference in relative concentrations allowed determinations of kinetic parameters in a procedure similar to that of Matthews and Laster (1965). The preparation of bound and free gossypol and the description of the amino acids were the same as in the in vivo experiment. Transport was determined using sacs of everted small intestine from 200–300 g white rats by the procedure of Wilson and Wiseman (1954). The rats were prestarved 18 hr before use. From the small intestine of each rat, four sacs were made, numbered 1 through 4 from the pyloric end. The sacs were each 7 cm in length and were each removed from their respective quarters of the gut length, with sac no. 1 being taken several centimeters distal to the ligament of Treitz at the duodeno–jejunal junction. The composition of the standard physiological media was (m*M*): NaCl, 118.5; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25; dextrose, 11.1. The $NaHCO_3$ was pregassed with 100% CO_2, and the combined media was gassed for 1 hr before use with 95% O_2 and 5% CO_2.

The medium in the everted sac was called the serosal fluid; that in the flask used for incubation of the sac was called the mucosal fluid. The terms "initial" and "final" were used for serosal and mucosal fluids before and after incubation, respectively. In all cases, the volume of the serosal fluid was 1 ml, and that of the mucosal fluid was 10

Table I. Effects of 0.5% Free or 0.5% Bound Gossypol on Lysine, Methionine, or Valine Removal of a 10 μmol Amino Acid Load from in Vivo Intestinal Sacs and on the Deposition of the Amino Acids in Intestinal Sac Tissue and Liver (Micromoles)

	Lysine			Methionine			Valine		
Gossypol treatment	Removed from sac	In sac tissue	In liver	Removed from sac	In sac tissue	In liver	Removed from sac	In sac tissue	In liver
None	7.72[a]	1.04[a]	2.27[b]	9.70	0.21	6.16	7.94	1.15	1.62
Free	8.16	1.01	2.18	8.54	1.27	3.45	7.74	1.56	1.59
Bound	7.20	1.12	2.88	9.63	0.21	5.62	9.25	0.43	2.34
LSD[c] ($P < 0.05$)	0.96	0.64	1.39	0.96	0.64	1.39	0.96	0.64	1.39
LSD ($P < 0.01$)	1.30	0.86	1.87	1.30	0.86	1.87	1.30	0.86	1.87

[a] Each value represents a mean of eight sacs from four rats. [b] Each value represents the mean of four rats. [c] When the difference between any two means equals or exceeds the given least significant difference (LSD), then the comparison is judged significant at the stated probability level.

Table II. Effects of 0.9% Free or 0.9% Bound Gossypol on Lysine, Methionine, or Valine Removal of a 10 μmol Amino Acid Load from in Vivo Intestinal Sacs and on the Deposition of the Amino Acids in Intestinal Sac Tissue and Liver (Micromoles)

	Lysine			Methionine			Valine		
Treatment	Removed from sac	In sac tissue	In liver	Removed from sac	In sac tissue	In liver	Removed from sac	In sac tissue	In liver
None	8.38[a]	0.857[a]	1.33[b]	9.33	0.400	4.74	7.96	0.592	1.25
Free	8.02	0.784	1.46	9.46	0.418	5.06	8.39	0.879	0.97
Bound	8.79	0.498	1.46	9.73	0.163	5.95	9.41	0.386	2.07
LSD[c] ($P < 0.05$)	0.82	0.350	0.72	0.82	0.350	0.72	0.82	0.350	0.72
LSD ($P < 0.01$)	1.11	0.470	0.97	1.11	0.470	0.97	1.11	0.470	0.97

[a-c] See footnotes a-c of Table I.

ml. The initial mucosal and serosal solutions were identical in all respects. Incubations were carried out in 25-ml erlenmeyer flasks specially prepared for continuous gassing with 95% O_2–5% CO_2, in a slowly oscillating water bath at 37°. The final serosal volume was measured at the end of 1-hr incubation time by draining each sac into a graduated centrifuge tube and lightly expressing as much adhering fluid as possible. Since only four rats could optimally be done each day, the order of treatments was at random.

The amino acid translocation from the mucosal to the serosal side of the intestine was expressed in the following two forms: (a) net transport per sac—the increase in moles of the amino acid inside the sac after incubation per 7-cm sac per hr; (b) *I/O* ratio—the ratio of the molar concentration of the amino acid in the final serosal fluid to that concentration in the mucosal fluid. The dry weight of the sacs was determined by trimming immediately inside the soft multistrand tie at each end of the sac and lyophilizing for 24 hr.

Each of the two amino acid translocation measurements was analyzed by the same analysis of variance described for the in vivo experiment. The net transport per sac was used as the measure of amino acid transport in the kinetic determinations. The velocity measurements were fitted to a regression curve. Michaelis–Menton kinetics (1913) could be applied to the transport system (Matthews and Laster, 1965), and values were determined for V_{max}, the apparent limiting rate of transport, and K_t, the concentration of amino acid at which half this rate was attained, and a value considered to be an indicator of the affinity between the transport system and the substrate.

Determination of Any Active Transport of Gossypol. Using the Wilson–Wiseman technique (1954), ^{14}C-labeled gossypol prepared according to Smith (1974) was used to determine if there was any active transport of the previously described sodium salt of gossypol. In the initial inquiry, levels of 100 and 1 μ*M* gossypol were included in serosal and mucosal initial fluids. All media and other procedures were as described.

A subsequent experiment was conducted to determine if micelles, characteristic of fat absorption in the gut, affected transport of labeled gossypol in the in vitro state. A technique similar to that of Strauss (1966) was employed using micelles made from sodium taurodesoxycholate, oleic acid, and monoolein by the method of Johnston and Borgström (1964). All preparations and procedures were as described except that calcium and magnesium were omitted from the physiological media. The ^{14}C-labeled sodium salt of gossypol was used at a 5 μ*M* concentration with a critical micellar concentration of either 1× or 5× using four rats with four sacs per rat at each critical concentration. Preparation of sacs and determination of transport were as described.

RESULTS AND DISCUSSION

In Vivo Study of the Effect of Free or Bound Gossypol on the Removal of Lysine, Methionine, or Valine from Intestinal Sacs. The locations of sacs 1 and 2, respectively, corresponded to the areas of mid to lower jejunum and lower ileum. Transport can be expected to occur and to be representative of the gut at these locations for the concentrations employed (Ramaswamy and Radhakrishnan, 1966; Larsen et al., 1964). All sacs were as close as possible to 7 cm in length.

Tables I and II present the results of the in vivo study of amino acid absorption as affected by two forms of gossypol at levels of 0.5 or 0.9% of the amino acid. Since statistical analysis showed no gossypol by sac interaction, the mean of the two sacs was used to evaluate the effect of gossypol on amino acid transport of sacs.

Although there is much precedence for basing transport on a sac weight value, it was assumed that the absorptive surface of the villi in carefully measured 7-cm lengths of in-

testine was more even over the length of the small gut than was the musculature at each point along the gut. The amount of the muscle mass would be the determining factor in any dry weight measurement (Ahmed and Walker, 1972; Fearon and Bird, 1967). For these reasons transport per unit length was chosen over transport per unit weight. The amino acids in the intestinal tissue of the sacs and in the liver were expressed in terms of an entire sac or the entire liver to maintain the same basis as the amino acid removed from the sacs. Such measurement allowed better visualization of the distribution of the 10-μmol load of [^{14}C]amino acid.

The amount of amino acid removed depended highly ($P < 0.01$) on what amino acid was involved at both the low and high gossypol level experiments. More methionine and valine were removed than lysine. This is in agreement with observations in the literature (Delhumeau et al., 1962; Larsen et al., 1964; Finch and Hird, 1960; Hagihira et al., 1962).

Table I indicates a decreased removal ($P < 0.05$) of lysine in the presence of 0.5% bound gossypol relative to the removal at the same level of free gossypol. Neither gossypol form showed statistically different removal from the control value. Methionine removal was less ($P < 0.05$) in the presence of 0.5% free gossypol than it was with either 0.5% bound gossypol or in the control case. Valine showed more removal ($P < 0.05$) in the case of both 0.5 and 0.9% bound (Tables I and II) than it did for either the control or with free gossypol. All treatments showed no difference in the case of lysine and methionine at the higher gossypol level of 0.9% presented in Table II.

The distribution of lysine or the metabolic products of lysine in the intestinal wall and the liver indicated that the decreased removal of lysine with 0.5% bound gossypol relative to 0.5% free gossypol was not followed by a similar significant decrease in sac tissue or liver. The results do not indicate that lysine, a basic amino acid, is hindered in its removal from the gut by either free or bound gossypol. No concomitant decrease of the compound or its products in the tissues was noted.

The intestinal tissue methionine at the low gossypol level (Table I) indicates that there is significantly ($P < 0.01$) more of the compound in the sac walls in the presence of free gossypol than there is with bound gossypol or the control. This is accompanied by an equally significant decrease of the compound in the liver in the case of free gossypol. The net result in the case of the low gossypol-level experiment is that there was less methionine removed from the sacs in the presence of free gossypol with more of it appearing in the sac wall and thus less in the liver. Methionine is absorbed very rapidly from the gut (Delhumeau et al., 1962; Finch and Hird, 1960; Adibi and Gray, 1967), and it appears that free gossypol hinders that absorption, causing the amino acid to accumulate in the intestinal wall. It may be that in the presence of free gossypol methionine is transferred through the brush border into the musocal cell wall but subsequent steps of the transport system are inhibited. Why bound gossypol does not affect methionine removal in the same way is a function of the transport system. The disposition of methionine noted at the low gossypol level is not observed at the high (0.9%) gossypol level. Bound gossypol at 0.9% did produce an increase in the ^{14}C found in the liver above that with free gossypol or the control. It is possible that the physiological mechanisms responsible for the alterations of methionine transport with 0.5% gossypol may be gossypol concentration dependent and are not in effect at a free gossypol level of 0.9%.

Tables I and II indicate that at both gossypol levels more ($P < 0.05$) valine-bound gossypol complex is removed from the sac, less appears in the sac wall, and more is found in the liver (although not significantly more on the low gossypol level) at the end of the hour than is the case of either

Table III. Transport by Everted Intestinal Sacs of L-Lysine, L-Methionine, or L-Valine at Four Different Relative Concentrations as Affected by Free or Bound Gossypol and the Inside-to-Outside Ratio Created by the Sacs at 1 hr

Gossypol treatments	Lysine at rel concn of				Methionine at rel concn of				Valine at rel concn of			
	1[a]	10	50	100	1[a]	10	50	100	1[a]	10	50	100
None												
Transport (μmol/sac per hr)	12[b]	118	623	660	94	1296	2049	2198	79	1025	2185	3237
I/O[c]	3.43	3.21	3.00	2.10	3.73	3.63	1.57	1.23	2.85	3.30	1.68	1.51
Free												
Transport (μmol/sac per hr)	13	152	618	826	166	1260	3881	3231	172	1675	2858	3975
I/O	3.85	3.99	3.37	2.11	5.64	3.84	1.97	1.33	6.34	4.59	1.89	1.45
Bound												
Transport (μmol/sac per hr)	15	177	501	833	180	1436	3679	2634	165	1520	5489	7153
I/O	4.21	5.08	2.87	2.28	5.61	4.02	2.00	1.26	5.22	4.65	2.89	2.02

[a] The initial actual concentration at RC = 1 for lysine was 10 μM and that for methionine and valine was 0.1 mM. [b] Each value is the mean of 16 determinations. [c] Ratio of moles of amino acid per milliliter of fluid inside sac to moles of amino acid per milliliter in surrounding medium at end of 1 hr with initial ratio being 1.

Table IV. Kinetic Values of Transport for Lysine, Valine, and Methionine under Control Conditions and with 0.5% Free or Bound Gossypol Derived from Means of Four Sac Locations over a 100× Concentration Range Involving Four Different Concentrations

	Lysine			Valine			Methionine, four-sac mean[a]		
Gossypol treatment	None	Free	Bound	None	Free	Bound	None	Free	Bound
K_t[b] (mM)	0.43	0.69	1.30	4.44	2.04	5.65	0.99	5.38	3.21
	±0.32[c]	±0.17	±0.56	±1.99	±0.92	±1.04	±0.37	±0.26	±0.01
V_{max}[d] (μmol/hr per sac)	1.01	1.41	1.90	4.52	4.51	11.32	2.48	8.05	6.04
	±0.30	±0.17	±0.51	±0.82	±0.60	±0.94	±0.28	±0.22	±1.20

[a] See text for explanation. [b] K_t = the amino acid concentration at which half-maximum transport was attained. [c] ± standard deviation. [d] V_{max} = the apparent limiting rate of transport.

Table V. Literature Values of the Kinetic Parameters of Intestinal Transport K_t and V_{max} for L-Lysine, L-Methionine, and L-Valine

Amino acid	K_t, mM	V_{max}[a]	Reference
L-Lysine	0.70	0.65 μmol/100 mg per 90 min	Larsen et al. (1964)
	0.55		Finch and Hird (1960)
L-Methionine	1.30		Hagihira et al. (1962)
	9.55		Jervis and Smyth (1959)
	5.30	6.5 μmol/100 mg per 90 min	Larsen et al. (1964)
	0.91		Finch and Hird (1960)
L-Valine	3.60		Hagihira et al. (1962)
	2.89	16.3 μmol/500 mg per 60 min	Reiser and Christiansen (1965)
	3.30	3.0 μmol/100 mg per 90 min	Larsen et al. (1964)
	2.10		Finch and Hird (1960)

[a] All rates were on a wet-tissue weight basis.

the control or free treatment, both of which behave similarly. Speculation as to why gossypol bound to valine increases the transport and assimilation of the amino acid would be based only on tentative suppositions. It does appear that the bound gossypol affects the transport of valine in such a manner as to increase the transmural flow.

Valine and methionine, both neutral amino acids and long believed to be transported by the same system, appear to be affected in a different manner by either free or bound gossypol, regardless of the gossypol concentration. A possible explanation of this observation may be found in the proposal of Christensen (1967) that neutral amino acid transport is accomplished by a series of multiple mechanisms with overlapping specificities. If the bulk of either methionine or valine transport was accomplished through mechansims affected differently by free or bound gossypol, then the present observations may be expected.

In Vitro Effects of Two Forms of Gossypol on the Kinetics of Transport of Lysine, Methionine, or Valine. The in vitro experimental design permitted an examination of the transport of amino acids in four locations of the small gut. However, all values were expressed as the mean of all four sac locations except where it became necessary to do otherwise due to unusual data produced in the case of methionine. The values used were the means of 16 determinations.

Net transport per sac per hour (hereafter called transport) was used as the parameter for determining kinetic values and for comparison purposes. Table III indicates the means for transport and the I/O ratio produced for each kind of amino acid–gossypol treatment, at different relative concentrations. The values at each relative concentration for each gossypol and amino acid treatment were used in a regression equation. The values obtained by regression for each transport rate were plotted against amino acid concentration. The statistical analysis conducted over all relative concentrations, amino acids, and all four sacs indicated a highly significant difference in both transport and I/O ratio due to the type of gossypol treatments.

Michaelis–Menton kinetics were then determined using the values at each relative concentration for each gossypol and amino acid treatment obtained by regression equation. Values were obtained for K_t, the substrate concentration at which half-maximum transport occurs and an indicator of the affinity between the mechanism and the substrate, and for V_{max}, the maximal rate of transport expected. The application of Michaelis–Menton kinetics to transport is pertinent, but precautions regarding too literal an interpretation of the values are in order. The kinetics relationship does not demonstrate the nature of the transport mechanism and should be taken as no more than a possible saturable rate-limiting step in the transport process. This step may be enzymatic or some other process such as adsorption (Matthews and Laster, 1965; Fisher and Parsons, 1953).

The kinetic values were obtained from Lineweaver–Burk (1934) plots of the original regression curves of amino acid concentrations vs. transport for each of the three gossypol forms. The resulting K_t and V_{max} values are presented in Table IV. The transport rates for lysine and valine covered four points over a 100-fold range of initial substrate concentration. At the highest relative concentration of methionine (10 mM), the transport, in the case of both free and bound gossypol, decreased from the value observed at 5 mM initial concentration although not significantly so when values at the two levels were tested by a t test. This same situation was encountered with lysine when it was used at the actual initial concentrations of methionine and valine, thus necessitating a tenfold decrease in each initial concentration for lysine, a phenomenon also observed by Jervis and Smyth (1959). A decrease in transport at high concentrations has also been noted in the rat gut for L-phenylalanine (Tillman and Kruse, 1962), histidine (Agar et al., 1954), and L-tryptophan (Spencer and Samiy, 1960). Due to the fact that the points for the 10 mM initial con-

centrations of methionine for both free and bound gossypol fell away from an asymptotic projection, only the three lowest points representing a 50-fold concentration range were used in the Lineweaver–Burk plots of methionine from which the methionine K_t and V_{max} values were derived. Even though the 10 mM control points for methionine did not behave as described above, it too was dropped for consistency.

Table V presents K_t and V_{max} values observed in the literature. The values obtained in the present study may not be considered out of line due to the fairly wide variation in K_t values evident in the literature, especially for methionine. The values for V_{max} cannot be readily compared due to the expression of velocity on a 7-cm sac length basis previously mentioned. Methionine and valine are known to be two of the most rapidly transported amino acids (Delhumeau et al., 1962; Finch and Hird, 1960; Adibi and Gray, 1967), while lysine is reported to be transported at a rate of $1/10$ to $1/20$ that of neutral amino acids (Finch and Hird, 1960), an observation confirmed by the present data.

The kinetic values in the presence of gossypol present a varied picture which must be considered on an individual amino acid basis. The K_t of lysine was increased over the control by both free and bound gossypol. Both K_t and V_{max} for lysine were not significantly different in the presence or absence of free gossypol. The difference appears when the two are compared to the lysine with bound gossypol. The K_t and V_{max} both increase on the order of twofold. It is known that binding gossypol to lysine involves the ϵ-amino group of the amino acid (Lyman et al., 1959). If the Schiff's base binding of gossypol to the amino acid alters its spatial arrangement or if the transport mechanism requires the ϵ-amino group for recognition, then an increased K_t may not be unexpected. The increased K_t indicates a decrease in transport affinity of lysine with bound gossypol. The in vivo data on lysine with bound gossypol at 0.5% only indicate a nonsignificant decrease in lysine removal (Table I) from the in situ sacs. Free gossypol was like the control in that the K_t was lower than in the case of bound gossypol.

The K_t for valine with bound gossypol is only slightly different than that for the control while the K_t for valine with free gossypol is lower than the control. There is a large increase in the V_{max} for the bound over the other two treatments. The higher transport maximum logically coincides with the greater observed removal of the valine-bound gossypol complex in the in vivo experiment. The kinetic manifestations of this observation are a K_t not greatly different from the control, but a V_{max} more than 2.5 times that of the control. Such a situation may be indicative of factors in the valine transport system which dictate the increased in vivo removal seen for valine-bound gossypol.

The pattern of the methionine data indicates that free gossypol increased both the K_t and V_{max} over the control and bound gossypol treatments. The methionine kinetic data were obtained over a 50-fold concentration range, employing only three actual concentration points.

The general observation of the effect of gossypol on the kinetics of methionine transport is that free gossypol reduces the affinity of the amino acid for the methionine transport system but that bound gossypol does not act in this way. Such an observation is in agreement with the results found in the low gossypol level in the in vivo study in which less methionine was removed from the sacs and appeared to be held up in the intestinal walls on the bound gossypol treatment. The correlation of the in vitro–in vivo data for methionine with free gossypol is parallel to the in vitro–in vivo correlation for lysine with bound gossypol. In both cases, the increase in the K_t and V_{max} of a certain gossypol treatment over the control values is coupled with an indication of decreased removal of the amino acid in the in vivo experiment in the case of the same gossypol treatment. High K_t and V_{max} values for the lysine-bound gossypol complex correlate with an indicated decreased removal of the complex from the in vivo sacs while the high K_t and V_{max} values of methionine with free gossypol correlate with decreased removal of the amino acid in the in vivo treatment.

Active transport has been postulated as a multistep process (Meister, 1973) with both passive and energy-requiring step(s). It is possible that K_t and V_{max} are primarily determined by factors attributable to one or the other of these steps. Alterations in these steps by compounds, including both forms of gossypol, could affect either or both K_t and V_{max}. In the present study, there are two cases in which the K_t and V_{max} are both raised over control values (the lysine-bound gossypol complex, and methionine with free gossypol). The corresponding low gossypol levels in in vivo experiments indicate a decreased removal of the amino acid in both cases. The present study also provides a case where the K_t is not essentially changed by a gossypol treatment but the V_{max} is more than doubled (valine with bound gossypol). The in vivo observation in this case is that the removal of the amino acid with bound gossypol is increased. The accumulated information in the present study tends to indicate different transport mechanisms for lysine, methionine, and valine. Each mechanism could easily have more than one step, with each step reacting differently to the gossypol treatments. Such a multistep process for individual amino acid transport systems could logically be implicated in producing the combined in vivo and in vitro results seen in the present study.

The examination of the transport of the sodium salt of gossypol alone indicated no active transport against a gradient for 100 or 1 μM gossypol or for 5 μM gossypol in the presence of a 1× or 5× critical concentration of micelles. This observarion rules out consideration of gossypol as a competitive inhibitor of amino acid transport since gossypol does not appear to be actively transported. Relative to this observation, Bressani et al. (1964) found that the total gossypol fed to dogs was almost quantitatively excreted in the feces, with little alteration in the amount of bound and some increase in the amount of free gossypol. They could not distinguish between unabsorbed gossypol and that which was absorbed and reexcreted by way of the bile. More recent work indicates the latter is the case (Smith and Clawson, 1965; Albrecht et al., 1972). Evidence from the present work indicates that lysine, methionine, or valine with bound gossypol can be transported actively across the gut wall. Bound gossypol possibly enters the body in this manner, but it is unproven as to whether free gossypol enters by diffusion in conjunction with micelles.

This present study was an attempt to determine the cause of an observed (Jones, 1974) decrease in nitrogen uptake from the gut and an increase in all amino acids present in the gut lumen 1 hr after the start of eating diets containing primarily bound but also some free gossypol. Some of the observations made in this study indicate there may be a decreased transport of lysine and methionine in the presence of bound or free gossypol, respectively, especially if the luminal concentrations of the amino acids are less than the level used in this investigation—a distinct possibility when considering normal diets.

The present study does not indicate any uniformity of action of free or bound gossypol on the transport of all amino acids. Different transport systems are observed to react differently. However, the simultaneous transport of all amino acids characteristic of the case under normal dietary conditions could be greatly affected by the gossypol alteration of one or more of transport systems. Delhumeau et al. (1962) have provided evidence that the pattern of the amino acids presented to the gut wall has a great effect on the percent of each amino acid absorbed. An amino acid pattern simulating egg albumen resulted in greater absorption of more amino acids than did a pattern representing

zein or casein. Thus, if gossypol altered the absorption of one or more amino acids from the protein of high quality cottonseed meal it could result in an alteration in overall absorption, a reduced absorption rate for many amino acids, a net decrease in nitrogen absorption, and thus a reduced protein value for the gossypol-containing meal.

Consideration must be given to at least two other factors besides an alteration of amino acid transport by gossypol which could cause the compound to reduce the total nitrogen removal from the intestine. Gossypol may increase the rate of passage of the chyme along the intestinal tract and reduce the time of exposure of the diet to digestion. The other possibility is that gossypol may have an action on the enzymes of protein digestion. Wong et al. (1972), Finlay et al. (1973), Lyman et al. (1959), and Tanksley et al. (1970) have shown that gossypol can react with the ϵ-amino groups of lysine in the pepsinogen, pepsin, and trypsin molecules, thus hindering their action. The above two actions of gossypol, coupled with alterations of amino acid transport, could produce a definitive decrease in amino acid absorption and explain the reduced nutritive value of gossypol-containing meals.

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Preparation of Optically Active 6-Chlorotryptophan and Tryptophan

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Optical resolution of racemic modifications of 6-chloro-DL-tryptophan and DL-tryptophan was studied in order to develop practical methods for the production of 6-chloro-D-tryptophan, a nonnutritive sweetening agent, and L-tryptophan, an essential amino acid. 6-Chloro-DL-tryptophan methanesulfonate and DL-tryptophan *p*-phenolsulfonate were resolved by preferential crystallization procedures. High yields of optically pure isomers of both amino acids were obtained. Industrial production of the isomers by these methods is considered promising if the appropriate synthetic methods for production of the racemic modifications are developed.

It has been reported that 6-chlorotryptophan (6-Cl-Trp) is useful as a nonnutritive sweetening agent (Kornfeld et al., 1970) and that it possesses interesting biological activities (McGeer et al., 1968; Peters, 1972; Pascalon et al., 1972). In the former report, the D isomer of 6-Cl-Trp was shown to be an exceedingly sweet compound. However, details of the preparation of the optically active isomer were not described and evaluations of the degree of sweetness were carried out with the DL form. The DL form was used in the latter biological studies because the optical resolution of 6-Cl-Trp was considered difficult. Fukuda et al. (1971) reported that 6-chloroindole is converted microbiologically into 6-Cl-L-Trp, but detailed physical data of the isolated 6-Cl-Trp were not included. Thus, neither a practical method for production of optically active 6-Cl-Trp nor

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