CHROMATOGRAPHIC EFFECTS OF GENETIC SEX-REVERSAL BY THE MUTANT TRANSFORMER IN DROSOPHILA MELANOGASTER

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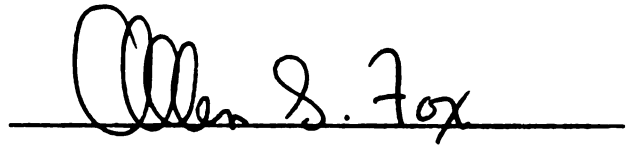
Approved by 

ABSTRACT

Previous work with *Drosophila melanogaster* by Fox (1956 a) demonstrated that males possess a peptide (the "sex peptide") which is absent in females, and that the two sexes exhibit quantitative differences with respect to individual constituents of the free amino acid pool in adults. Genetic analysis disclosed that these differences were not attributable to the presence or absence of the Y chromosome, and it was suggested that an examination of the effects of the mutant *transformer* could serve to determine whether the differences were attributable to the sex-determining system of genes. This thesis reports the results of such an examination.

By means of two-dimensional chromatography, the free ninhydrin-positive materials present in *ŷv/Y;tra/tra* intersexes and in their female sibs *ŷv/Y;tra/Cx* were compared with the patterns exhibited by the males and females of the wild Oregon R stock. A total of twenty-two different ninhydrin-positive substances were observed, but only twenty of them were present in both sexes. The presence of the sex peptide in males and its absence in females was confirmed. In addition, spot 1, probably a peptide, was found only in females. All of these substances have been previously reported except phenyl alanine. Two spots remain unidentified.

The patterns of *ŷv/Y;tra/tra* intersexes were qualitatively identical to those of Oregon R males and *ŷv/Y;tra/Cx* females exhibited qualitative identities to Oregon R females. The quantity of glutamine was higher in the females of the two stocks used than in the wild males, while that the intersexes occupied an intermediate position with respect to this trait. β-alanine and tryptophane-proline were present in higher quantities in Oregon R males than in Oregon R females. The inter-

sexes occupied an intermediate position with respect to these substances also.

Stock differences are responsible for the higher quantity of β-alanine in $\widehat{yv}$/Y;tra/Cx females than in Oregon R females. A stock difference may also exist with respect to taurine-serine-glycine (8-6-9), but other factors obscure this situation. None of the genotypes studied can be considered to differ significantly with respect to the quantity of glutamic acid. Visual comparisons of the remaining substances indicated possible stock differences with respect to cystine and/or cysteine, threonine, methionine, the valines, the leucines, and two of the unidentified substances, all of which appeared larger or more intense in the transformer than in the Oregon R stock.

It is concluded that both qualitative and quantitative differences between the sexes are due to the differences in dossage of the sex-determining loci present in the X chromosomes. The presence of the sex peptide in the intersexes suggests that this substance may be a sex hormone and that the transformer mutant alters the metabolism of small peptides. The work reported also supports the conclusion that the sex-reversed, sterile males produced by tra are truly intersexual in nature.

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I. INTRODUCTION

The existence of such a gene as transformer at least modifies Bridges' (1922,1925,1932) explanation of sex-determination in Drosophila. This hypothesis, known as the balance theory of sex-determination was applied by Bridges to Drosophila, and previously had been developed by Goldschmidt (review in 1934, 1938) as an interpretation of his studies in Lymantria dispar intersexes. Goldschmidt's general conclusions state that sex depends on a balance or unbalance between sex-determiners for one sex, located in the X chromosomes, and sex determiners for the other sex, located outside the X. The factor (s) for maleness are symbolyzed M, for femaleness F. Therefore, the sex of each individual depends upon the quantitative relation of both F and M, that is, on the so called F/M balance result of the sex-determiners. This picture was complicated by the discovery of autosomal genes with effects on sex, but Goldschmidt found that these were concerned with special developmental processes related to sex-differentiation, and considered them as modifiers of the primary sex determiners mentioned. He also originated the concept of a turning point mechanism which occurs in individuals that begin their development with their gametic sex, then switch over to that of the other sex at some later stage; the result is an intersex, the degree of which will depend on the position in time of the turning point.

Bridges in turn (Ibid., also Dobzhansky and Bridges, 1928) observed that the subtriploid aneuploids in Drosophila develop as intersexes; he concluded that the X chromosome has a net female tendency due to the presence of a majority of female determining genes and that there are also male determining genes located mainly in the autosomes. The end result of sex differentiation will depend on the interplay between the female and the male determiners. In the way Bridges expressed it: X/A = 1, product is a female; X/2A = 0.5, product is a male; 2X/3A = 0.6, product is an intersex (A = set of autosomes).

Transformer, a third chromosome recessive gene (Sturtevant,

1945), has the effect of causing flies with the same number of sets of X's as of autosomes (2X/2A tra/tra), which otherwise would be females, to become sterile males; these actually, if described exactly, are extreme female intersexes. They are frequently larger than normal males, have the same developmental rate of normal females and the sex-linked mutants which show sexual-dimorphism, such as Bar, eosin, scute-3 and facet, behave in them as in normal females. The rest of their characters are male-like and they mate with females, but the testes are reduced in size. 3X/3A tra/tra/tra resemble 2X/2A tra/tra. 3X/2A tra/tra individuals are affected by transformer in that their genitalia look masculine and sex combs are present, although these have only half the normal number of teeth. Males are not morphologically affected by transformer.

This single mutant therefore shifts the sex differentiation reaction almost completely to the male side, thus opposing the 2X/2A condition established at fertilization.

Other autosomal mutants with major effects on sex have been found in the family Drosophilidae. Sturtevant (1920) studied a second chromosome recessive in D. simulans, which causes females to become intersexes and males to be sterile. In D. virilis, Lebedeff(1939) found a gene in the third chromosome, the recessive ix^m (intersex; the m refers to maleness), with no effect on males but converting diploid females into several degrees of intersexes, from female-like through hermaphrodites, to complete sex-reversed sterile males. After selection through several generations, Lebedeff isolated four lines which gave predominantly only one type of intersex; this showed that the original range of intersexuality was due to suppressors of the ix^m gene, complete sex-reversal occurring when modifiers of ix^m were lacking. The embryological studies of these intersexes classify ix^m as a sex-modifier.

Dobzhansky and Spassky (1941) found a dominant gene in D. pseudoobscura but could not locate it. Its action brought about intermediate types of intersexes. The authors supposed that the gene caused a reduplication or splitting of the genital imaginal disc yielding different intersexual types.

Gowen (1942) and Sui-Tong and Gowen (1957 a) have reported a dominant third chromosome gene, Hr (Hermaphrodite) in D. melanogaster, which does not affect males morphologically or physiologically, while females become sterile and acquire parts of external male genitalia with corresponding internal changes. The latter exhibit great variability such as either well-developed or rudimentary ovaries; other times rudimentary testes accompany the ovaries; also, male accessory glands, abnormal sperm pump, female and male duct systems, and yellow pigmented tissue associated with the gonads are usually present. Size is not affected and sex combs are always present. Transplantation experiments by the same authors (1957 b) disclosed that wild type and Hr gonads have pigment producing potentialities, the effect being greatest in the males but similar in females and Hr hermaphrodites. The authors do not recognize the existence of a turning point in Hr intersexes. They suggest that the simultaneous presence of homologous sex organs of both sexes, such as ejaculatory ducts and oviducts in the same individual, support organ autonomy under the control of specific genes better than development in one direction followed by a change. However, Lebedeff (Ibid.) observed the same characteristics in his hermaphroditic intersexes. Studies of the early development of the gonads disclosed that these organs start their development as ovaries, after which a shift occurs (the turning point) and they continue as ovotestes. No examinations of such early stages have been made by Gowen and Sui-Tong.

In D. virilis, Newby (1942) studied the effect of the second chromosome dominant Ix^B (Intersex; the B refers to the Blanco stock where it was discovered) on the embryology of the intersexual forms that it caused. He concentrated on the sequence of development of the sexual organs of these flies and found two imaginal discs. His conclusion was that Ix^B caused the primary imaginal bulb to develop into an incomplete male system and the secondary disc to form some parts of the female system. He indicated that the condition of intersexuality was a response to the developmental influence of both sexes and that his intersexes did not exhibit development with a turning point, contrary to what Goldschmidt (Ibid.) found in Lymantria and to Dobzhansky and Bridges' observations in Drosophila intersexes.

Every one of the single mutations mentioned above have an effect on sex-determination that is extended to the development of all the sexual organs. To those who think of all mutant loci that can produce shifts in sex as sex-determiners, their existence is enough evidence to support the theory that the interaction of a few main genes, with or without modifiers, decides the sexual fate of an individual, in opposition to Bridges' balance theory. On the other hand, these single mutants may be considered as modifiers of the sex-determiners within the balance theory, this is, not as affecting the original direction of sex-determination set at the time of fertilization, but as affecting sexual developmental processes later on. If we maintain this definition of modifiers then genes such as $\underline{ix}^{m}$, Hr, and tra are probably modifiers; $\underline{ix}^{m}$ fits this definition most satisfactorily. The hermaphrodites caused by Hr are genetic females but show a mixture of male and female characters, either rudimentary or well developed. From this fact it can be deduced that their development probably started as that of normal females, and that during the course of action of Hr a differentiation of male characters took place.

The nature of tra is more difficult to ascertain since the sex-reversal caused by this gene is almost complete. Analyzing the unchanged female characters of the tra intersexes it is noticeable that the preservation of the faster developmental rate of the female, in a body possessing male gonads, causes the relation between the growth of the gonads and the growth of the rest of the body to be the same as in the females. This could explain why the testes in the tra intersexes are rudimentary for the ovary in normal females is smaller at all stages than the testes of normal males. It would therefore seem probable that the action of tra is such as to change the gonad from ovary to testis without influencing its relative growth rate. The relative growth rates of gonad and body would seem to be determined by the F/M ratio very early in development. There are evidences from embryological studies of Drosophila and of the Diptera in general, that long after the period of embryonic determination, which is extremely precocious in this order, there is a second period when the imaginal discs are determined. It would be at this second stage when sex-modifiers

could interfere with the originally determined sex-differentiation, their success depending on the degree of their strength and on the regulation of the genital embryonic field. According to this it can be inferred that transformer acts at a time later than the sex-determiners, this being a satisfactory prerequisite to consider it a sex-modifier. Suggestions on ways to test this assumption will be given later in this paper.

Among other evidences that support the balance theory in Drosophila, besides Bridges observations on subtriploid intersexes, the work of Dobzhansky and Schultz (1934) showed that there are numerous female determining sex factors in various parts of the X chromosomes and only a few male determiners. Also, Pipkin (1940) found that multiple sex genes with additive effects are located in the X; in general the feminizing effect of extra sections is proportional to the size of the fragment. She pointed out that a single primary sex-gene, which acts regardless of dossage of any lesser modifier, could not exist because none of the eight short sections of the X, when added to 2X/3A intersexes or to 3X/3A females, produced a marked shift toward femaleness.

Patterson et al.(1937) investigated the effects of aneuploidy of short and long segments of the X chromosome. The effects agreed with those observedd by Pipkin, except that they indicated the possibility that if a single female sex-determiner existed it was restricted to the garnet-pleated region, but this was not sufficiently tested.

Bridges' affirmation that the male determining genes are located in the autosomes is made less plausible by the lack of detection of multiple sex factors there. Bridges himself (1922) found that haplo-,diplo-, and triplo-IV did not affect sex or grade of intersexuality. The rest of the studies in this field (Patterson et al., 1940; Pipkin, 1947) have provided excellent information about other phenomena involving genic balance, such as viability and fertility, but they failed to show that any section of the second or third chromosomes had a marked effect on the sex of diploid aneuploids. The wild alleles of Hr and tra could be considered as sex factors since Hr and tra are capable of causing maleness in a

2X/2A fly. This view is suggested by Sui-Tong and Gowen (1957 a) and had previously been regarded by Patterson et al. (1937) with respect to the wild allele of ix^m . If their proposition proves true it is likely that the methods used in searching for autosomal sex-genes have been inadequate.

The work of Fox (1956 a, b) on chromatography of free amino acids and peptides of normal males and females disclosed another important difference between the sexes, namely the presence of a substance, so called "sex peptide", in males but not in females. Fox attributed this condition to the difference in the number of X's between the male and the female, and strongly suspected that the differences in dosage of the sex-determining loci in these chromosomes were the ones responsible for the effect observed. Since the transformer mutant changes a large number of the traits which normally are exhibited by a female to those of a male, therefore acting as a strong modifier of the sex-determining loci present in the X, it was likely to think that it would also reverse the chromatographic pattern of a female to that of a male. The study of this problem and its results are related below. Fox also found that there were possible quantitative differences between the sexes with respect to free ninhydrin-positive substances, but he only used visual comparisons of the spots to detect these differences. By means of two-dimensional chromatography the forthcoming experiments test the presence of the sex peptide in tra female intersexes, study the quantitative differences between the sexes with more accurate methods, and set the position of the female intersexes with respect to the normal males and females as regards their chromatographic pattern and the quantities of free amino acids and peptides.

II. MATERIALS AND METHODS

The stocks used were the transformer stock, tra/Cx;yv/w^a, from California Institute of Technology and the wild Oregon R stock from our laboratories (Table 1). In the transformer stock the females have attached X's carrying yellow and vermilion, and are heterozygous for transformer and the Curlex inversion in their third chromosomes. The males are apricot and also heterozygous for transformer and Curlex, which makes the stock self-maintaining.

The flies were grown on standard Drosophila medium at 25° C and were then collected for aging. Preliminary work disclosed that age (3 to 28 days) does not seem to alter the chromatographic patterns of free amino acids and peptides found by Fox (1956), but an aging period between 7 and 10 days was chosen before using the flies for chromatography. The procedures and solvents described by Fox were followed except that twelve females or tra IS or fourteen males were squashed at the point of origin of the two-dimensional chromatograms.

In order to assure more compactness in the spots to be obtained, sheets were hydrated (Block et al., 1955) for 10-15 minutes in a steam chamber immediately before the first development in 80% phenol. Triple distilled water was used in the preparation of the solvents and these were always freshly made for each run. After the second development in the butanol-acetic-water solvent (BAW), the sheets were dried and then sprayed with 0.2% ninhydrin in iso-propyl alcohol; then they were kept in the dark for 24 hours.

The spots were identified by depositing 5-7 gamma of particular amino acids and available peptides on the squashed flies before development. The spots whose color became more intense was considered identical to the amino acid or peptide added in each case. Chromatograms of the amino acids alone were used as controls. Even though this method is not absolutely discriminating, it offers a sufficient degree of certainty for our purposes.

It was necessary to preserve the ninhydrin-positive spots for later quantitative analyses. For this purpose, a solution of copper nitrate and 10% nitric acid in ethanol (Block et al., ibid) was

sprayed on the spots which acquired an orangish-pink color, except proline which became very pale pink. After this fixation each spot was scanned with a Photovolt densitometer, to which a 505 millimicron filter was adapted; the latter eliminates variation in the copper nitrate background. The total density of each spot was recorded as an absorption curve on graph paper. The area under each curve was measured by a compensating polar planimeter (# F 4236, serial # 55777, Keuffel and Esser Company). The amounts of the several spots were expressed as the ratio between the quantity of each spot as recorded from the curve by the planimeter, over the total sum of the ninhydrin-positive material of the respective chromatogram. This procedure was adopted to reduce the possible error caused when different quantities of flies of each sex were taken to approximate their dry weights and to eliminate the variations which could occur among sheets obtained from different chromatographic runs. These variations arise from small differences in temperature and time of development and of drying, and may affect the intensity of the spots obtained.

III. QUALITATIVE RESULTS

A. Wild-type Patterns

The two-dimensional chromatograms of OR males are illustrated in Figure 1. The point of origin is situated in the lower right hand corner of each figure. The chromatograms of OR females are not shown because, except for possible quantitative differences, they exhibit the same pattern as Cx females (Figure 3). Fox's system of numeration of the free ninhydrin-positive spots was used throughout this work.

By chromatographic separation, a total of 22 different substances were found. 17 of them are amino acids or peptides, which appear to be qualitatively the same in the two sexes. However, some of them could not be specified. These were spots 5, produced by either cystine or cysteine or both; 12, made up by histidine and/or arginine; 18-19, consisting of one or more of valine, norvaline and phenyl-alanine (this spot is named with two numbers because Fox found them separated, identifying 18 as valine and 19 as norvaline). The same can be said about spot 22, in which the amino acids leucine, isoleucine and norleucine were detected.

Spot 2 is the only one in the list which was identified as a peptide, i. e., glutathione. Kaplan (1957) reports that this substance is present in small concentration in two-dimensional chromatograms of several strains of D. melanogaster. Perhaps glutathione is equivalent to the peptide "pupine" found by Hadorn and Mitchell (1951) in one-dimensional chromatograms of pupae and imagoes.

It seems that spots 20, 24, and 25 are the same in males and females but they have not been identified. Spot 20 is very faint and elongated. It might be the "front peptide" reported by Hadorn and Mitchell (Ibid.).

It should be indicated that the position of Fox's spots 15 and 22 (which he respectively identified as methionine and the leucines) are comparable to the positions of

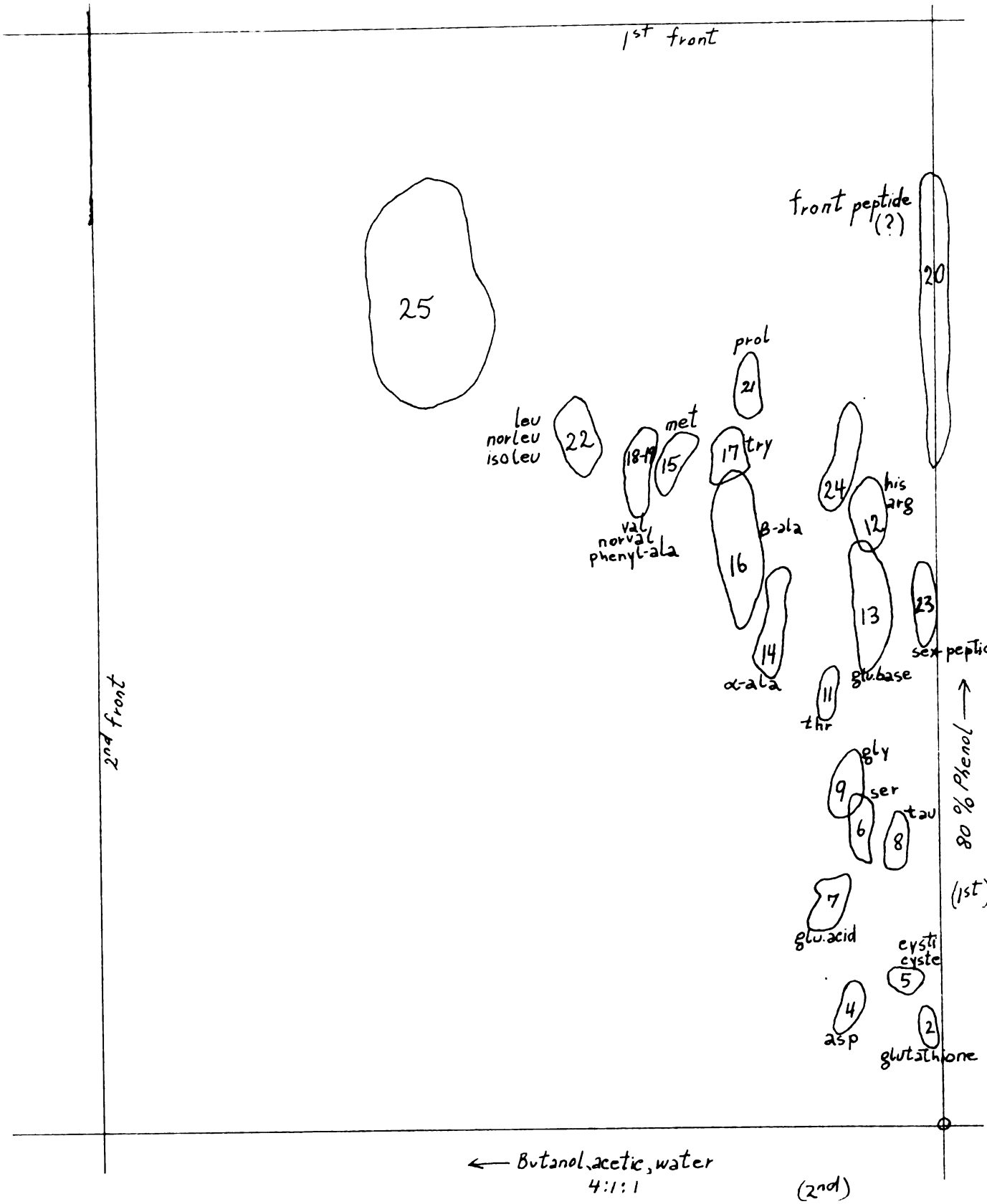

Figure 1. Two dimensional chromatograms of free ninhydrin positive substances in fourteen Oregon R males

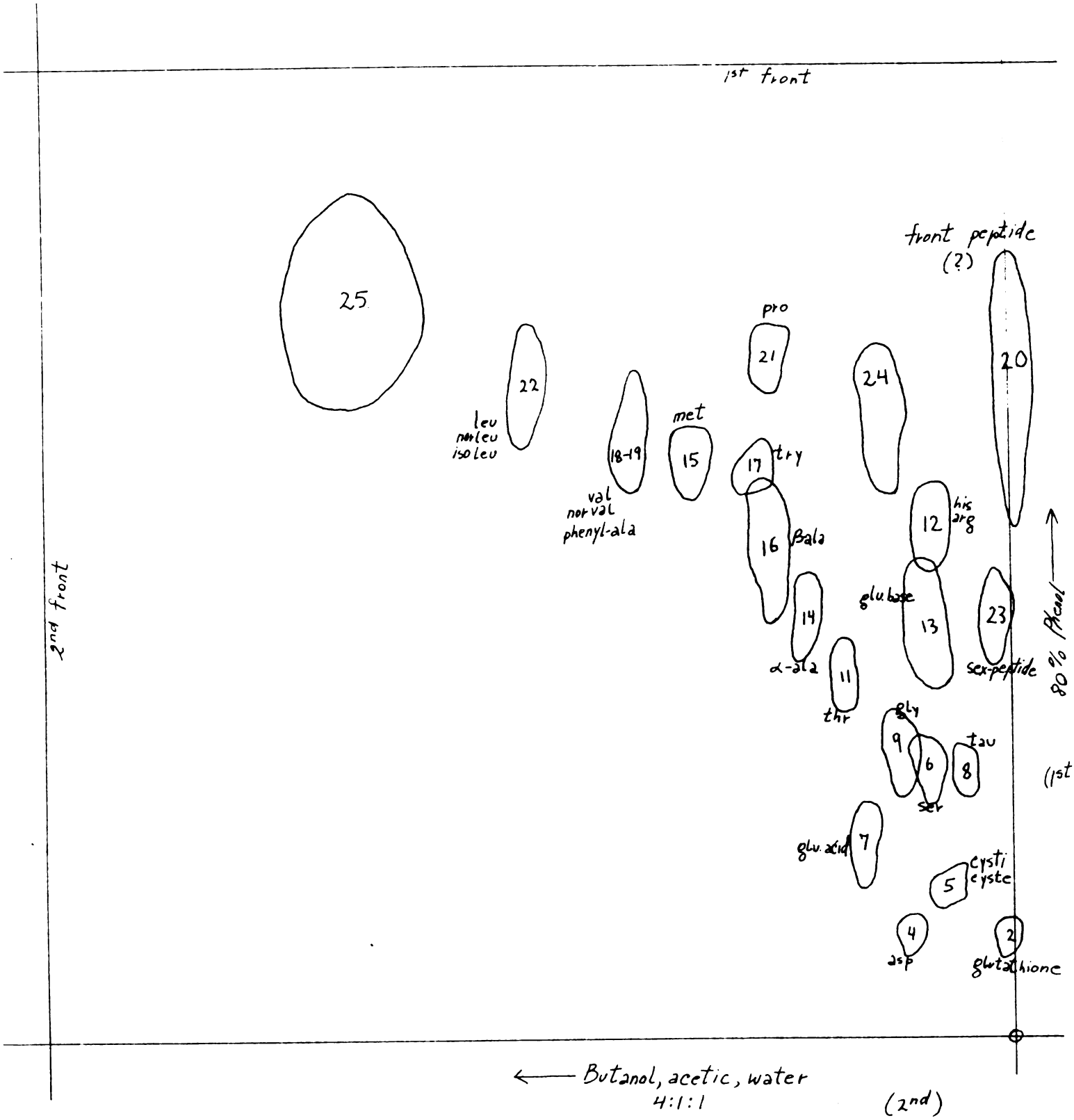

Figure 2. Two dimensional chromatograms of free ninhydrin positive substances in twelve "transformed" females

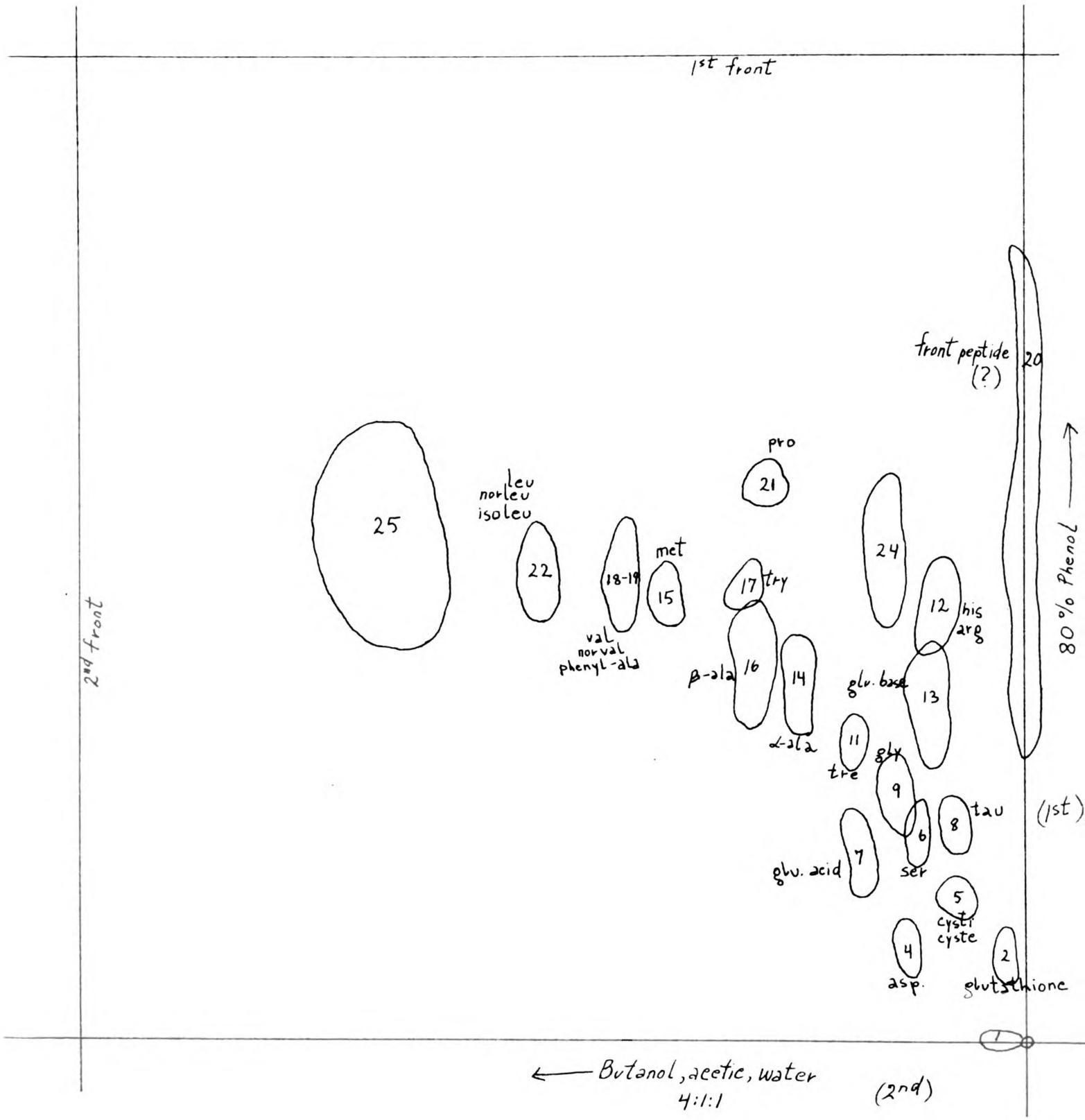

Figure 3. Chromatographic pattern of free ninhydrin positive substances in twelve Curlex inversion females

24 and 25 found in these experiments; but as can be seen from the figures, our results about their identities disagree. Consideration of relative Rf values and of the results of co-chromatography make it improbable that spot 24 is methionine or that 25 is any of the leucines. They may be peptides.

Spot 1 was observed only in the females but not in the males. Again my results disagree with Fox's, since he found this substance was present in both males and females. This effect could be considered a qualitative sexual difference, unless the amount of spot 1 in OR males is so small that it cannot be detected with my techniques.

It was previously discovered by Fox (1956), that spot 23 is found only in chromatograms of male genotypes. Having demonstrated this substance to be a peptide, he entitled it the sex peptide. The present results confirm the conclusion that spot 23 was present only in males.

Besides most of the substances found in this experiment, other workers have also reported the following: mixed ornithine and lysine (Stumm-Zollinger, cited by Hadorn, 1954) in two-dimensional chromatograms of unhydrolyzed hemolymph of larvae. Kaplan (1957) reports that free ethanol amine phosphate is present in high concentrations in two-dimensional chromatograms of several strains of D.melanogaster; also tyrosine and asparagine, but in lesser quantities. Fox found that lysine was the amino acid corresponding to spot # 10. None of these substances were found in my chromatograms. However, Mead (1957) observed that even though lysine and tyrosine were present in his two-dimensional chromatograms of twenty imagoes, their quantities were so small as not to be recorded by densitometry; therefore the number of flies used in my work may account for the failure to find these amino acids.

Fox reports an unidentified substance, spot 3, which was never observed in any of the chromatograms developed in this work. Mead does not think he found this substance either, but his methods, although similar, made use of buffers and the configuration of his patterns were quite different from Fox's and the ones obtained in this work.

B. Patterns in Female Intersexes

As it can be seen in Figure 2, the pattern of two-dimensional chromatograms of $\widehat{yv}$/Y;tra/tra intersexes is qualitatively identical to that of OR males. This indicates an effect of the tra mutant on the amino acid and peptide metabolism of a genetic female, so that its chromatographic pattern resembles that of a male.

C. Patterns in Curlex Females

The chromatographic pattern of $\widehat{yv}$/Y;tra/Cx females (Figure 3) exhibits the same qualitative identities as the pattern in OR females. Therefore such genic substitutions as yellow and/or vermilion, the Curlex inversion, and one dose of tra alone do not have qualitative effects on the pattern of free amino acids and peptides.

IV. QUANTITATIVE RESULTS

A. Rf Values

No exact measurements of Rf values have been made. However, visual comparisons performed among the four studied genotypes disclose the existence of consistent differences:

When 80% phenol is used as the solvent the order of Rf values, except for spots 2, 4, 5, 7, may be expressed as, OR males > tra IS > OR females = Cx females.

The Rf values in BAW follow the opposite trend except for spots 5,12, 20, 22, 24, and 25; thus, OR males < tra IS < OR females = Cx females. The important exception here refers to one of the unidentified substances, spot 24; it is well separated from spot 12 in Cx females and tra IS, but not in OR males and OR females; of course this means that its Rf value in BAW is higher in males of the transformer stock than in OR males or females. Therefore this difference can be considered a stock difference, whose causes, as well as those of the other Rf differences mentioned, are unknown since substances of identical chemical constitution should not differ in these values. Fox (1956), citing Block *et al.* and Berry *et al.*, suggests that the different relative concentrations of the different substances in the two sexes, and also the differences in the quantity of salts as well as in pH's, may be factors which affect the Rf values of the same substances. It would be necessary to add that when other genic or chromosomal changes occur, beside the changes regarding the sex- determining loci, these factors might also be altered. Thus differences in Rf's could be used with profit in studies of taxonomy, as has already been pointed out by Fox (1956 c).

B. Quantitative Measurement of Ninhydrin-Positive Substances

The lack of a convenient separation between certain spots added to the fact that the quantity of

ninhydrin-positive material of some substances was not high enough to be measured by densitometry, did not allow the quantitative investigation of all free ninhydrin-positive materials present in the chromatograms obtained.

The only measurable spots were those which (1) presented a relatively convenient separation from the neighboring substances (2) were relatively concentrated, and (3) exhibited attributes (1) and (2) in all the chromatograms available for each of the genotypes studied. These substances were: glutamic acid (7); taurine-serine-glycine, which were measured as one (8-6-9); glutamine-histidine and/or arginine (13-12), also measured as a single spot; β-alanine (16); and tryptophane-proline, measured together as a single spot (17-21); the sex peptide (23), whose quantity could be checked only in OR males and in the tra IS.

Ten of the best chromatograms developed for each genotype, were selected and treated as previously described. The mean proportion of the measurable substances and their standard errors are shown in Table 2. In order to compare the results from OR females and Cx females with those from either OR males or tra IS, the ratios obtained for the spots above named, with respect to the total amount of ninhydrin-positive material measured omitting spot 23, were used. When the results from OR males and tra IS were to be compared, ratios were calculated using the total ninhydrin- positive material measured in their chromatograms (including that of spot 23).

Tables 3, 4, 5, 6, 7, and 8, contain the differences of the mean proportions and their probabilities of occurrence by chance among all the genotypes studied.

C. Quantitative Differences in Free Ninhydrin-Positive Substances Attributable to Sex-Determination

Quantitative effects of the balance of sex-determiners and of tra are best examined by comparing OR females with OR males. Significant quantitative differences between these two genotypes with respect to any of the ninhydrin-positive materials

would be indicative of an effect of sex-determiner balance, and the influence of tra on these substances could then be determined in tra IS. OR females and OR males exhibit significant quantitative differences with respect to glutamine-histidine and/or arginine (13-12), β-alanine (16), and tryptophane-proline (17-21).

The quantities of glutamine-histidine and/or arginine in the four examined genotypes is as follows:

OR females $\geq$ Cx females $\geq$ tra IS > OR males. It appears that glutamine by itself contributes most importantly to these differences. The difference between OR females and OR males is highly significant ($P \ll 0.001$), indicating a real difference between normal males and females. The similarity of Cx females and OR females in this regard corroborates such a conclusion. The intermediate position of tra IS shows that the sex-determiners are responsible for this difference, and emphasizes the intersexual rather than the male nature of these flies.

The order of the four genotypes with respect to tryptophane-proline is as follows:

OR males > tra IS $\geq$ OR females $\geq$ Cx females. Again a sexual difference is exhibited, and while tra IS does not differ significantly from either males or females, their intermediate position supports conclusions similar to those reached in the case of glutamine-histidine and/or arginine.

The same conclusions are supported by the results obtained for β-alanine. The order of genotypes is:

OR males $\geq$ tra IS $\geq$ OR females > Cx females. The situation is similar to the preceeding one, except the Cx females have significantly less β-alanine than OR females. This is a reflection of a stock difference, and is discussed below.

Thus, in every case where a quantitative difference is found between males and females, tra IS are intermediate. The quantitative differences between the sexes are therefore attributable to the sex-determiners, and the effect of tra is to produce true intersexes.

D. Quantitative Differences in Free Ninhydrin-Positive Materials Attributable to Non-Sexual Factors

Aside from the sexual differences discussed above, additional quantitative differences have been observed which are attributable to the differing genetic backgrounds of the Oregon R and the transformer stocks. These are best demonstrated by comparing OR females with Cx females.

There is a significantly greater amount of β-alanine in OR females than in Cx females, and the sexual difference described above makes the difference between OR males and Cx females even larger. Thus, the existence of a quantitative stock difference for β-alanine is particularly clear.

Cx females possess significantly more taurine-serine-glycine (8-6-9) than do OR females. In this case, however, OR males and tra IS are intermediate and do not differ significantly from either type of female. The order of the genotypes is nevertheless suggestive:

Cx females $\geq$ tra IS $\geq$ OR males $\geq$ OR females. A stock difference in taurine-serine-glycine may very well exist, obscured, however, by other factors.

The only other significant difference observed is that between Cx females and their tra IS sibs with respect to glutamic acid. The order of genotypes is as follows:

Cx females $\geq$ OR males $\geq$ OR females $\geq$ tra IS. Since there is a lack of stock consistency, and since the difference between Cx females and tra IS is of borderline significance (P between 0.02 and 0.05), this observation is probably of no particular importance.

In addition to these densitometric comparisons, visual comparisons among the remaining spots were made to obtain more information on quantitative effects. The most consistent differences observed were as follows: Spot 1 is larger in Cx females than in OR females. Spots 5, 11, 15, 18-19, 22, and 24 are larger in both tra IS and Cx females than in either OR males or females. It seems that these effects may also be caused by differences between the two types of stocks used.

V. DISCUSION AND CONCLUSIONS

A. Consideration of Qualitative Differences

Two qualitative differences between males and females have been observed in this work. Males possess the sex peptide previously reported by Fox (1956 a), while spot number 1 has been observed only in females. Since Fox observed spot 1 in both sexes, discussion will be confined to consideration of the sex peptide. The fact that $\widehat{yv}$/Y;tra/tra intersexes possess the sex peptide gives a clue to the nature of the genetic mechanisms responsible for qualitative differences between the sexes, but first the known facts about tra will be discussed.

In normal circumstances, sexually dimorphic characters are the products either of the sex-determiners and sex-modifiers, or of loci whose action is affected by the different developmental circumstances prevailing in males and females. In the latter case, the end products of gene action exhibit sexual-dimorphism just as if they were directly affected by sex-genes. Examples of this sort (Bridges and Brehme, 1944) are the effects of such sex-linked genes as eosin (darker in females than in males), scute-3 (lethal in females but not in males), facet (more extreme in males than in females), and Bar (number of facets is larger in males than in females).

Transformer reverses many of the effects of the sex-determining loci, which if left to their fate would produce a female when contained by a 2X/2A individual. For the sake of review, the tra non-affected and the tra affected female characters will be listed:

Non-affected	Affected
i. Size	i. Rest of sex-dimorphic characters, including
ii. Developmental rate	ii. Two-dimensional chromatographic pattern of free amino acids and small peptides
iii. Rhythm of gonad development	
iv. Sex-linked mutant genes showing sex-dimorphism	
v. Sex-linked mutants whose expression is identical in both sexes	

As indicated in the introduction, the failure of tra to affect size, developmental rate, and the rhythm of gonad development suggests that it is a sex-modifier rather than a sex-determiner. If this is correct, its failure to affect the action of sex-linked mutants exhibiting sex-dimorphism can be attributed to the fact that the developmental rate of the intersex remains as in females.

Considering the genes in category v., to which the majority of genes in the X belong, it could be postulated that their action would not be interferred with by the primary developmental sexual differences and the identical result would be achieved in the male and the female. For example, the apricot (w^a) mutant, which belongs to this group, will produce the same effect when present in one dose in a male as when present in two in the female if the developmental processes controlled by the sex-determiners run parallel to the developmental changes caused by the change in dosage of w^a. This view is essentially the same as the one held by Goldschmidt (1953).

On the other hand, Muller (1950) has constructed the idea of dosage compensation to explain the lack of sex-dimorphism for loci in category v.; he postulates the existence of modifiers at other sex-linked loci which compensate for the difference in dosage in males and females of the loci of this category. Since, for mutants like w^a, the phenotype of tra IS is the same as that of normal females, he concludes that the dosage compensating loci really exist and are different than the sex-determining loci of the X chromosomes.

It seems to me that Muller's test was inadequate to prove what it attempted, since there is still a preponderance of female sex determiners in what Muller thought was a completely transformed female. Even though their action is not fully exerted, due to transformer, they are able to determine the size and developmental rate of the tra IS, which remain as in females. In other words, the failure of tra to affect a given trait does not demonstrate that development of the trait in question is not influenced by the sex-determining loci.

The qualitative differences in the chromatographic pattern of males and females, however, are affected by tra. In this case, the converse conclusion is justified. Since tra appears to be a sex-modifying gene, and since it changes the chromatographic pattern of genetic females, that pattern is to be attributed to the action of the sex-determining system of loci.

The presence of the sex peptide in the body fluids of a tra IS suggests that it could be a hormone (Fox, 1956a). Of course, its size is small in comparison to the molecular size of known polypeptide hormones but it may turn out to be one. Otherwise it may be concluded that the tra gene is altering amino acid and peptide metabolism, but unfortunately the relation of the pool of amino acid and small peptides to protein synthesis is unknown in this case. Immunogenetic studies of tra IS would be useful, especially since antigenic differences between the sexes have already been investigated by Fox (1958) and by Fox and Yoon (1958).

During the course of my experiments some observations were made which may offer more information concerning transformer. A few cultures of the transformer stock had a tendency to produce what looked to me like "semi-transformed females"; they were yellow and vermilion and had sex combs like their intersexual sibs, but their genitalia were neither female nor male. There was a fold in place of the anus and no signs of anal plates were seen. Anteriorly to and separated from this fold there was an elongated opening on a crest-shape structure. All these deformities were displaced to the left side of the abdomen which had risen above its standard level, while the right side was abnormally flat. Individuals like these lived only about two days. These results may be taken as to indicate the effect of incompletely dominant suppressors on the action of tra, in a simmilar manner to that found in the case of ix^{m} (Lebedeff, 1939).

This work has suggested but not proven the sex-modifying nature of tra. This can only be done by studies of the early development of the imaginal genital disc in the intersexes

caused by tra. Chromatographic studies of a large developmental series of tra IS and of normal male and females might also yield an answer to this question. Such studies could also furnish information of the time in which the qualitative differences are established in the sexes. The last aspect of the second problem has already been attacked by Chen and Hadorn, and Stumm-Zollinger (reported in addendum of Fox, 1956 b), who found differences among larvae, pupae and imagoes concerning γ-amino butyric acid.

B. Consideration of Quantitative Differences

The real differences between the sexes with respect to the quantity of glutamine can be definitely attributed to the result of the different dosage of the sex-determiners. The intermediate positions of the tra IS with respect to the quantity of glutamine emphasizes the intersexual rather than the male or female nature of these flies.

Since glutamine is a key amino acid, taking part in most transamination reactions, it is interesting to speculate on its quantitative sexual difference in reference to amino acid and protein metabolism. It is known that glutamine is a requirement in the synthesis of inosinic acid, a precursor of the purines. The higher quantity of glutamine in the female Drosophila could be taken to indicate that the rate of nucleoprotein synthesis is higher in the female than in the male. The size of the body cells as a whole is larger in the female than in the male and from the speed of development it can be inferred that the rate of protein synthesis of the female is higher than the male, at least during all the pre-imaginal stages.

The quantitative differences between males and females with respect to β-alanine and tryptophane-proline, together with the observation that the tra IS are intermediate in both cases, demonstrates that these are also controlled by the sex-determining loci. It is interesting to note that β-alanine is also affected by other genic substitutions, as is evidenced by the stock

difference, but identification of these loci is not possible in this material.

Green (1949) has reported that the vermilion mutant causes an accumulation of free tryptophane. Since this mutant is present in tra IS and Cx females, a similar observation might have been expected here. Failure to detect the anticipated effect is probably attributable to its small size and to the use of only twelve flies in the preparation of the chromatograms.

No difference in principle should probably be assigned to the qualitative effect of tra on the sex peptide and its quantitative effects on glutamine, β-alanine, and tryptophane-proline. In the case of the sex peptide, a sharp threshold probably translates a quantitative effect into a qualitative difference.

VI. SUMMARY

1. By means of two-dimensional chromatography, the free ninhydrin-positive materials present in $\widehat{yv}$/Y;tra/tra intersexes and in their sibs $\widehat{yv}$/Y;tra/Cx females are compared with the patterns exhibited by the males and females of the wild Oregon R stock.

2. A total of twenty-two different ninhydrin-positive substances have been observed, but only twenty of them are present in both sexes. The presence of the sex peptide in males and its absence in females has been confirmed. In addition, spot 1, probably a peptide, is found only in females. All of these substances have been previously identified by Fox (1956 b), except for phenyl alanine. Two spots remain unidentified.

3. The patterns of $\widehat{yv}$/Y;tra/tra intersexes are qualitatively identical to those of Oregon R males and $\widehat{yv}$/Y;tra/Cx females exhibit qualitative identities to Oregon R females.

4. The quantity of glutamine is higher in the females of the two stocks used than in the wild males, while that the intersexes occupy an intermediate position with respect to this trait. β-alanine and tryptophane-proline are present in higher quantities in Oregon R males than in Oregon R females. The intersexes occupy an intermediate position with respect to these substances also.

5. Stock differences are responsible for the higher quantity of β-alanine in $\widehat{yv}$/Y;tra/Cx females than in Oregon R females. A stock difference may also exist with respect to taurine-serine-glycine (8-6-9), but other factors obscure this situation.

6. None of the genotypes studied can be considered to differ significantly with respect to the quantity of glutamic acid.

7. Visual comparisons of the remaining substances indicate possible stock differences with respect to cystine and/or cysteine, threonine, methionine, the valines, the leucines, and two of the unidentified substances, all of which appear larger or more intense in the transformer than in the Oregon R stock.

8. It is concluded that both qualitative and quantitative differences between the sexes are due to the differences in dosage of the sex-determining loci present in the X chromosomes.

9. The presence of the sex peptide in the intersexes suggests that this substance may be a sex hormone and that the transformer mutant alters the metabolism of small peptides.

10. The work reported supports the conclusion that the sex-reversed, sterile males produced by tra are truly intersexual in nature.

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Table 1

Genotypes Studied and Their Abbreviations

A. Oregon R Stock
   1. X/Y = OR males
   2. X/X = OR females

B. Transformer Stock
   1. $\hat{yv}$/Y;tra/Cx = Cx females
   2. $\hat{yv}$/Y;tra/tra = tra IS

Table 2

Mean Proportions of Free Ninhydrin-Positive Substances With Their Standard Errors

A. In Oregon R Stock

Spot No.	Males (+23)	Males (-23)	Females
7	0.099+0.007	0.111+0.008	0.106+0.012
8-6-9	0.153+0.013	0.177+0.012	0.161+0.010
13-12	0.208+0.013	0.240+0.015	0.363+0.013
16	0.313+0.013	0.362+0.016	0.304+0.015
17-21	0.093+0.006	0.107+0.007	0.073+0.006
23	0.133+0.009		

B. In Transformer Stock

Spot No.	IS (+23)	IS (-23)	Cx females
7	0.094+0.004	0.105+0.004	0.122+0.006
8-6-9	0.164+0.010	0.184+0.010	0.205+0.008
13-12	0.271+0.013	0.305+0.013	0.347+0.016
16	0.289+0.010	0.326+0.013	0.254+0.010
17-21	0.071+0.007	0.082+0.008	0.072+0.007
23	0.111+0.030		

Table 3

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Oregon R Males and Females

Spot No.	Difference (Males-Females)	P
7	0.005	>0.50
8-6-9	0.015	0.50-0.10
13-12	-0.124	<<<0.001
16	0.058	0.02-0.01
17-21	0.034	0.01-0.001

Table 4

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Oregon R Males and Female IS

Spot No.	Difference (Males-IS)	P
7	0.006	0.50-0.10
8-6-9	-0.010	0.50-0.10
13-12	-0.064	0.01-0.001
16	0.024	0.50-0.10
17-21	0.022	0.05-0.02
23	0.022	0.50-0.10

Table 5

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Oregon R Males and Cx Females

Spot No.	Difference (Males-Cx Females)	P
7	-0.011	0.50-0.10
8-6-9	-0.029	0.10-0.05
13-12	-0.107	$<<$0.001
16	0.108	$<<<$0.001
17-21	0.035	0.01-0.001

Table 6

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Oregon R Females and Female IS

Spot No.	Difference (OR Females-IS)	P
7	0.001	$>$0.50
8-6-9	-0.023	0.50-0.10
13-12	0.059	0.01-0.001
16	-0.022	0.50-0.10
17-21	-0.009	0.50-0.10

Table 7

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Oregon R Females and Cx Females

Spot No.	Difference (OR Females-Cx Females)	P
7	-0.017	0.50-0.10
8-6-9	-0.044	0.01-0.001
13-12	0.017	0.50-0.10
16	0.050	0.02-0.01
17-21	0.001	>0.50

Table 8

Differences in the Ratios of Free Ninhydrin-Positive Substances Between Female IS and Cx Females

Spot No.	Difference (IS-Cx Females)	P
7	-0.017	0.05-0.02
8-6-9	-0.021	0.50-0.10
13-12	-0.042	0.10-0.05
16	0.072	<0.001
17-21	0.010	0.50-0.10