XY Females Caused by X-Irradiation

There have been many attempts to influence the sex ratio in the offspring of organisms whose sex is determined by an *XY* mechanism. We should like to report some new results which we have obtained from experiments with the wild livebearing fish *Platypoecilus maculatus* from the Jamapa River in Mexico[1]. These animals have a distinct sexual dimorphism. The sex of an animal can be easily determined by observing the structure of its anal fin. In the female, this fin is normally constructed, whereas in the male it is transformed into a gonopodium. In addition, the ovaries can be seen in the female because of the transparency of the fish.

Sex determination has been proved to be of the *XY* type[2]. The sex chromosomes are distinctly heteromorphic[3]. In our stock the *X* chromosome carries either the genes *Sd* (Spotted dorsal) and *Dr* (Dorsal red) or *Sp* (Spotted). The *Y* chromosome carries *Sr* (Stripe-sided) and *Ar* (Anal red). Both male and female animals have in addition a homozygous autosomal gene *Sh* (Shoulder spot), which only manifests itself in the absence of the *Y* chromosome[4]. The gonosomal constitution can be identified as easily as the functional sex simply by observing these distinctive characteristics. Under normal conditions functional sex and gonosomal constitution never differ, indicating that the *XY* mechanism of sex determination in the wild *P. maculatus* is a very stable one.

With regard to these facts, it is surprising that functional females with the heterosomal male constitution *XY* appear after irradiation (1000–2500 R) of embryos with X-rays. The greatest percentage of *XY* females was obtained after irradiation with a dosis of 2000 R. In this series of experiments, all irradiated pregnant females ($n = 12$) gave birth to young with different numbers of *XY* females. In all, 54.4% of the *XY* offspring were females. Irradiation of pregnant females ($n = 13$) with 1500 R produced 33.1% *XY* females (see Table). In one group of young of this series, all *XY* offspring ($n = 12$) became females.

All of these *XY* females are normal individuals, displaying normal sex characteristics and functions. When these females are mated with nonirradiated *XY* males, *XX* females and *XY* and *YY* males in the expected ratio of 1:2:1 are produced. From these results, we can conclude that the occurrence of *XY* females after X-irradiation seems to be physiologically rather than genetically determined.

Determination of *XY* embryos to females by X-rays

Dose	No. of irradiated pregnant females	Offspring irradiated in the embryonic stage				
		Total No.	*XX* ♀♀	*XY* ♂♂	*XY* ♀♀	% of *XY* ♀♀ offspring
1500 R	13	233	103	87	43	33.1
2000 R	12	111	54	26	31	54.4
	25	344	157	113	74	

Zusammenfassung. Bei *P. maculatus* werden *XY*-Embryonen durch Röntgenstrahlenbehandlung (1000 bis 2500 R) physiologisch zu normalen Weibchen umdeterminiert.

A. ANDERS, F. ANDERS and S. RASE

Genetisches Institut und Institut für Biophysik im Strahlenzentrum der Justus-Liebig-Universität, 63 Giessen (Germany), 2 May 1969.

[1] The strain used in this investigation was derived from a collection made in 1939 by MYRON GORDON. Some years later it was introduced into the laboratories of CURT KOSSWIG, and it has been cultured in our laboratory for 10 years.

[2] M. GORDON, *Fishes as Laboratory Animals* (Ed. E. FARRIS, New York 1950), p. 345.

[3] W. FÖRSTER, Dissertation Giessen (1969); A. ANDERS, F. ANDERS, K. KLINKE, W. FÖRSTER and S. RASE, Verhandl. Zool. Ges. in Würzburg, in print (1969).

[4] P. MACINTYRE, Genetics *46*, 575 (1961).

Amino Acids as Stimulating Agents of DNA-Replication in Melanomas[1]

I. Stimulation in Explants

As we know from the work of KOSSWIG[2], GORDON[3] and HÄUSSLER[4], certain platy-fishswordtail hybrids (Poeciliidae) develop tumours. Two years ago[5] this was interpreted as misled regulation of genes controlling production of pterinophores and melanophores (colour genes). We would like here to present some new evidence for this interpretation.

Colour genes. The genes investigated in this research, the pterinophore gene *Dr* (dorsal red) and the macromelanophore gene *Sd* (spotted dorsal), are closely linked on the *X*-chromosome of the wild species *Platypoecilus maculatus* from Mexico. This linkage is reflected by the participation of certain pterines as cofactors in melanophore differentiation and melanin synthesis[5,6]. In the pure-bred species, these 2 genes are phenotypically responsible for a specific colour pattern, in which small black spots (*Sd*) appear in a reddish background (*Dr*) on the dorsal fin.

Repression and derepression. Crossing wild *P. maculatus* of *Dr-Sd* genotype with wild *Xiphophorus helleri*, which

[1] This publication is dedicated to Dr. CURT KOSSWIG on his 65th birthday.

[2] C. KOSSWIG, Z. indukt. Abstamm.- u. VererbLehre *44*, 253 (1927); *59*, 61 (1931); Copeia *1964*, 65 (1964).

[3] M. GORDON, Genetics *12*, 253 (1927); *Pigment Cell Biology* (Academic Press, New York 1959), p. 215.

[4] G. HÄUSSLER, Klin. Wschr. *27*, 1561 (1928).

[5] F. ANDERS, Experientia *23*, 1 (1967).

[6] Compare I. ZIEGLER, Ergebn. Physiol. *56*, 1 (1965); N. KOKOLIS and I. ZIEGLER, Z. Naturf. *23b*, 860 (1968).

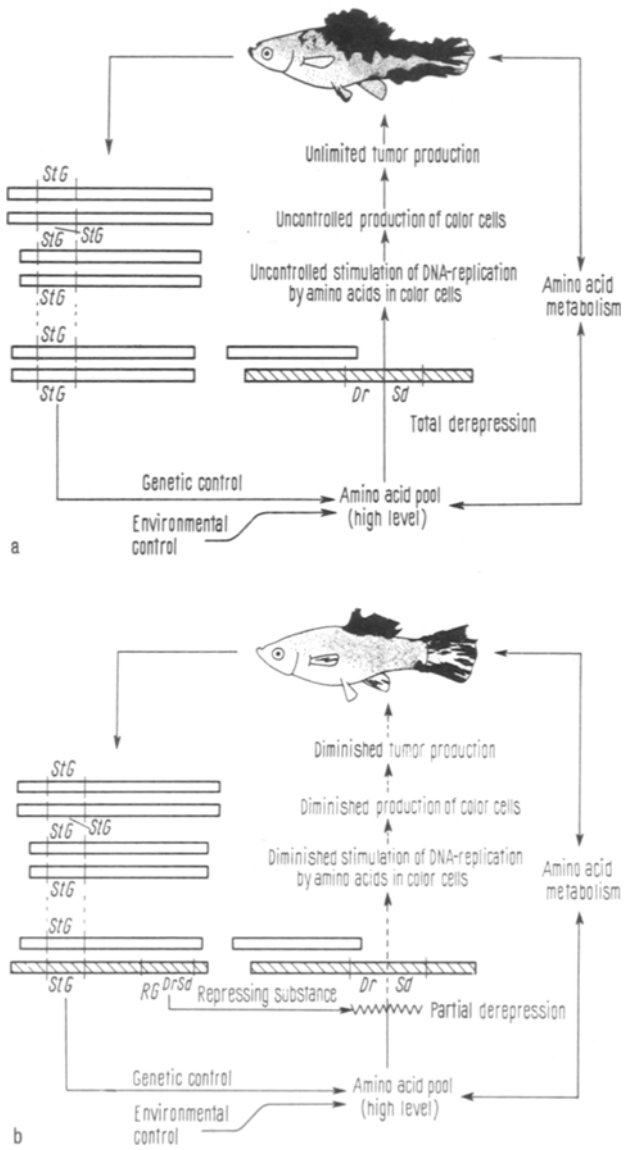
does not have *Dr* and *Sd*, followed by repeated backcrossing with *X. helleri*, may lead to the 2 genetic situations demonstrated respectively in Figures 1a and b.

In Figure 1a *Dr* and *Sd* from *P. maculatus* are affected by the genome of *X. helleri*. Under the influence of this genome, the production of pterinophores and macromelanophores proceeds uncontrolled, causing the appearance of a general red colour and large, black melanomas in areas adjacent to the dorsal fin. As a result of this tumour production these fishes die young. In Figure 1b, *Dr* and *Sd* are affected by the *X. helleri* genome and a specific autosome contributed by *P. maculatus*. In this case, red colour production and tumour growth is considerably diminished, never becoming lethal. Therefore, we can assume that the autosome contributed by *P. maculatus* carries a gene (RG^{DrSd}) which represses both *Dr* and *Sd* through the production of a repressing substance. Since RG^{DrSd} does not seem to influence colour genes other than *Dr* and *Sd*, it may be colour-gene specific[7].

In order to prove that the difference between these 2 types of control (Figures 1a and b) can be attributed exclusively to one repression gene-carrying chromosome, we crossed the genotype of Figure 1b with *X. helleri*. The offspring of this cross having the colour genes could be divided into 2 equal-sized groups, those having totally (Figure 1a), and those having partially derepressed colour genes (Figure 1b). The exact ratio obtained in our experiments was 729 totally derepressed: 713 partially derepressed animals. This 1:1-ratio proves that control of the colour genes depends upon a single repression gene-carrying chromosome contributed by *P. maculatus*.

This special system of genes can be used further to investigate the mechanism of tumour prevention. One can, for instance, introduce a double dose of RG^{DrSd} (see Figure 1c) by crossing a female of the partially derepressed type (Figure 1b) with a male of the same regulation type. Here, the double dose of RG^{DrSd} represses *Dr* and *Sd* so strongly, that the production of pterinophores and macromelanophores is limited to a small number so that tumour formation never takes place.

Stimulation. When the 2 colour genes are completely derepressed, the uncontrolled production of pterinophores and macromelanophores increases gradually with dependence upon hereditary and environmental conditions[5].



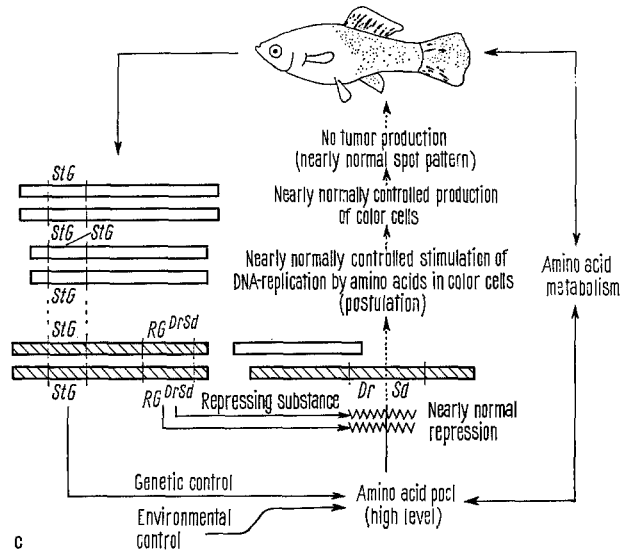


Fig. 1a–c. Three states of regulation of colour cell production. See text. Striped bars = chromosomes contributed by *P. maculatus*; white bars = chromosomes contributed by *X. helleri*; *Dr* and *Sd* = colour celldifferentiating genes; *StG* = stimulating genes; RG^{DrSd} = repression gene.

[7] In addition to RG^{DrSd}, there are other repression genes; for example those limiting the areas of the body where colour cells can assemble. They seem to be distributed on all chromosomes of *P. maculatus*, including the sex chromosomes (F. Anders and K. Klinke, Verh. dt. zool. Ges. in Göttingen *1966*, 391). However, these genes seem to have a function which is less important in the control of tumour formation than that displayed by the gene RG^{DrSd}. – The idea of repression and derepression of colour genes in platyfish-swordtail hybrids was first published by H. Breider, Strahlentherapie *88*, 618 (1952).

This indicates that stimulation exists. Furthermore, KOSSWIG[2] and GORDON[3] have already acknowledged the presence of gene systems responsible for such stimulation. The stimulating genes, termed *StG* (see Figure 1), seem to be identical with those determining the qualitative and quantitative constitution of the cellular amino acid pools[5,8], because the hereditary change in the level of amino acids in hybrids is almost exactly correlated with the rate of colour cell formation in nearly all derepressed colour genes that we have investigated (22 different genes of 8 different species and subspecies). This strongly suggests that free amino acids are a link in the functional mechanism of the stimulating genes (see Figure 1). In contrast to the repression genes, however, the *StG*-system acts upon the expression of nearly all derepressed colour genes tested, indicating that it is colour-gene non-specific. In addition, it is known that all environmental conditions which make the amino acid level increase stimulate tumour formation in these totally derepressed systems[5,9].

In order to determine more accurately the way in which amino acids stimulate tumour production, an investigation of their effect upon DNA-replication, which determines tumour production, was necessary. This was accomplished by incubating halves of *Dr-Sd* melanoma explants, consisting of pure colour cells and a thin layer of epidermis, for 15 h in the medium which produced the best results with fish tissue culture (Medium 199, DIFCO, and calf serum, DIFCO, as is used for mammal tissue culture; dilution with distilled water 8:3; roller tube; 27 °C). The level of free amino acids present in this standard medium is lower than that found in the tumours (Compare 1st and 2nd column in Table I). The other half of each of these tumours was incubated in the same medium containing an additional amount of amino acids. H^3-thymidine (5 μC/ml; specific activity = 16.5 μC/m*M*) was added and mean values of labelling indices were determined, from which the stimulating effect was measured. Melanomas from albinos were used in these particular experiments, to eliminate the histological and cytological difficulties encountered when working with normal, melanin-containing melanomas. Except for the albino gene and its chromosome, the genetic constitutions of the fishes examined were those of Figures 1a and b.

In the situation involving total derepression (Figure 1a), we found that the labelling indices increase with the free amino acids present in the medium. The highest indices were found in tumours cultured in a medium containing about 10 times as much total free amino acids as present in the standard medium or 3 times as much as found in the tumours (Compare the upper part of Table II. For the exact content of amino acids, see Table I.) When this level is exceeded, the labelling index begins to decrease. These results suggest that amino acids indeed stimulate thymidine incorporation. In many areas of the melanomas, the effect of the additional amino acids is so strong that the labelling index may be 5 times higher than that determined from the controls (Compare Figures 2a and b).

[8] F. ANDERS and K. KLINKE, Z. VererbLehre *96*, 49 (1965); F. ANDERS, Zentbl. VetMed. B *15*, 29 (1968).

[9] F. ANDERS, F. DRAWERT, K. KLINKE and K. H. REUTHER, Biol. Zentbl. *81*, 45 (1962); Experientia *19*, 219 (1963).

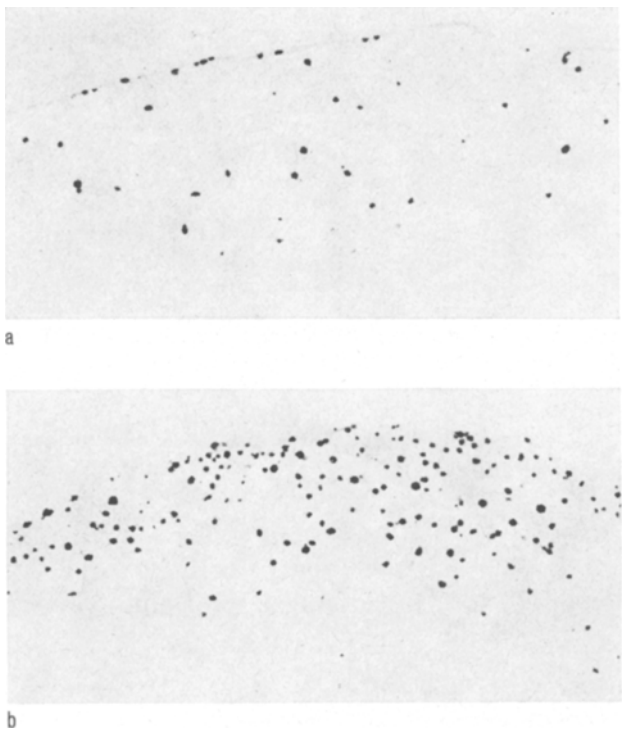


Fig. 2. H^3-thymidine autoradiographs of comparable regions of one tumour of the totally derepressed type (Figure 1a); (a) cultured in the standard medium (for amino acid content see 2nd column in Table I); (b) cultured in the medium containing additional amino acids (for amino acid content see 4th column in Table I).

Table I. Concentration of free amino acids

Amino acids	mg amino acids/100 g wet weight of melanomas[a]	mg amino acids/100 ml standard medium	mg amino acids added to 100 ml of the standard medium to obtain the total amino acid content given in column 4	mg amino acids/100 ml of the medium in which the highest stimulation of DNA-replication was observed
Glu	34	9.55	68	77.55
Gly	24	3.47	48	51.47
Ala	22	3.49	44	47.49
His	20	1.41	40	41.41
Asp-NH_2	15	–	45	45.00
Glu-NH_2	15	6.06	45	51.06
Asp	14	3.63	42	45.63
Lys	14	4.66	42	46.66
Ser	10	3.27	30	33.27
Arg	9	4.50	27	31.50
Leu	6	8.12	18	26.12
Try	6	–	18	18.00
Thr	6	3.79	18	21.79
Val	5	3.43	15	18.43
Ileu	4	2.42	12	14.42
Pro	3	2.60	9	11.60
Tyr	3	2.42	9	11.42
Phe	3	3.35	9	12.35
Total	213	66.17	539	605.17

[a] Mean values taken from reference [9] and A. ALTMAIER, Giessen (personal communication).

Until now, one could have argued that this increase in labelling index is caused exclusively by nutritional factors. However, we know now that a nutritional effect is only possible in the absence of the repression gene RG^{DrSd} (Figure 1a). In the situation involving partial derepression (Figure 1b), it can be seen that the repression gene hinders thymidine incorporation and additional amino acids indicate no stimulating effect (see the lower part of Table II).

In conclusion, it can be stated that amino acids have a general ability to stimulate DNA-replication in melanoma cells. However, they are allowed to stimulate excessively only if the repression gene RG^{DrSd}, which specifically represses the genes *Dr* and *Sd*, is absent.

These results agree with other investigations we have undertaken about thymidine-incorporation in in-situ melanomas[10]. They also agree with the findings that, not only the genetic, but also the environmental factors which effect the amino acid level, stimulate tumour formation in fish having totally derepressed cell-differentiating genes[5,9]. We do not believe that the state of total derepression is the only case in which amino acids are allowed to stimulate DNA-replication. However, stimulation of DNA-replication in repressed colour cells would be very difficult to prove, since the repression genes hinder the very thymidine-incorporation which indicates stimulation.

Concluding remarks. After considering our new results, we have decided that our former interpretation concerning the stimulating effect of amino acids, needs to be corrected. Two years ago[5] we assumed that amino acids stimulate the activity of the derepressed colour genes which in turn control differentiation of colour cells. Furthermore, we thought that the degree of melanoma formation depends upon the degree of colour gene activity. However, our recent results indicate that the colour genes do not have different degrees of activity, but only of derepression. Derepressed colour genes and free amino acids coordinate in causing tumour formation. The colour genes themselves are not stimulated by amino acids but, in the derepressed state, they allow amino acids to stimulate DNA-replication excessively in those cells which they determine. This colour gene-directed stimulation of DNA-replication, which is controlled by the stimulating gene-system *StG*, environmental conditions and amino acid metabolism[5,9], leads to abnormally accelerated colour cell production, causing the formation of melanomas. When the colour genes are repressed amino acids are hindered in their general ability to stimulate DNA-replication (Compare Figures 1a, b, c). Therefore, tumour formation depends upon 2 different events: (1) derepression of cell-determining genes and (2) stimulation of replication in cells which they determine.

Finally, we would like to mention that the artificially produced genetic system used in this research may not be representative of tumour formation in general. Nevertheless, it is a system which, by its simplicity, may yield new information concerning the principle of tumour formation. The main advantage of our system is that we can compare the reaction of similar tissues, the cells of which differ only in the absence or presence of a specific chromosome which carries a tumour-preventing gene[11].

Table II. Labelling indices in 8 melanomas (H^3-thymidine incorporation; labelled nuclei/total No. of nuclei in 6 areas of 3 chosen regions of every 50th section of each tumour; region *a* had the highest labelling index and *c*, the lowest; half of each of the tumours was incubated in the standard medium; the other half was incubated in a medium containing an additional amount of total amino acids

Tumour genotype *DrSd*	Tumour	Tumour region	Incubation: Standard medium (for amino acid content see 2nd column of Table I) Average of 6 areas	Standard medium Mean values	Medium with additional amino acids giving highest labelling indices (for amino acid content see 4th column of Table I) Average of 6 areas	Medium with additional amino acids Mean values
Without repression gene RG^{DrSd} (See Figure 1a)	I	a	0.218	0.198	0.414	0.268
		b	0.198		0.227	
		c	0.179		0.163	
	II	a	0.281	0.197	0.606	0.379
		b	0.172		0.314	
		c	0.139		0.217	
	III	a	0.297	0.200	0.622	0.497
		b	0.160		0.276	
		c	0.142		0.213	
	IV	a	0.167	0.132	0.863	0.530
		b	0.133		0.522	
		c	0.097		0.204	
With single dose of repression gene RG^{DrSd} (See Figure 1b)	I	a	0.053	0.050	0.066	0.054
		c	0.048		0.042	
	II	a	0.067	0.053	0.051	0.043
		c	0.040		0.036	
	III	a	0.084	0.072	0.097	0.069
		c	0.061		0.042	
	IV	a	0.029	0.025	0.036	0.028
		c	0.021		0.020	

Zusammenfassung. Es wird über den Fortgang früherer Arbeiten[5] berichtet. Bei Zahnkarpfen-Melanomen, die durch total dereprimierte zellendeterminierende Gene bedingt sind, vermögen freie Aminosäuren den H^3-Thymidineinbau zu stimulieren. Ein spezifisches Repressionsgen, das in einfacher Dosis das Tumorwachstum stark vermindert und in doppelter Dosis vollständig unterdrückt, hemmt den Stimulationseffekt der Aminosäuren. Tumorbildung erfolgt in zwei Schritten: 1. Derepression zellendeterminierender Gene und 2. Stimulation der Replikation in Zellen, die von diesen Genen determiniert werden.

F. Anders, M. Sieger and K. Klinke

Genetisches Institut der Justus-Liebig-Universität, 63 Giessen und Anatomisches Institut (Lehrstuhl II) der Justus-Liebig-Universität, 63 Giessen (Germany), 31 January 1969.

[10] M. Sieger, F. Sieger and F. Anders, Verh. dt. zool. Ges. in Innsbruck *1968*, in print; M. Sieger, Dissertation der Universität Giessen 1968; F. Sieger, Diplomarbeit der Universität Giessen *1968*.

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