Properties of the acetates from Croton oil

	From column		m. p.	$[\alpha]_D^{20°}$ 1% in ethanol	Analysis		R_f (Zaffaroni)	I. R. abs. in CH_2Cl_2	U. V. abs. in ethanol
	fraction	eluted with			found %	calculated %			
Acetate A ($C_{23}H_{30}O_8$)	1–10	Benzene	198–200° or 178°	−138°	C 63·29 H 6·86 O 29·40 (C)CH_3 15·5 (CO)CH_3 13·6 M. W. 389	63·58 6·96 29·46 3·45 (one) 434·5	0·94	OH at 2·96 μ C=O at 5·76 μ 5·81 μ	233 mμ (ε = 8,000) 324 mμ (ε = 53)
Acetate B ($C_{21}H_{28}O_7$)	12–16	Benzene-20% ether	118–120°	+ 69°	C 64·13 H 7·38 O 28·22 (C)CH_3 15·6 (CO)CH_3 9·3 M. W. 360	64·27 7·19 28·54 3·82 (one) 392·4	0·65	OH at 2·82 μ 2·95 μ C=O at 5·76 μ 5·85 μ	233 mμ (ε = 4,000) 330 mμ (ε = 51)
Acetate C ($C_{21}H_{28}O_7$)	22–36	Ether-50 % $CHCl_3$ to pure $CHCl_3$	119–121°	− 65°	C 63·98 H 7·35 O 28·76 (C)CH_3 15·9 (CO)CH_3 9·7 M. W. 324	64·27 7·19 28·54 3·82 (one) 392·4	0·41	OH at 2·82 μ 2·95 μ C=O at 5·76 μ 5·83 μ	238 mμ (ε = 5,400) 338 mμ (ε = 49)

acetates *B* or *C* led to acetate *A* exclusively. None of the acetates reacted with 3,5-dinitrophenylhydrazine, and the Legal test was negative, but acetate *B* (possessing weak activity in the leucocyte migration test) reduced Fehling's solution, and acetate *A* exhibited soda fluorescence on paper[11]. It is assumed that in 'phorbol' an additional molecule of ethanol is present (though this cannot be removed on drying) leading to a formula of $C_{19}H_{26}O_6$ for the skeleton of the molecule. It will be noticed that a free OH group is still present in acetate *A*, as indeed there is in the original resin (I. R. absorption at 2·93 μ).

In the croton resin contained in the naturally occurring oil, the polyhydroxy-compound from which these acetates are derived is esterified by saturated fatty acids from C_{10}–C_{16} (even) and acetic acid, but not by C_4–C_8 acids. This was shown by converting the esterifying acids to the corresponding hydroxamic acids by treatment of the resin itself with hydroxylamine and potassium hydroxide, and chromatography on paper by two different methods[12]. It thus seems that the polyester called 'Croton resin' contains three esterified OH groups, two by acetic acid and one by an acid containing 10–16 carbon atoms, one of the acetates being much more difficult to hydrolyze. There is also a free hydroxyl group that is not readily esterifiable, leaving only two oxygen functions in the resin and one in the saponified skeleton unaccounted for.

The biological testing was carried out by Dr. B. Schär in the CIBA (Basle), biological department (Direction Prof. R. Meier). Thanks are extended to Dr. R. Neher and Mr. E. von Arx for assistance with the paper chromatography. Analyses were carried out under the direction of Dr. H. Gysel.

Noted in proof: It has recently been reported that a tumor-promoting principle containing nitrogen has been isolated from croton oil (W. Lijinsky, 375th Meeting of the Biochemical Society, Sheffield, 1958).

A. F. Thomas and A. Marxer

Research Laboratories of CIBA Limited, Pharmaceutical Department, Basle, June 16, 1958.

[11] I. E. Bush, Recent Progress Hormone Res. *9*, 321 (1954).

[12] F. Micheel and H. Schweppe, Angew. Chem. *67*, 136 (1955).– E. Bayer and K. H. Reuther, Angew. Chem. *68*, 698 (1956).

Zusammenfassung

Durch Hydrolyse des aus Krotonöl gewonnenen Krotonharzes wurde eine empfindliche, wasserlösliche Polyhydroxyverbindung gewonnen, die teilweise als das bereits bekannte Alkohol-Phorbol abgeschieden werden konnte. Von ihren 4 Hydroxylgruppen scheinen im Krotonharz 2 mit Essigsäure verestert zu sein. Nur eine der Acetylgruppen ist verseifbar. Die dritte Hydroxylgruppe ist mit einer höheren Fettsäure von 10–16 C-Atomen verestert, die vierte scheint frei vorzuliegen. Zwei der Hydroxylgruppen im Verseifungsprodukt sind acetylierbar. Im Grundskelett der Formel $C_{19}H_{26}O_6$ ist daher eine Sauerstoff-Funktion noch ungeklärt.

A New Evidence for Induction of Respiration Deficiency in Yeast by Acriflavine

Various chemicals have been known to increase the frequency of respiration-deficient variants in yeast. To prove the actual induction by the drug, one must successfully exclude the possibility that the variant cells contained in the inoculum, or spontaneously occurring during growth, might have selective advantage over the normal (respiration-sufficient) cells in a population growing in the presence of the drug. This critical examination was achieved by: (1) Following the time course of population changes and finding the accumulation of variant cells at a rate faster than that of the variant culture alone, as performed by Ephrussi, L'Heritier and Hottinguer[1] on acriflavine; (2) placing a normal mother cell in a medium containing the drug, detaching the buds formed in the presence of the drug by micromanipulator, transferring them separately to drug-free medium, and finding variants to be the majority of thus detached daughter cells, with the mother cell still remaining normal, as demonstrated by Ephrussi and Hottinguer[2] on euflavine

[1] B. Ephrussi, P. L'Heritier, and H. Hottinguer, Ann. Inst. Pasteur *77*, 64 (1949).

[2] B. Ephrussi and H. Hottinguer, Nature *166*, 956 (1950).

Increased frequencies of respiration-deficient variant cells in glycerine medium containing acriflavine. Shaking culture for 24 h. Initial inoculum: ca. 500 cells per ml.

Culture	Concentration (parts/million)	Total colony number per ml of culture	Variant colonies		Variegated and sectored colonies	
			Number per ml	%	Number per ml	%
14061 (Haploid)	None	$0{\cdot}2 \times 10^7$	$1{\cdot}2 \times 10^4$	0·1	0	0
	0·5	$2{\cdot}9 \times 10^5$	$1{\cdot}7 \times 10^5$	58·7	$7{\cdot}0 \times 10^4$	24·4
	1	$2{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^5$	50·9	$6{\cdot}6 \times 10^4$	32·3
	3	$1{\cdot}8 \times 10^5$	$1{\cdot}0 \times 10^5$	57·5	$3{\cdot}7 \times 10^4$	20·6
	5	$1{\cdot}1 \times 10^5$	$3{\cdot}6 \times 10^4$	33·3	$2{\cdot}8 \times 10^4$	25·9
	10	$1{\cdot}2 \times 10^4$	$3{\cdot}5 \times 10^3$	30·4	$3{\cdot}0 \times 10^3$	26·1
N 560 (Haploid)	None	$1{\cdot}5 \times 10^7$	$<1{\cdot}5 \times 10^4$	< 0·1	0	0
	0·5	$3{\cdot}2 \times 10^5$	$1{\cdot}7 \times 10^5$	53·0	$1{\cdot}0 \times 10^5$	32·1
	1	$2{\cdot}8 \times 10^4$	$1{\cdot}4 \times 10^5$	48·6	$8{\cdot}6 \times 10^4$	30·8
	3	$1{\cdot}1 \times 10^5$	$3{\cdot}2 \times 10^4$	28·0	$4{\cdot}5 \times 10^4$	39·4
	5	$1{\cdot}1 \times 10^5$	$3{\cdot}2 \times 10^4$	30·0	$2{\cdot}8 \times 10^4$	25·8
	10	$7{\cdot}1 \times 10^3$	$1{\cdot}5 \times 10^3$	22·0	$9{\cdot}5 \times 10^2$	13·5
N 579 (Haploid)	None	$1{\cdot}4 \times 10^7$	$9{\cdot}9 \times 10^4$	0·7	0	0
	0·5	$2{\cdot}3 \times 10^5$	$1{\cdot}5 \times 10^5$	65·3	$7{\cdot}9 \times 10^3$	3·5
	1	$1{\cdot}4 \times 10^5$	$5{\cdot}6 \times 10^4$	40·0	$4{\cdot}1 \times 10^3$	2·9
	3	$1{\cdot}3 \times 10^5$	$5{\cdot}2 \times 10^4$	41·6	$4{\cdot}6 \times 10^3$	3·7
	5	$6{\cdot}3 \times 10^4$	$3{\cdot}2 \times 10^4$	50·5	$3{\cdot}4 \times 10^3$	5·4
	10	$3{\cdot}6 \times 10^4$	$1{\cdot}4 \times 10^4$	38·4	$2{\cdot}1 \times 10^3$	5·8
F–2 (Diploid)	None	$1{\cdot}2 \times 10^7$	$<1{\cdot}2 \times 10^4$	< 0·1	0	0
	0·5	$4{\cdot}8 \times 10^5$	$2{\cdot}6 \times 10^5$	53·5	$1{\cdot}0 \times 10^5$	21·7
	1	$1{\cdot}5 \times 10^5$	$5{\cdot}8 \times 10^4$	39·2	$6{\cdot}0 \times 10^4$	40·6
	3	$1{\cdot}3 \times 10^5$	$4{\cdot}1 \times 10^4$	31·8	$3{\cdot}5 \times 10^4$	27·2
	5	$7{\cdot}2 \times 10^4$	$2{\cdot}1 \times 10^4$	30·0	$2{\cdot}0 \times 10^4$	27·3
	10	$1{\cdot}4 \times 10^4$	$2{\cdot}5 \times 10^3$	17·8	$1{\cdot}5 \times 10^3$	10·7
11294 × 11296 (Tetraploid)	None	$1{\cdot}7 \times 10^7$	$<3{\cdot}4 \times 10^4$	< 0·2	0	0
	0·5	$4{\cdot}8 \times 10^5$	$2{\cdot}6 \times 10^5$	54·5	$9{\cdot}4 \times 10^4$	19·6
	1	$3{\cdot}5 \times 10^5$	$1{\cdot}8 \times 10^5$	53·1	$8{\cdot}8 \times 10^4$	26·5
	3	$2{\cdot}5 \times 10^5$	$9{\cdot}7 \times 10^4$	38·0	$7{\cdot}7 \times 10^4$	30·2
	5	$2{\cdot}4 \times 10^5$	$6{\cdot}2 \times 10^4$	25·8	$8{\cdot}2 \times 10^4$	34·2
	10	$6{\cdot}0 \times 10^3$	$1{\cdot}3 \times 10^3$	20·8	$1{\cdot}1 \times 10^3$	18·3

(active component in commercial acriflavine); or (3) growing separate clonal populations in two lots with and without the drug and applying the Luria-Delbruck test, as performed by LASKOWSKI[3] on triphenyl tetrazolium chloride. The present information deals with a new, simpler approach to prove the induction.

Respiration-deficient yeast cells do not grow in nutrient medium in which sugar is substituted with glycerine, whereas normal cells do. Since a small number of variant cells contained in the inoculum or occurring during growth do not undergo further proliferation in glycerine medium, the proportion of variants in the population tends to decline below the initial level. If an increase in variant cells, both in number and proportion, has occurred in glycerine medium containing the drug, it should be clear evi denoe for the induction. It may well be said that the aforementioned procedure (1) is simplified by elimination of one fundamental factor, self-multiplication of variant cells. Such a situation was found in the following experiment with a glycerine medium containing acriflavine.

Nutrient medium contained glycerine: 20 ml; $MgSO_4 \cdot 7H_2O$: 0·5 g; KH_2PO_4: 1 g; $(NH_4)_2SO_4$: 1·5 g; peptone: 1·8 g; dry yeast extract: 2 g dissolved in 1000 ml of distilled water. Sterilized stock solution of acriflavine (1:2500) was added to make the concentrations 0·5, 1, 3, 5 and 10 parts/million, respectively. Erlenmeyer flasks of 200 ml capacity containing 40 ml of medium were respectively inoculated with five normal strains chosen from the Carbondale breeding stocks. Each inoculum contained ca. 20000 cells and fewer than 1% variant cells. Flasks were incubated at 30°C under shaking. Samples were taken after 24 h and quantitatively plated on ordinary glucose-peptone-agar medium. Sample cultures were scored for frequency of variant cells by the tetrazolium overlay technique[4] after 2 days of incubation. The results summarized in the table indicate a significant increase in frequency of variant cells in the presence of acriflavine, while the frequency in the control flasks was low as the result of disadvantageous selection. On the tetrazolium-overlaid sample plates, many sectored or variegated colonies (counterpart of 'scalloped' colonies in EPHRUSSI's description) were seen besides normal and variant colonies as shown in the table. These colonies seem to indicate clustering in sampled cells or delayed manifestation of the induction. A similar lot of flasks were inoculated with ca. 500 cells per ml of previously isolated variant subcultures. They did not grow more than 1000 cells per ml. The hardly conceivable possibility that acriflavine added to glycerine medium might enhance the growth of variant cells was thus excluded.

This new method proved the induction by acriflavine far more efficiently than methods hitherto used. It will be

[3] W. LASKOWSKI, Heredity *8* (Part 1), 79 (1954).

[4] M. OGUR, R. ST. JOHN, and S. NAGAI, Science *125*, 928 (1957).

generally applicable to other supposed inducers. This work was supported by a grant from the Illinois Division of the American Cancer Society.

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Zusammenfassung

Mit Hilfe einer Nährlösung, in der 2prozentiges Glyzerin den Zucker ersetzte, wurde ein neues und vereinfachendes Verfahren gefunden, die durch Acriflavin bedingte Induktion der Atmungsunfähigkeit von Hefe zu prüfen. Während die normalen, das heisst atmungsfähigen Zellen sich in dieser Nährlösung vermehren, tun dies die atmungsunfähigen Zellen nie. Eine bemerkenswerte Zunahme der atmungsunfähigen Zellen wurde bei normalen Hefekulturen in Glyzerin-Nährlösung nach Zusatz von verschiedenen Konzentrationen von Acriflavin trotz der nachteiligen Bedingungen beobachtet.

Produktion von Acetylmethylkarbinol durch Aktinomyzeten

Aus unserer früheren Arbeit[1] geht hervor, dass der Aktinomyzet *Streptomyces erythreus* während der Submerskultivierung eine geringe Menge von Acetylmethylkarbinol produziert, die durch den Zusatz von Arsenit wesentlich gesteigert wird. Die Erhöhung der Acetylmethylkarbinolproduktion führt dabei zu einer intensiven Störung der Biosynthese des Erythromyzins. Um festzustellen, ob die Fähigkeit, das Acetylmethylkarbinol zu bilden, unter den Aktinomyzeten allgemein verbreitet ist, studierten wir seine Produktion in An- und Abwesenheit von Arsenit bei den Aktinomyzeten *Streptomyces noursei, S. griseus, S. antibioticus, S. rimosus, S. aureofaciens, S. coelicolor* und *S. olivaceus*.

Die Bestimmung des Acetylmethylkarbinols, der Brenztraubensäure und der Fähigkeit der Fermentationsflüssigkeit, Bisulfit zu binden (ausgedrückt in mg von Acetaldehyd), wurde ebenso wie in den vorhergehenden Arbeiten durchgeführt[2].

Die Tabelle zeigt, dass das Acetylmethylkarbinol, unter den von uns angewandten Bedingungen, in Abwesenheit von Natriumarsenit nur von den Aktinomyzeten *S. erythreus, S. antibioticus, S. aureofaciens* und *S. olivaceus* produziert wird. Bei *Streptomyces aureofaciens* war die Acetylmethylkarbinolkonzentration kleiner als 50 µg/ml, bei *S. erythreus, S. antibioticus* und *S. olivaceus* wurde das Acetylmethylkarbinol je nach dem Stamme in Konzentrationen von 150 bis 360 µg/ml synthetisiert. Die Anhäufung grösserer Mengen von Pyruvat und «Acetaldehyd» wurde dabei nur bei *S. erythreus* festgestellt.

In Anwesenheit von Arsenit wurde die Produktion von Acetylmethylkarbinol und die Anhäufung von Pyruvat und «Acetaldehyd» durch *S. erythreus, S. antibioticus, S. aureofaciens* und *S. olivaceus* wesentlich erhöht. Bei den Aktinomyzeten *S. noursei, S. griseus, S. rimosus* und *S. coelicolor* erhöhte der Zusatz von Arsenit die Anhäufung von Pyruvat und «Acetaldehyd» mehrfach und induzierte die Biosynthese von Acetylmethylkarbinol.

Zur maximalen Anhäufung von Pyruvat und «Acetaldehyd» kam es bei allen untersuchten Aktinomyzeten vor der maximalen Produktion von Acetylmethylkarbinol, und bei der Erhöhung der Acetylmethylkarbinolsynthese

[1] V. MUSÍLEK und V. ŠEVČÍK, Naturwissenschaften *45*, 215 (1958).

[2] V. MUSÍLEK und V. ŠEVČÍK, Naturwissenschaften *45*, 215 (1958); *45*, 86 (1958); Čs. mikrobiologie *3*, 205 (1958).

Produktion von Acetylmethylkarbinol durch verschiedene Aktinomyzeten

Typen der benutzten Nährmedien:
A: Glukose, Maisextrakt, $CaCO_3$. *B:* Glukose, Sojamehl, Maisextrakt, $CaCO_3$. *C:* Glukose, Laktose, Maisextrakt, $CaCO_3$.
D: Sacharose, Melasse, Maisextrakt, $CaCO_3$. *E:* Glukose, Stärke, Maisextrakt, $CaCO_3$.
Kultivierung auf einer reziproken Schüttelmaschine bei 28° C.

Stamm	Typ des Nährmediums	Zusatz	Maximale Konzentration von		
			Acetylmethylkarbinol µg/ml	Brenztraubensäure mg/ml	«Acetaldehyd» mg/ml*
Streptomyces erythreus	*A*	—	150	2,8	0,05
		Arsenit $4 \cdot 10^{-4}$ *M*	2300	5,9	1,69
Streptomyces noursei	*B*	—	—	0,35	0,1
		Arsenit $2 \cdot 10^{-5}$ *M*	210	0,95	0,92
Streptomyces griseus	*A*	—	—	0,08	0,09
		Arsenit $5 \cdot 10^{-4}$ *M*	90	0,45	0,33
Streptomyces antibioticus	*C*	—	175	0,05	0,07
		Arsenit $1 \cdot 10^{-3}$ *M*	500	0,08	0,37
Streptomyces aureofaciens	*D*	—	40	0,09	Spuren
		Arsenit $1 \cdot 10^{-5}$ *M*	400	1,55	1,03
		K_2HPO_4 0,03%	350	0,07	Spuren
Streptomyces rimosus	*E*	—	—	0,18	Spuren
		Arsenit $7{,}5 \cdot 10^{-4}$ *M*	50	0,60	0,33
Streptomyces coelicolor	*C*	—	—	0,03	0,18
		Arsenit $5 \cdot 10^{-4}$ *M*	100	0,3	0,29
Streptomyces olivaceus	*E*	—	360	0,36	0,15
		Arsenit $1 \cdot 10^{-3}$ *M*	1800	2,0	0,95

* Die Fähigkeit der Fermentationsflüssigkeit, Bisulfit zu binden, ist in mg von Acetaldehyd ausgedrückt.