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THE APOCAROTENOID SYSTEM OF SEX HORMONES AND PROHORMONES IN MUCORALES

J. D. BU'LOCK, B. E. JONES and N. WINSKILL

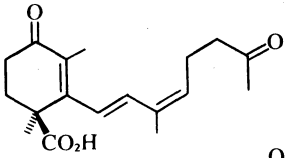
Weizmann Microbial Chemistry Laboratory, Department of Chemistry, The University, Manchester M13 9PL, UK

Abstract—The sexual process in Mucoraceous fungi is mediated by a system of C_{18} apocarotenoid substances. Of these, the prohormones are mating-type-specific products from a branching pathway of metabolism, and each type can be converted into the trisporic acid hormones only by the mating-type in which it is not formed. The trisporic acids are the actual effectors of sexual differentiation and they also depress the production of the prohormones from which they arise. The presence or absence of particular reaction steps in each mating-type is shown to be consistent with this picture, and the structural evidence for the individual prohormones is presented. It is shown that the trisporic acids exert their effects by way of transcriptional regulation of new RNA synthesis, and that their action is at least partly mediated by adenosine cyclic monophosphate.

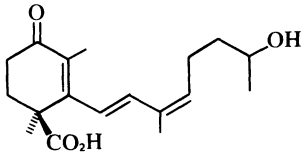
INTRODUCTION

One of the most interesting sidelines in the study of natural carotenoids has been the chemical, biochemical, and physiological study of the trisporic acid system of fungal sex hormones. In our own experience this has led us from simple biosynthetic problems into fundamental questions of general mycology, and for the topic as a whole it is difficult to define suitable limits for an account such as the present. For example, we have published short notes on the chemical structures of the prohormones of this system[1] and on the biochemical basis of their sexuality;[2] both rest upon an extensive background of chemical and mycological data that is difficult to present in terms of either discipline alone. In the present account we have attempted to present the chemical and biochemical aspects, reviewing earlier work where necessary and supplementing it with an adequate account of our own most recent results; the mycological and genetic aspects will only be dealt with in barest outline, but they have been similarly up-dated elsewhere.[3]

We are concerned with fungi of the Mucorales (Zygomycetes) including well-known genera such as *Mucor*, *Phycomyces*, and *Blakeslea*, which have in common a mechanism of sexuality which appears to be truly primitive and hence of rather wider interest.[4] With increasing precision over the last few years[5–8] it has become clear that their sexual development is mediated by hormones, *viz* the trisporic acids I–IV. Of these, 9-*cis*-trisporic acid B, I, is the most active and is probably the "true" hormone, as will become apparent. There are two mating-types, *plus* and *minus*, but sexual development is at least superficially the same in both types and is in response to the same hormone. Simultaneous and cooperative metabolism in both mating-types is however essential for hormone production. It is trying to understand the cooperative synthesis of trisporic acids that the greatest progress in understanding the biochemistry of this sexual system has been achieved, and this will be the main theme of the present account.



Trisporic acid B (I)
(also 9-*trans*) (II)



Trisporic acid C (III)
(also 9-*trans*) (IV)

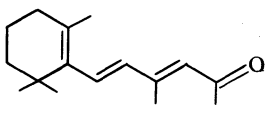
THE PROHORMONES

Trisporic acids are produced in relatively large amounts (0.2–1.0 g/l) by mixed cultures of the two mating-types of, e.g. *Blakeslea trispora*[9], and it is abundantly established[10–12] that their biological activity is shown towards *both* mating-types of test species such as *Mucor mucedo*. In contrast, the *prohormones* are substances which are produced when the mating-types are grown separately, but only in very small amounts. They are demonstrably precursors of trisporic acids, but in a quite unique sense: prohormones from the *plus* cultures—usefully designated P^+—are only converted into trisporic acids by the *minus* cultures, and the P^- prohormones, from *minus* cultures, are only converted into trisporic acids by *plus*. Correspondingly, P^+ only give positive bioassays when tested with *minus M.mucedo* and P^- only with the *plus* test. Neither set of prohormones can, on this evidence, be a precursor of the other, but both are precursors of trisporic acids. In terms of biosynthetic pathways such a situation is, to say the least, unusual.

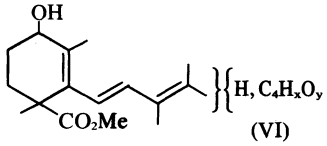
The existence and significance of these prohormones, and their occurrence amongst the neutral metabolites released into the culture medium of single-strain cultures, was largely made apparent by the work of Sutter *et al.*[13–15] in the United States, and of van den Ende *et al.*[16,17] in Holland; we had independently developed the somewhat tortuous concept of their existence during our work on the overall biosynthesis of the trisporic acids from β-carotene.

Characterisation of P^+

Cultures of *plus B.trispora* (grown submerged on 5% malt extract) afford 2–5 mg/l of an ether-extractable neutral fraction in the medium, which is revealed by TLC as a very complex mixture. Even when the more relevant constituents are selectively labelled from trace additions of the C_{18} ketone V, which is a general precursor for the trisporic series of C_{18} and C_{15} apocarotenoids,[18,19] this



(V)



(VI)

complexity remains. However, systematic differences between this mixture and the corresponding mixture from *minus* cultures encouraged us to attempt some overall studies.[2] Some 20 separate zones from TLC plates of the *plus* neutrals were examined by u.v. and mass spectrometry and after excluding the data for adventitious materials such as steroids and carotenoids (from fragmented mycelium) and trace phenolics (from the malt extract) a clear pattern in the remaining products emerged. The size and composition of the molecular ions showed that nearly all were variously oxygenated derivatives, either of the same C_{18} skeleton as the trisporic acids, or of the C_{15} skeleton to which the latter are eventually degraded in *B.trispora*, and for the C_{18} derivatives (actually all C_{19}) a general part-structure, VI, could be written on the following grounds: the u.v. absorption, λ_m 285 nm, was unchanged by $NaBH_4$ and consistent with this chromophore; the -OH group could be acetylated, adding 42 mass units (or a multiple thereof) to the molecular ion; the carbomethoxy group was required by the u.v. and mass spectral data and confirmed in selected samples by transesterification with NaOEt, which adds 14 mass units to the molecular ion and correspondingly alters the fragmentations; fragmentations generally similar to those of known trisporic acid derivatives were found throughout.

Even at this stage in our investigations it had become apparent how the prohormones—which were somewhere in this mixture—could function;[2] we could now attend specifically to any biologically active components in the mixture. The standard bioassay procedures[12] using *plus* and *minus Mucor mucedo* could be applied directly, using silica gel scraped from the TLC plates; nothing with hormone-like activity on *plus M.mucedo* could be detected, but there was a single zone with trisporic acid-type activity towards the *minus* strain. In fact, P^+ from *B.trispora* consists almost entirely of a single substance to which we can now assign the structure and stereochemistry shown, VII.

About 1 mg/l. of purified P^+ was obtained from *plus B. trispora* cultures, either by TLC or, more conveniently, by high-pressure LC (in 1:1 ethyl acetate-heptane on Porosil A silica), which gives an excellent resolution of the major neutral metabolities. The gross structure follows from spectroscopic data: molecular ion $C_{19}H_{28}O_4$, giving a monoacetate, M^+/e 362, and convertible into the ethyl ester, M^+/e 334; the u.v. spectrum [λ_m 283 nm (log ϵ 4.18) with triene fine structure] is consistent with VII and unchanged on conversion by $NaBH_4$ to the 13-dihydro-derivative, M^+/e 322. Sufficient material was obtained for an NMR spectrum which fully confirmed structure VII including the 9-*cis*-double bond; we have not detected any significant amount of the 9-*trans*-isomer. The NMR (and CD) spectra were also important for the stereochemical assignment (see below).

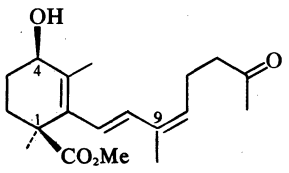
It is noteworthy that the *plus* prohormone VII is a 13-keto-compound like the trisporic acids B, I and II; the corresponding 13-hydroxy compound VIII has been detected in the total neutral materials but it is a minor component. Some special features attach to the chemistry of the 13-hydroxy compounds and are discussed later.

The configuration of VII at C(1) follows from its relationship with the trisporic acids, whose stereochemistry is established,[20] but that at C(4) was not known *a priori* and was only tentatively (and wrongly) assigned in our preliminary account.[1] To establish it securely, recourse was had to the 13-hydroxy analogues which are readily obtained by the reduction of the methyl ester of 9-*cis*-trisporic acid C, III, or its acetate, with $NaBH_4$. This affords the epimers VIII and IX (R = H, R′ = H or Ac); The reduction products are well-separated on TLC or in the LLC system, and this gave us a "fast" and a "slow" epimer in 2:1 ratio. These were shown to be VIII and IX respectively by the following arguments.

First, given the probable conformation of the ring in the methyl trisporates[2] the predominant product of borohydride reduction, i.e. the "fast" epimer, is predictably[21] VIII.

Second, whereas in the NMR spectrum of the "fast" epimer the signal for the carbinol proton at (C(4) is a rather broad one at 5.86 τ, that in the "slow" epimer is a sharper signal at 6.02 τ; this suggests that the former is quasi-axial and the latter quasi-equatorial. Now in the preferred conformation of the methyl trisporates the carbomethoxy group is quasi-axial,[20] so if this conformation is retained in the reduction products the "fast" epimer with its quasi-equatorial hydroxyl group will be VIII. This conformation would incidentally make VIII less polar than IX, in chromatography, because the hydroxyl group of VIII will be rather effectively hindered by the adjacent C-Me group.

Third, the configurations at C(4) can be assigned independently of that at C(1) (and of conformational arguments) from circular dichroism. The CD spectra of the epimeric alcohols themselves are distinctively different (Fig. 1) but not directly interpretable since their overall shape, with a single positive Cotton effect, is mainly due to interactions between the triene chromophore and the chirally-placed carbomethoxy group. However, an approach suggested to us by Nakanishi proved more fruitful. In the CD spectra of the derived *p*-methoxybenzoates (Fig. 1) the effects of the triene and *p*-methoxybenzoate chromophores (λ_m *ca.* 285 and 255 respectively) interact strongly (Davydov splitting). For such cases the configuration of the chiral centre—here C(4)—can be assigned, in terms of the



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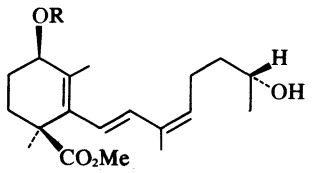

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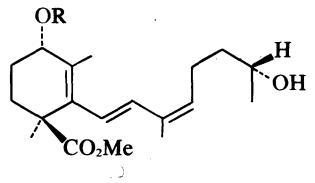

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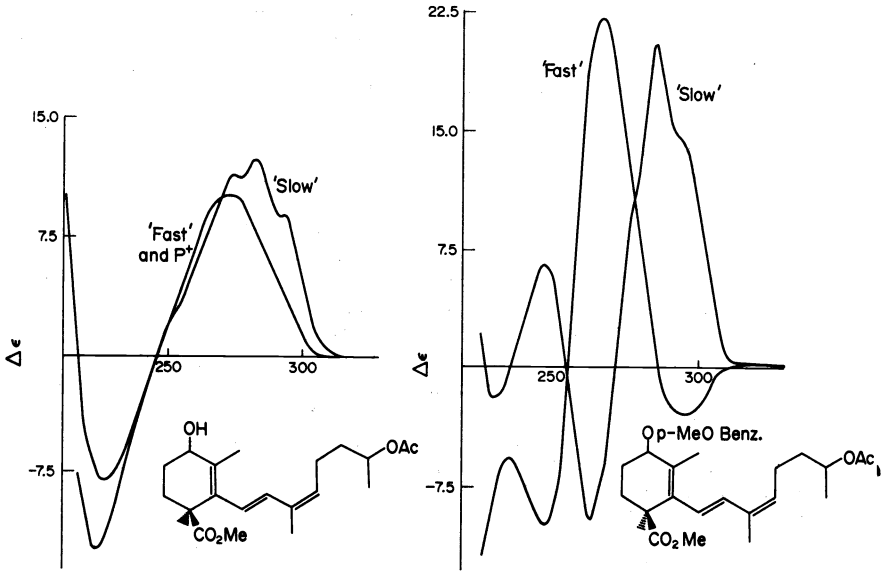


Fig. 1. CD spectra of (left) the epimeric reduction products from Me trisporate C acetate and of natural *plus* prohormone, and (right) the *p*-methoxybenzoates of the epimeric products.

chirality of the relation between the two chromophores, from the sign of the first (longest wavelength) Cotton effect, which for the "fast" epimer is negative (296 nm) and for the "slow" epimer is positive (285 nm). Hence the configurations at C(4) are 4R (VIII) and 4S (IX) respectively. The CD spectra of the epimeric benzoates were also measured, and justify the same assignments, but are less unambiguous in the lower-wavelength region, where the chromophore of the benzoyl group (λ_m *ca.* 225 nm) coincides with the negative maximum in the CD spectrum of the triene system.

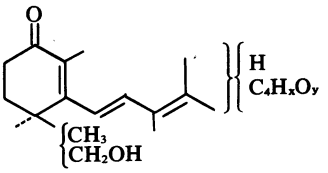
The 4*R*-configuration of natural P^+, VII, follows quite simply; its CD spectrum is virtually identical with that of VIII (Fig. 1) and its NMR spectrum shows the same signal for an axial proton at C(4) (5.85 τ). In the bioassays against *minus M.mucedo* (Table 1), the 4*R*-analogue VIII is some 30–50 times more active than is IX, which again implies that VIII has the "natural" configuration at C(4); this is finally confirmed by the rapid conversion of added VIII into VII by both *plus* and *minus B.trispora* which is noted below.

Our isolation of P^+ was from *plus B.trispora*; subsequently[22] Nieuwenhuis and van den Ende have confirmed that VII is the active prohormone from *plus M.mucedo*, apparently accompanied by rather more of the 13-hydroxy-analogue VIII in this case.

Identification of P^- substances

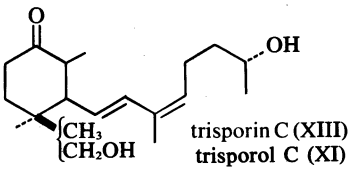
Our study of the *minus* prohormones from *B.trispora* followed similar lines. As with the *plus*, the cultures afforded less than 5 mg/l of a complex mixture of neutral metabolites. The relevant components again showed systematic similarities; in this case all were C_{18} and C_{15} compounds and the C_{18} compounds were all conjugated trienones, with λ_m 290–298 nm shifting to 250–260 nm on treatment with $NaBH_4$. The u.v. and mass spectral data justified the partial structure X for the series.[2]

Bioassays of the *minus* neutrals against *minus M.mucedo* were negative, but after TLC two well-separated regions contained materials active towards the *plus* strain. In this case we found that we were dealing with compounds which had already appeared in some of

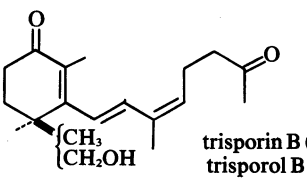


(X)

our previous work[10] so that the identifications were rather simpler. The most polar component was identified by chromatography, TLC, mass spectrum, and conversion to a diacetate, as trisporol C, XI, which we first encountered[23] as a minor metabolite in mixed *plus-minus B.trispora* cultures (for which it is obtained predominantly as the 9-*cis*-isomer XI; none of the P^- components has been obtained in sufficient quantity to confirm this stereochemistry in the single-strain products). From the same region of the chromatograms we could also isolate and similarly characterize the somewhat less polar trisporol B, XII, which we had obtained together with trisporol C in the earlier transformation studies.[10] The same earlier work also enabled us to recognize in the less-polar biologically-active region of the chromatograms trace amounts of the *gem*-dimethyl analogue trisporin C, XIII, and an isolable quantity of trisporin B, XIV (we prefer the trivial name trisporin, now introduced by Niewenhuis and van den Ende,[22] to the name trisporene which we have previously employed).



trisporin C (XIII)
trisporol C (XI)



trisporin B (XIV)
trisporol B (XII)

Although all these compounds show significant P^- activity, XII is the most active, XI having about 1/4 of its activity and XIV about 1/8 (Table 1); insufficient XIII has been available for detailed bioassay but it is probably the least active component. However, Niewenhuis and van den Ende have since reported that the trisporins XIII and XIV are the only P^- components in *M.mucedo*,[22] so that their prohormone activity is biologically significant.

Biological effectiveness

At this point it is convenient to comment upon the assay method used for biological activity.[12] The compound to be tested is placed in a well a few mm in advance of the colony margin of a *plus* or *minus* culture of *M.mucedo* on an agar plate, and after some 12 hr the number of sexual initials that have been induced can be counted. Whenever possible the compound is tested at several different levels to give a better quantitative basis for the assay, and because the response may vary considerably from one set of cultures to another it is usual to include a series of assays of a standard hormone preparation on each occasion.

At various times over the last eight years we have carried out comparative bioassays on the compounds so far described; it is not easy to bring all these data together in absolute terms, but by referring them to common standards valid and useful comparison can be drawn. In Table 1 this has been done taking the most active hormone, I, as the standard to which the arbitrary value of 1000 has been attached. The tabulated data are probably correct to within ±20% or better, which is quite adequate for the conclusions we wish to draw; to give some idea of the absolute effectiveness of these compounds, one might add that in the test situation—in which less than half the added hormone reaches the test mycelium—0.1 μg of 9-*cis*-trisporic B will induce the formation of some zygophores in our test strain of *minus M.mucedo*.

It is clear from Table 1 that the prohormones are all quite significantly active, as well as being wholly specific for one mating-type. However on a quantitative basis they are all signficantly less active than 9-*cis*-trisporic acid B, which on present evidence we must accept as the actual chemical agent causing sexual differentiation in these fungi. As will be shown, the prohormones are effective precisely to the extent that they can be converted into trisporic acids, and in this respect the data for the methyl trisporates are interesting. Conversion of the P^+ prohormones, e.g. VII, into trisporic acids requires both oxidation at C(4) and hydrolysis of the carbomethoxy group, and we may then ask which of these steps confers the mating-type specificity? The methyl trisporates are appreciably more active towards *minus M.mucedo* than towards *plus*, and a mating-type difference in ester hydrolysis may be suspected. Moreover, we have never found any esters among the metabolites of *minus B.trispora*. However, this difference in the effectiveness of the methyl trisporates towards the two mating-types of *M.mucedo* is by no means absolute (Table 1). On the other hand the 4-dihydrotrisporate esters are *wholly* specific in their activity and moreover it is only the 4*R*-epimer that is active. This suggests that it is the ability to convert the 4-hydroxy compounds into 4-keto-compounds that is really characteristic of the *minus* mating-type and which is critically absent in the *plus*.

Similarly, for the P^- prohormones, the fact that trisporal B is the most active P^- substance in the *M.mucedo* bioassay, even though Niewenhuis and van den Ende have found only the trisporins in this species, suggests that the really characteristic feature of the *plus* mating-type is the ability to oxidise the $-CH_2OH$ group of XII to carboxyl, and it is this which is critically absent in the *minus*.

However, such "less critical" features as the presence of the ester group in P^+, and the existence of the trisporins as well as (or in place of) the trisporols, may be significant in particular cases, especially for the mode of diffusion of the pro-hormones between *plus* and *minus* mycelia, for their penetration into the cells, or for certain differences between species. Work by van den Ende *et al.*[24] has implicated volatile prohormones in the "zygotropic" phenomenon[6] shown by many of these fungi, including *M.mucedo*, in which the developing zygophores of the two mating-types grow towards each other aerially. Our own experiments suggest that while both the P^+ ester VII

Table 1. Effectiveness in zygophore induction (determined with *Mucor mucedo*, taking 9-*cis*-trisporic acid B as a standard)

	On *minus*	On *plus*
Compounds effective on both mating-types		
9-*cis*-trisporic acid B(I)	1000 (std)	1000 (std)
9-*trans*-trisporic acid B(II)	500	600
9-*cis*-trisporic acid C(III)	600	600
9-*trans*-trisporic acid C(IV)	450	600
Compounds with some mating-type specificity		
methyl 9-*cis*-trisporate B	800	400
methyl 9-*trans*-trisporate B	400	200
methyl 9-*cis*-trisporate C	350	200
methyl 9-*trans*-trisporate C	250	50
Compounds with P^+ activity		
methyl 4-*R*-4-dihydrotrisporate B(VII)	150	<1
methyl 4-*R*-4-dihydrotrisporate C(VIII)	50	<1
methyl 4-*S*-4-dihydrotrisporate C(IX)	<1	<1
Compounds with P^- activity		
trisporol B(XII)	<1	300
trisporol C(XI)	<1	75
trisporin B(XIV)	<1	40

and the trisporin XIV might be sufficiently volatile to fulfil such roles, neither the 4-dihydrotrisporic acids nor the trisporols would. An untested, but possibly more volatile, P^+ substance is noted later. Here molecular properties which are based on structural features other than those directly corresponding to the mating-type are clearly important. Again with *M.mucedo*, Jockusch[25] has described mutants of the *minus* strain which show normal sexual development when mated with wild type *plus* but do not respond to trisporic acids; we have ourselves examined some of these mutants and find that they do respond normally both to methyl trisporates and to the P^+ ester VII. This suggests that in *M.mucedo*, at least, while there may be a special mechanism for the uptake of trisporic acids, in the absence of this mechanism (e.g. in the mutants) the esters (including the prohormone VII) still have access to the cells. A somewhat similar situation probably exist in *Phycomyces blakesleeanus*, in which sexual development is also most probably controlled by trisporic acids; this species cross-reacts with *M.mucedo* very well, but with *B.trispora* rather poorly (observations of R. P. Sutter, private communication). We believe that the basic prohormone/hormone mechanism in *Phycomyces* is not significantly different from that in *Mucor*, but that this does not preclude species variations in the actual prohormone structures.

Formation and transformation of the prohormones

The overall biosynthetic origin of the prohormones is of course the same as that of the trisporic acids of which they are precursors, and this has been adequately established in *Blakeslea trispora* by a series of incorporation studies, carried out at various times by ourselves and our co-workers,[10,19,23] some results of which are assembled in Table 2. For all these, and for subsequently-noted experiments, the general techniques have been fully described;[19] incubations were in 150 ml cultures grown in 500 ml shake-flasks on 5% malt extract medium; products were recovered and purified by TLC and HPLC and identified and quantified spectrophotometrically. The data lead to the following conclusions. The C_{18} compounds are formed from mycelial β-carotene by cleavage, quite possibly (though not certainly) by initial formation of a C_{20} (retinyl) derivative, and very probably by way of the "β-C_{18} ketone", V, which we have found is an excellent precursor for all the hormones, prohormones, etc., both in mixed *plus-minus* cultures and in single strains. The good incorporation of the 4-hydroxy-derivative of V, and its acetate, even though these were racemic compounds, suggests that hydroxylation of the C_{18} skeleton at C(4) is also quite an early step in the biosynthesis. However, it is soon after this step that the pathways to specifically *plus* and *minus* prohormones must diverge, and the overall pathway to trisporic acids in the mixed cultures, which is what was studied in these experiments, must to that extent be accepted as ambiguous.

It is, however, difficult to carry out really valid tracer experiments on the biosynthesis of the prohormones in single strains because these are formed in such small amounts that any addition of precursors must usually be accounted a perturbation of the system—and indeed its liability to such perturbations seems to be an essential part of its biochemical function. For example, when labelled β-C_{18}-ketone V was incubated with *minus B.trispora*, some 25% of its radioactivity was recovered, after 8 hr, in trisporol-C, XI; however, this had exactly the same specific activity as the precursor, so that no endogenous synthesis had occurred and this was an experiment on the transformation of a substrate, which strictly cannot be interpreted as a tracer experiment on an already-operating pathway. Indeed, in a similar experiment with *plus B.trispora*, 13% of the activity was recovered, undiluted, in trisporic acids—which normally are only produced in quite negligible amounts by this strain. Details of this experiment are summarized in Table 3; the reasons why trisporic acids are produced by the *plus* mycelium under these conditions are not entirely clear to us, but the products were fully authenticated (as the methyl esters, by TLC, UV spectroscopy and mass spectra) and their isolation is an incidental proof of the quite important point that *under appropriate conditions* the *plus* mycelium is capable of carrying out *all* the reactions involved in hormone synthesis (see below).

Table 2. Precursors of trisporic acids in mixed *plus-minus B.trispora*

Precursor	%Incorporation into trisporic acids	Dilution ratio in trisporic acids
2-^{14}C-acetate	13	2700
2-^{14}C-mevalonate	1.8	4400
G-^{14}C-β-carotene	3.4	220
10-^{14}C-retinol	18	34
10-^{14}C-C_{18} ketone V	38	9 (5 in trisporol C)
10-^{14}C-*RS*-4-acetoxy-V	29	7
10-^{14}C-*RS*-4-hydroxy-V	33	not done

Table 3. Conversion of 10-^{14}C-β-C_{18}-ketone V by *plus B.trispora*

Fraction	% Incorporation	Yield	Dilution
Total neutral from medium	44		
Total acids from medium	15		
Trisporic acid B(I + II)	8	*ca.* 1.3 mg	1.0 ± 0.1
Trisporic acid C(III + IV)	5	*ca.* 0.8 mg	1.0 ± 0.1

A total of 16 mg. of the labelled ketone (1.14×10^8 dis/min mmol^{-1}) was fed to 5×150 ml of cultures which was worked up after 5 hr incubation.

In a second experiment, 0.75 mg of V per 150 ml gave, after 3.5 hr, 42% incorporation into neutrals (9% as VII) and 9% into acids.

UNIT TRANSFORMATIONS

The formation of the prohormones, and their activities as precursors of trisporic acids, are more clearly interpreted[2] in terms of unit reaction steps, as set out in Fig. 2. Here we have made certain presumptions for the sake of simplicity: first, that the trisporate side-chain, with its "extra" 2H at C(11)–C(12), is elaborated before this group of reactions occurs, so that the last common intermediate is of the type shown; second, that the side-chain difference between the 13-keto (B) and 13-hydroxy (C) series is not immediately relevant (i.e. that the two are interconvertible in both mating types); third, that enzyme reactions at one centre, e.g. at C(4), can occur whatever the stage which has been reached by the transformations at the other centre, e.g. at C(1)-Me, though not necessarily with quantitatively equal facility. In these terms, the scheme of Fig. 2 is our simplest representation of the relevant reaction systems, and the status of the different reaction steps in it can now be examined on the basis of the various kinds of evidence available for the two species about which most is known, i.e. *B.trispora* and *M.mucedo*. This allows us to see both mating-type and species differences, as summarized in Table 4, in considering the individual steps one by one. Most of the data derive, except as otherwise noted, from recent work in our own laboratory.

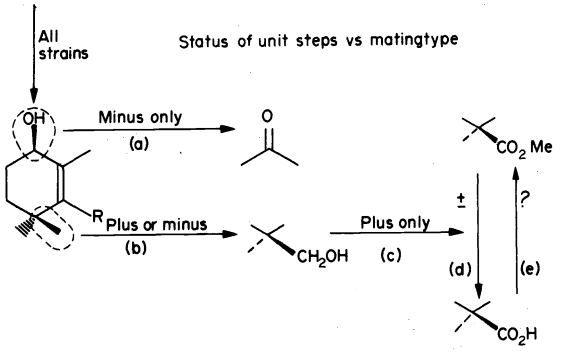
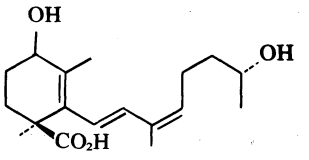


Fig. 2.

Step (a). Conversion of 4-R-*hydroxy- to* 4-*keto*

This is the reaction which we believe specifically characterizes the *minus* mating-type. Both *minus B.trispora* and *minus M.mucedo* produce prohormones with the 4-keto-group, and in bioassays the *minus* mating-type of *M.mucedo* responds to 4-*R*-hydroxy compounds of the P^+ type, presumably by converting them into trisporic acids. The interspecific cross-reactions by which the mating-types are defined throughout the Mucorales further extend this inference.

Direct evidence for the reaction in *minus B.trispora* comes from experiments in which labelled 4-hydroxy compounds are converted into trisporic acids. When ^{14}C-labelled 4*RS*-hydroxyacid XV (from trisporic acid C) was incubated with *B.trispora* (3.5 mg/l. 8 hr), the *minus* strain afforded some 36% of neutral products, almost exclusively the 4-keto compound trisporol B, XII; in the parallel experiment with the *plus* strain, only 4-hydroxy-compounds were recovered. A more detailed study was made with the corresponding ester VIII (mainly 4*R*),



XV

which was incubated for 0.5–6 hr at a concentration of 7 mg/l. With *plus B.trispora* less than 3% incorporation (4% of total recovered activity) into 4-keto-compounds (identified as the methyl esters of I and II) was obtained in 1 hr. Some complications in this experiment are discussed separately, below.

Table 4. Units steps in hormone synthesis (cf. Fig. 3); summary of experimental evidence

Step	Data	Conclusion
(a) 4*R*-HO→4-keto	(i) 4-keto only in *minus* products	
	(ii) P^+ with 4*R*-HO active on *minus M.mucedo*, not *plus*	
	(iii) *ca.* 40% conversion by *minus B.trispora*, but under 3% by *plus*	Characteristic of *minus* mating-type
	(iv) significant conversion by *minus* independent of new protein synthesis	
(b) 1-Me→1-CH_2OH	(i) P^+ and P^- formation implies this step in both *plus* and *minus B.trispora*	Varies with species; not necessarily linked to mating-type
	(ii) may be absent in *minus M.mucedo*	
	(iii) P^- activity of trisporins in *plus M.mucedo*	
(c) 1-CH_2OH oxidation	(i) P^+ structures imply this step in *plus M.mucedo* and *plus B.trispora*	
	(ii) trisporols active on *plus M.mucedo*, not *minus*	Characteristic of *plus* mating-type
	(iii) *ca.* 20% conversion by *plus B.trispora*	
(d)/(e) ester formation/hydrolysis	(i) esters found in *plus B.trispora* and *plus M.mucedo*	
	(ii) ester hydrolysis fast in *minus B.trispora*, slower in *plus*	Uncertain
	(iii) Me trisporates more active on *minus M.mucedo* than on *plus*	

Using partly-purified preparations of P^+, presumably mainly VII, Werkman and van den Ende[17] observed *ca* 22% conversion of labelled material into 4-ketoacids in 24 hr with *minus B.trispora*; with *minus M.mucedo*, Niewenhuis and van den Ende[22] obtained *ca.* 50% conversion of purified, but unlabelled, VII, into trisporic acids in 42 hr. Similarly Sutter *et al.*[15] obtained *ca.* 15% conversion of a partly-purified (labelled) P^+ into trisporic acids in 2.5 hr with *minus B.trispora.* Neither *plus M.mucedo* nor *plus B.trispora* gave significant conversion in either case.

The enzyme system which carries out the 4*R*-hydroxy→4-keto conversion is fully stereospecific as we have seen. It is present constitutively in the minus strain, i.e. at a significant level independently of the enzymic induction mechanisms that are so important in the reaction system as a whole, as will be described later. In one of the earlier papers describing what we now recognise as the prohormones, Sutter showed that the conversion of physiological levels of P^+ into trisporic acids by *minus B.trispora* proceeded equally well when the incubations were carried out in the presence of sufficient cycloheximide to ensure over 95% inhibition of protein synthesis,[13] and we subsequently made similar observations using 5-fluorouracil as the inhibitor (with D. J. Winstanley; unpublished). Indeed, Werkmann and van den Ende (private communication, 1973) provided evidence that at least part of this conversion may even be due to extracellular enzymes in the medium of *minus B.trispora* cultures.

The fact that this conversion is mating-type-specific, and does not normally occur with *plus* strains, is attributable, in our view, to a block in the synthesis of this enzyme in the *plus*, rather than to deletion of the corresponding gene. We base this belief, which is at least consistent with the available genetic evidence,[3] upon the well-authenticated cases of the production of trisporic acids, i.e. of 4-keto-compounds, in small but measurable amounts, by *plus B.trispora*: in our own work, for example, in 3% yield from labelled VIII in 5 hr and in 9–13% yield from labelled V in 3.5–5 hr (as already noted). Moreover in the careful work of Sutter *et al.*,[14] up to 0.05 mg/l. of trisporic acid was detected in the culture medium of a pure *plus B.trispora* culture.

In our study of this reaction using the 4-hydroxy ester VIII, with the 13-hydroxy side-chain (which is only a very minor component of "natural" P^+ in *B.trispora*), we have encountered a major complication which is not yet fully explored and whose biological significance is still uncertain. After 0.5 hr incubation of VIII with *minus B.trispora*, some 24% can be recovered either as an anhydro-acid or as the corresponding ester. On longer incubation these

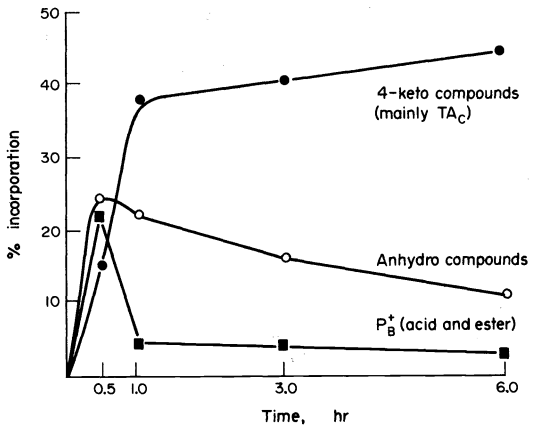
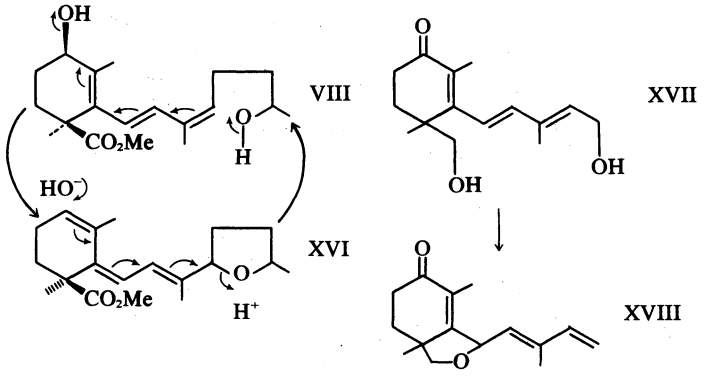


Fig. 3. Conversions of the hydroxy-ester VIII into anhydro- and 4-keto-compounds by *minus B. trispora.*

compounds are largely removed, as the yield of 4-keto-compounds builds up (Fig. 3). We could show that the anhydro-compounds were actually being converted into trisporic acids by isolating the anhydro-ester and re-incubating a sample with *minus* mycelium; in 5 hr, virtually all was converted. The anhydro-ester is a rather non-polar substance with M^+/e 304. The chromatographic properties, u.v. spectrum (λ_m 283 nm), and fragmentations in the mass spectrum, suggest that the most plausible formula for the anhydro-ester is XVI, formed from VII as shown and reverting to VIII by the reverse process.

We hope to obtain enough of this anhydro-ester for full chemical characterisation and for bioassays in the near future. Meanwhile we consider its direct relevance to the "natural" situation in *B.trispora* somewhat doubtful. Examination of the time-course data (Fig. 3) suggests that in the first hour after adding VIII to *minus B.trispora* there are two competing reactions, one (perhaps by way of the 4-hydroxy-13-ketoacid) rather directly to trisporic acids and one to the anhydro-ester, the latter then going more slowly by way of the reverse reaction XVI→ VIII to trisporic acids. The formation of XVI can only occur with the 13-hydroxy-side-chain, but in the "natural" P^+ this is a very minor component compared with the 13-keto-compound VII, and in the bioassay with *minus M.mucedo* VII is considerably more active than VIII (Table 1). Again, in forming XVI the asymmetric centre at C(4) is lost, yet only one of the epimers at this centre is biologically active. Moreover, we have found that the formation of the anhydro-ester from VIII is not mating-type specific, and indeed in *plus B.trispora* (when conversion into trisporic acids is not competing) we



recovered nearly 50% of added VIII (at 7 mg/l) as XVI after 5 hr. We do not exclude the possibility that formation of the anhydro-compound may be non-enzymic; trisporone (XVII), a C_{15} degradation product of the trisporols which occurs in the neutral fraction from mixed *plus-minus B.trispora* cultures, readily undergoes a related dehydration to anhydrotrisporone, XVIII[26] (though this transformation is not recorded as being reversible) and we had previously encountered a substance which we now recognise as the anhydroester XVI as an apparent *in vitro* decomposition product of VIII.

The existence of this quite non-polar molecule, which appears to be fully interconvertible with a known *plus*-prohormone may be quite relevant in diffusion- or uptake-controlled situations, as for example in the zygotropic effect, and this aspect awaits investigation.

Step (b). Hydroxylation of C(1)-Me

This step is presumed to be the first in the sequence by which the carboxy and carbomethoxy groups are formed and so it must occur in the *plus* strains; this is also directly shown by the positive P^- bioassay activity of trisporin B(XIV) (Table 1) and by the conversions of (unlabelled) XIII and XIV by *plus M.mucedo* reported by Niewenhuis and van den Ende[22] (3 mg/l, 42 hr; 40–50% conversion to trisporic acids). The existence of the trisporols XI and XII as major P^- prohormones in *B.trispora* shows that this reaction also occurs in the minus strain of this species, so that while their apparent absence in P^- from *M.mucedo* may imply that this is a feature which can vary between species, we do not regard it as one which is intrinsic to the mating-type phenomena.

Step (c). Oxidation of C(1)–CH_2OH

So far as the mating-type system is concerned, this reaction is the complement of step (a), in that it is distinctive for the *plus* strains. Nothing is known about the component reactions of this step, which starting from the $-CH_2OH$ group formed by step (b) clearly involves more than one reaction. We have never (knowingly) encountered the aldehydes that might be intermediates, though they could be present as trace components in the neutral metabolites of *plus B.trispora*. Nor do we know whether the final oxidation reaction in the *plus* strain generates a free carboxyl group, that is subsequently methylated, or a reactive acyl derivative which might afford an acid either directly or by way of the methyl ester. Hence in Fig. 2 the product of this step has been left unspecified and the reactions of methylation and hydrolysis are considered separately [steps (d) and (e), below].

Quite apart from the production of prohormone esters by *plus M.mucedo* and *plus B.trispora* (and of traces of trisporic acids by the latter), direct evidence for step (c) comes from the prohormone activities of the trisporols in *plus M.mucedo* (Table 1) and from the conversion of labelled trisporol C, XI (14 mg/l, 24–48 hr) by *plus B.trispora* into trisporic acids in 18% yield.[10] In the parallel experiment with *minus B.trispora*, however, appreciable conversion into acids was also reported;[10] we must acknowledge that this particular (single) experiment was performed some years ago, with less critical techniques than are now available, and we hope to obtain more definitive results in the near future. Meanwhile the fact that the trisporols have no detectable prohormone activity in the bioassay with *minus M.mucedo* (Table 1) is to us more convincing.

Evidence that the failure of step (c) in *minus* is due to repression of a gene rather than its absence (cf. the failure of step (a) in *plus*, above) is not strong; Sutter *et al.* were barely able to detect trisporic acids in highly-concentrated extracts of the medium from *minus B. trispora*.[14] However, this hypothesis does provide a simpler explanation of the genetic evidence than any other.[3] An abilility to carry out step (c) is constitutive in the *plus* strain, the evidence being the same as that for step (a) in *minus* (above).

Step (d). Ester hydrolysis

The rates of hydrolysis of labelled methyl trisporate C (30 mg/l.) by *plus* and *minus* mycelium of *B. trispora* were measured by Winstanley; the hydrolysis was *ca.* 10 times faster with the *minus*, but was by no means negligible in the *plus* (*ca.* 500 μg of ester hydrolysed in 12 hr). Bioassay data (Table 1) indicate that a similar situation exists in *M.mucedo*, the esters being some 2–5 times more active towards the *minus* strain but still significantly active towards the *plus*.

There was a more marked difference when the labelled hydroxyester VIII was incubated with *B.trispora*; less that 3% was recovered as acids after 5 hr with *plus*, but nearly 40% after 1 hr with *minus*. Equally the hydroxyester VII present in *plus B.trispora* cultures is not accompanied by significant levels of the corresponding acid. However, both these observations, in effect, relate to the "steady state" of the P^+ reaction system and not to ester hydrolysis alone. We suspect that differences in the ability to carry out ester hydrolysis are not directly linked to the mating-type, but they may be involved in species-specific details of hormone transfer and prohormone uptake.

Step (e). Formation of methyl esters

In unperturbed systems, methyl esters are only formed in the plus strains since only these produce the ester-precursor [whether this is the acid or some more active acyl derivation; see above, step (c)]. When the labelled 4-hydroxy acid XV was incubated with *plus B.trispora* (1.5 mg/l) some 30% was recovered in neutral metabolites, presumably esters (9% as labelled VIII), after 8 hr. In the parallel experiment with the *minus* strain no esters were recovered, but they could have been further transformed by reduction, see below. Therefore the status of the methylation "step" remains somewhat obscure.

Back-reactions in single strains

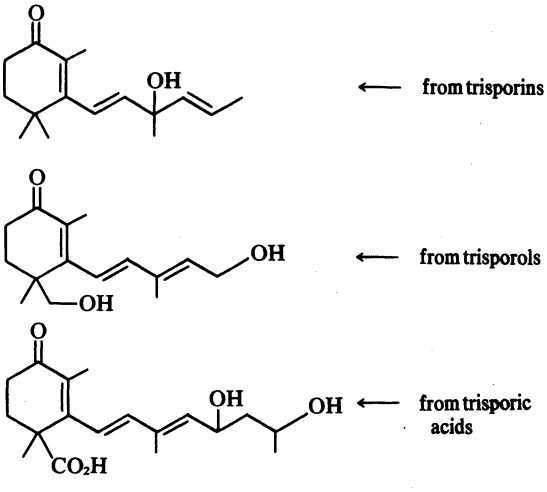
In several experiments we have observed back-conversions of trisporic acids or esters to the prohormone type corresponding to the strain used. These back-reactions seem generally slower that the "forward" reactions so far discussed but we believe they are biologically important as a "damping" mechanism, as a result of which temporary exposure to the opposite prohormone produces first a short-lived pool of active hormone which is then taken into the endogenous prohormone. Thus in our earlier experiments[10] some 5% of methyl trisporates administered to *minus B.trispora* was recovered as P^- compounds XI–XIV; in our recent work, 29% of labelled 4-hydroxy acid XV was recovered as the 4-ketoalcohol XII after 8 hr incubation with the *minus*. To an organic chemist, these conversions of C(1)-carboxy- to C(1)-hydroxymethyl-derivatives are striking; they can hardly involve reduction of the carboxylic acids themselves and they can hardly be regarded as adventiti-

ous processes, which is why we suspect that these back-reactions may be biologically significant. Back-conversions by *plus B.trispora* also occur but are less spectacular; ester formation from acids has been noted above, and we have also seen apparent conversions, not fully-checked, of methyl trisporate C into the 4-hydroxyacid XV and its ester VIII.

Side-reactions

The considerable range of biologically inactive but biosynthetically related co-metabolites found with the prohormones arises from the range of side-chain transformations which occur in both mating-types of *B.trispora* (and also in mixed cultures) which we believe represent the ultimate destination of both endogenous and exogenous prohormones; such inactivation processes are an essential part of any system of chemical communication. We have never studied these reactions in detail, but Table 5 lists some of the "inactive" side-chain types we have encountered; in single-strain metabolities these side-chains are found with the same ring-structures as the endogenous prohormones.

Table 5. Examples of inactivation products



CONCLUSIONS

Our analysis of the "unit steps" of Fig. 2, with the results which are summarized in Table 4, explains the mating-type specificity of prohormone production and fully justifies our conclusion that prohormone activity correspondingly depends on the mating-type-specific conversion of prohormones into trisporic acids. The minimum mating-type difference upon which this system rests is seen to involve just two reaction steps: (a) oxidation of 4-hydroxy- to 4-keto- in *minus*; (b) oxidation of 1-methyl- to 1-hydroxymethyl in *plus*. The relationship between this picture and the genetic evidence is one we have discussed elsewhere;[3] briefly, we suggest that the two alleles, *plus* and *minus*, of the single mating-type gene determine, *inter alia*, which one of two other genes—for the enzymes effecting step (a) or step (b) respectively—is strongly repressed and which is constitutively expressed. We have also shown that a slight modification of this picture[3] will explain the situation in the homothallic Mucorales, fungi in which very similar sexual mechanisms operate between phenotypically differentiated portions of a genetically homogeneous mycelium, mediated by what is now known to be the same system of prohormones and hormones.[27]

Effects of trisporic acid on prohormone production

The mechanism so far described explains how each mating-type produces prohormones which the other can convert into trisporic acids, i.e. a qualitative explanation of trisporic acid production. However, it does not explain the levels of trisporic acids found in mixed *plus–minus* cultures.

For example, we regularly find *ca.* 0.5 mg/l of P^+, mostly VII, in *plus B.trispora* cultures, and similar levels of P^-, mostly XI, in our *minus*, but up to 200 mg/l. of trisporic acids in mixed *plus–minus* cultures that are otherwise comparable. Similarly Sutter *et al.*[14] found that the level of prohormone production in their *plus B.trispora* was only *ca.* 2% of the level of trisporic acid production in mixed *plus–minus*.

A basis for explanation of this effect was provided by the important observations of van den Ende and Werkman who were able to obtain 7- to 10-fold increases in P^+ production, and 3-fold increases in P^- production, by repeated addition of trisporic acids to single strains.[17] These increases were not due to back-conversion (the labelling techniques used preclude this explanation), but they were blocked by 5-fluorouracil and are therefore mediated by new protein synthesis.

The total amount of trisporic acids used in these experiments was rather large, and the experiments might therefore be criticised on that account. However, it is extremely difficult to imitate, by multiple additions of exogenous trisporic acids to a single-strain culture, the effect of continued trisporic acid production within the mycelium from prohormone released by adjacent mycelium, which is what probably occurs in the mixed *plus–minus* cultures. Using a simpler experimental arrangement, we added *ca.* 1 mg/l of unlabelled P^+ extract in one lot to acetate-labelled *minus B.trispora* mycelium, and over the subsequent 8 hr we observed *ca.* 85% increase in the labelling of purified P^- compounds, as compred with unstimulated controls. We regard this as confirming the view of van den Ende *et al.* that trisporic acids derepress prohormone synthesis in both mating-types (i.e. P^+ synthesis in *plus*, and P^- synthesis in *minus*). Since prohormone synthesis in either mating-type effectively includes the steps which will convert the prohormone of the other mating-type into trisporic acids, the magnitude of the total effect in mated cultures is explained; for example, the *plus* mycelium in such a culture (i) is increasingly derepressed for the production of P^+ (which is then removed by the *minus* partner), (ii) is at the same time correspondingly derepressed for the conversion of P^- into trisporic acids, and (iii) is meanwhile receiving an increasing amount of P^- as a result of the corresponding situation in the *minus* partner.

On the other hand, derepression (by either trisporic acids or by prohormones) of the step whose blocking we have said is characteristic of the mating-type has never been observed, either in our own work, which has included experiments specifically designed to show such effects (e.g. of trisporic acids on the labelling of compounds by ^{14}C–C_{18} ketone) or in experiments by other workers in which such effects might have been apparent had they occurred.[3] In other words, the mating-type repression we have postulated (above) is not among the

spectrum of regulatory effects which the trisporic acids can alter.

The scope of depression by trisporic acids

There is insufficient evidence to enable us to conclude which particular steps in prohormone synthesis are affected by trisporic acids, but clearly more than one step in the overall pathway is thus affected; this has been clear since the trisporic acids were first discovered—not as hormones of sexual development but as promoters of β-carotene accumulation in *Blakeslea trispora*.[28–31] This effect, which is very marked in mixed *plus-minus* cultures, is very striking and it frequently, though not invariably, also occurs quite locally in the "mating zone" between adjacent colonies of other members of the Mucorales if they are of opposite mating-type. The main accumulations are, usually, of β-carotene and so are very visible, and in the older literature the relationship between carotene accumulation and sexuality in these fungi was often discussed.

The action of trisporic acids on carotenoid accumulation was shown by Goodwin and co-workers to be a derepression effect dependent on *de novo* protein synthesis and to affect the production of sterols as well;[32] they concluded that synthesis of an early rate-limiting enzyme, such as hydroxymethylglutaryl-CoA reductase or mevalonate kinase, was derepressed, and drew attention to the ensuing shift in the relative proportions of the different carotenoids. Apparently when the total input into carotenoids is increased, steps become rate-limiting which were not so previously, so that the steady state balance of intermediates changes. We have shown[33] that this applies equally to the balance between all the different isoprenoids in *B.trispora*—carotenoids, sterols, long-chain prenols, and ubiquinones—all of which are increased in mixed *plus-minus* cultures but to quite different degrees.

It is becoming clear that the normal regulation of carotene synthesis in *Phycomyces*, which is the best investigated species in this respect, is itself rather complex, and it is not yet possible to link the picture that is emerging[34,35] with our view of the regulatory effects of trisporic acids, except in a very tentative way. For example, from recent work of Sutter[36] it seems likely that one type of mutation, which results in over-production of β-carotene through defective feedback, also directly blocks the conversion of β-carotene to prohormones. However, since the increase in trisporic acid production in mixed cultures of *B.trispora* is far greater, in relation to the level of prohormone production in single strains, than is the increase in β-carotene accumulation (which in any case is always present in excess) we conclude that there are specific effects of trisporic acids on the later stages of prohormone synthesis, i.e. after β-carotene, in addition to the effects on isoprene synthesis generally. For example, since we have not detected significant quantities of either retinyl derivatives or of the C_{18} ketone either in single strains or in mixed cultures of *B.trispora*, we would conclude that the cleavage of β-carotene is one of the rate-limiting later steps that is promoted by trisporic acids, and also that it remains rate-limiting even in stimulated mycelium.

In any case it is *a priori* likely that trisporic acids derepress a considerable number of genes, either directly or indirectly. In terms of developmental biology, this follows from their role as the initiators of sexual differentiation. That process has been frequently described by observational mycologists, for whom the Mucorales are text-book examples, but we are only at the very beginning in attempting to explain it in biochemical terms. Even so, it is quite apparent that a considerable number of genes must be involved[3,6–8]—and incidentally that very few of these genes are at all directly affected by the mating-type, an interpretation which is connected with the relative simplicity of the sexual process in these organisms.

As an instance of the coordinated regulation of a multiplicity of genes by a single effector of low molecular weight, the trisporic acid system now takes on further interest, of a character quite different from that which first brought us into this field, and as an experimental test-bed for hypotheses about the fundamental mechanisms that control morphogenesis and cellular interactions it has considerable potential. We conclude the present account with some recent observations which seem to us to open a new chapter in the story.

First we have shown that the response of *B.trispora* to trisporic acids, in "physiological" concentration, includes a stimulation of RNA synthesis which is of such a magnitude, and which occurs sufficiently soon after addition of the hormone, as to allow us to suppose that it is a direct and early part of the chain of events the hormone brings about.

In terms of effect on steroid synthesis, etc., in *minus B. trispora* we could observe about half the maximum response with 15–30 mg/l trisporic acid C and within 4–5 hr after adding it to the cultures. Table 6 shows, for comparison, our measurements of RNA synthesis in *plus* and *minus B.trispora* following ghe addition of 7 μg/ml of trisporic acid C.

Table 6. Effect of trisporic acid on RNA synthesis in *B.trispora*

hr from TA addition	1	2	4	6	8
^{14}C in RNA in *minus* (%)	52†	125	270	92	91
^{14}C in RNA in *plus* (%)	95†	99†	110	100	100

Data are for ^{14}C incorporation into RNA as % of controls without trisporic acid (see text); [^{14}C]uracil was added at −1 hr and trisporic acid C(7 mg/ml) at 0 hr.

†For these reductions in ^{14}C incorporation as consequences of faster RNA synthesis, see text and Ref. 37.

The synthesis of RNA was followed by measuring the ^{14}C content (per unit weight of mycelium) of total RNA (hot $HClO_4$-soluble fraction), derived from a single addition of ^{14}C-uracil made one hour prior to the addition of trisporate, and the results are expressed as a percentage of the corresponding ^{14}C incorporation in cultures without addition hormone. As has been observed in other similarly-designed experiments on hormone-stimulated transcription,[37] the immediate effect of increased RNA synthesis is on precursor pools, in such a way as to *reduce* precursor incorporation relative to the controls, but after 2–4 hr the expected increase in ^{14}C incorporation is clearly apparent. The overall data also suggest that the hormonal effect is on the rate of synthesis, rather than on the turnover half-life, of the RNA. The *plus* we used is in nearly all respects less "responsive" than the *minus*, and this is correspondingly true for the RNA response, but though the effect in the *plus* is far smaller it is quite distinct and has the same time-course. Half-maximum responses are obtained at 5 mg/l or less, of trisporate C.

This burst of RNA synthesis is clearly consistent with the idea that trisporic acids cause the transcription of genes hitherto wholly or largely repressed, and the effect is of course similar to a wide range of better-documented effects of hormones from animal systems—but reassuringly so. Let us pursue the analogy.

Some vertebrate hormones affect gene transcription rather directly. Others are not themselves taken into the nuclear apparatus but act indirectly, and many of these act through the "second messenger"—cyclic adenosine 3′,5′-monophosphate (cAMP). Figure 4 shows some of our measurements of cAMP levels in the mycelium of adjacent *plus* and *minus* colonies of *Mucor mucedo*, using the competetive binding assay (Boehringer).

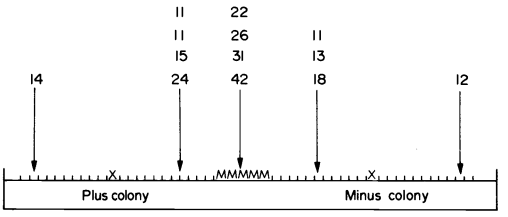


Fig. 4. cAMP levels (picomoles/mg protein) in mating colonies of *M. mucedo*. Strips of mycelium were removed from the locations indicated (see text); the original inocula were at X, X.

The colonies were grown on "Cellophane" overlays to facilitate manipulation. For the assays, *ca.* 5 cm^2 of mycelium was ground under liquid N_2, and extracted with $HClO_4$ in aq. EtOH; the perchlorate was precipitated with KOH and the freeze-dried supernatant used for the assay. Results are referred to the mycelial protein content estimated by the Lowry method.

Values for the normal colony "front" (extreme L and R of Fig. 4) are similar to those immediately behind the zone where the colonies meet (centre L and R), but in the central zone, where significant numbers of sexual structures in all stages of development are also found, they are 2–3 times higher.

We have also shown that similarly elevated cAMP levels can be induced directly by physiological levels of trisporic acids placed in front of single colonies, as shown in Table 7. The addition leads to a "burst" of cAMP production with a time-scale remarkably coincident with that for RNA synthesis in *B.trispora* (compare Table 6): in this case, too, we were using a relatively weakly-responding *plus* strain and the figures for cAMP levels show a correspondingly smaller, but qualitatively quite similar, effect.

Whether the effects of trisporic acids are directly upon a cAMP-mediated system, or only indirect, or indeed whether some of the effects are cAMP-mediated and others not, are some of the many problems which remain for future work on this topic, which has already carried our own work well beyond the limits of the carotenoid studies with which we began. We need to investigate special aspects of the prohormone system in other species, to study homothallic species from the chemical viewpoint, to investigate the problems of permeability and of hormone receptor sites, to learn much more about the role of cAMP and similar regulators in the mycelium, and to study some of the specific chemical and macromolecular features of the sexually-differentiated structures. Some progress in these matters has already been made; for example, we have experimental data showing that added *N,N*-dibutyryl-cAMP at quite low concentrations will stop vegetative extension-growth of *M.mucedo*, and at higher levels it promotes both septum formation, abnormal branching, and marked carotene accumulation in the swollen or segmented hyphae. Other preliminary studies in our laboratory have shown the appearance of specific lectin-binding wall components in the early stages of zygospore formation, and suggest that such steps may be more widely open to investigation. However, these are truly preliminary studies, and we can only anticipate how far these problems will yield to the molecular approach.

Table 7. Effect of trisporic acid on cAMP levels in *M.mucedo*

hr from TA addition	0	2	4	6	8
cAMP in *minus*	2	13	90	14	12
cAMP in *plus*	3	9	18	2	

The trisporic acid (50 μg) was distributed along a 5-cm strip in advance of the colony front at o hr; cAMP levels (see text) are in picomoles/mg protein.

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