SMOOTH MUSCLE CONTRACTOR ACTIVITY OF DUST AQUEOUS EXTRACT *IN VITRO* IV - HEAT TREATMENT UNDER MILD CONDITIONS REDUCES ITS RAT FUNDUS CONTRACTOR ACTIVITY; IMPLICATIONS FOR DETOXIFICATION OF RAW COTTON

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Abstract

On the basis of reduction in endotoxin content, a number of studies have suggested that cotton dust may be "detoxified" through heat treatment (typically, 252°C,20 sec.). Attempts to separate those components of cotton dust aqueous extract (CDE) responsible for the contraction of the rat stomach fundus (RF) by thin layer chromatography (TLC), prompted an investigation into the susceptibility of these components to autoxidation. In freeze-dried form, 80% of the RF activity was lost at 100°C (1hr.) and 100% at 120°C. However, boiling solutions of CDE did not result in significant loss of potency, suggesting that the RF contractor agent(s) are more heat-stable in solution. Whist stable in freeze-dried form, in the presence of air or oxygen in solution, at least the major RF contractor agent is prone to autoxidation at room temperature. Aeration of the aqueous solution resulted in loss of potency. Following application to TLC plates, the RF activity of CDE was lost even though the maximum temperature used in the procedure was 60°C (in the "rotavap" drying stage). Silica and alumina gels are known to promote autoxidation, providing further evidence of the susceptibility of these components. Some parallels have been demonstrated between fractions of CDE active on some smooth muscle tissues and acute *in vivo* responses in guinea pigs. Further investigation may reveal the possibility of a measure of "detoxification" through mild treatments without significant damage to fibre quality.

Introduction to Part IV

In parts I, II and III of this series of reports it was shown that, *in vitro*, depending on the preparation (in terms of both tissue and preparation technique), cotton dust aqueous extract (CDE) can elicit pharmacological responses via mechanisms, directly or indirectly, involving 5-HT receptors, histamine receptors, and eicosanoids. In the isolated rat fundus (RF) the response was found to consist of at least a two-component mechanism.

A number of attempts were made to separate the chemical components in CDE by various chromatographic methods, including TLC. In the latter case, following elution with pure 1,4-dioxan on a silica gel plate and the extraction of the eluted sections with water, only one of the sections exhibited RF contractor activity. This was much lower than an equivalent concentration of CDE. All attempts at further separation led to total loss of activity. These results prompted the stability studies described here.

Methods and Results

The left ventral quartile of the rat stomach fundus was prepared and the responses were measured isotonically, as described in part I of this series of reports.

A) Volatility and Dry-State Stability

In our preliminary studies, a stream of air was passed through open tubes containing freeze-dried CDE (50mg, 24hrs). There was no reduction in the rat fundus contractor potency.

B) Thermal Stability (fig. 1)

Two Sets of four ignition tubes, one heat-sealed and the other open to the atmosphere, both containing dry CDE (100mg), were heated (120°C) in an oven (1 hr). The contents of both sets of tubes darkened in colour. All of the rat fundus activity was lost in the tubes (0(± 0)%). Therefore, the contractile agents are unstable at 120°C and the result cannot be attributed to volatility.

In a similar experiment, using polypropylene centrifuge tubes in a boiling water bath, both sets of samples were again discoloured. Most of the rat fundus potency was lost in both sets (20(± 6)% of control). Since the dose-response curve is approximately logarithmic, if there were to be only a single rat fundus contractor agent in CDE, then such a drop in potency would result from a 90-95% loss of active agent. However, it is possible that some active components are stable at 100°C.

Boiling solutions of CDE (10 min) did not result in significant reduction in potency (91(± 5)%, of control, $p>0.05$) (fig. 1). This suggests that the active agents may be more heat-stable in solution.

C) Susceptibility to Autoxidation

Experiment 1

Sets of four test tubes containing solutions of CDE (10mg/ml,0.2ml) were allowed to stand open to the atmosphere for 1,2, & 3 days. Each was diluted to produce CDE-equivalent concentrations of 5 and 15 μg/ml in the bath.

The responses were compared with a control CDE dose (5 g/ml). After one day, there was a significant reduction in response (88(±1.5)%), this being equivalent to a greater than 10-fold drop in potency (fig. 2).

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After a further, approximately 2-fold drop in response after 48 hours, a plateau appeared to have been reached; no further reduction in response being observed on the third day. Experiments 2, 3 and 4 confirmed that this result was not due to bacterial growth (see below).

Experiment 2

A set of four test tubes containing solutions of CDE (10mg/ml,2ml) were allowed to stand open to the atmosphere for 1 day. Each was tested on the rat fundus. There was no significant change in response relative to control (95.3(± 5.6)%) (fig. 1). The only difference between this and the previous experiment was the surface/volume ratio of the samples. Therefore, this must be the factor contributing to the loss of potency in the previous experiment. Growth of micro-organisms would not be expected to be affected so greatly by surface/volume ratio, whereas autoxidation, on the other hand, would. The following experiment aims to support this.

Experiment 3

This experiment was carried out in order to confirm that the results of autoxidation experiment 1 were not due to free enzymatic processes or endogenous micro-organisms.

CDE (10mg/ml,3ml) was boiled for ten minutes. Aliquots (0.2ml) were transferred to 3 sets of 4 test tubes. These were allowed to stand for 1,2, & 3 days respectively and then tested on the rat fundus.

The results for this experiment were not significantly different from those of autoxidation experiment 1, with a reduction in response to 16(±4)% of control after 24 hours and 7(±5)% of control after 72 hour (fig. 2).

These findings indicate that the loss of potency was not due to any endogenous substance that was susceptible to boiling.

Experiment 4

Solutions of CDE (10mg/ml,2ml) were transferred to two sets test tubes. One group was aerated with 5% CO2 in oxygen (1hr) at room temperature.

There was a 39.5(±7)% drop in response, equivalent to an approximate 3-fold loss in active material (fig. 2), confirming that, in aqueous solution, free oxygen is responsible for loss of at least two-thirds of the rat fundus contractor potency. (compare with dry CDE; part (A), above)

Summary of Conclusions

In the freeze-dried state, the rat fundus contractor agents in CDE are neither volatile nor readily susceptible to autoxidation.

When freeze-dried CDE was heated to 100°C for one hour, a five-fold drop in potency was observed. It is probable that much of the remaining activity was due to a second, more heat-stable RF-contractor agent.

When dry CDE was heated to 120°C for one hour, the residual rat fundus contractror activity was abolished. Given that chemical [1] and pharmacological [2] evidence suggests that endogenous 5-HT may be the RF spasmogenic agent in CDE, it is interesting to note that pure 5-HT has a melting point of 167-168°C [3]. The creatinine sulphate salt has a melting point and decomposition temperature of 216-219°C [3].

The potency was not significantly affected by boiling for a relatively short period (10 min). This suggests that the active agents may be more heat-stable in solution than in freeze-dried form.

On standing at room temperature, in solution, only when the surface/volume ratio is high, RF spasmogenic potency was reduced by more than one log unit after 24 hours. This finding, along with aeration studies and experience with TLC as well as other experiments indicate that at least the major component of the rat fundus contractor agents in CDE is susceptible to autoxidation at room temperature, in solution.

Discussion

Similarities between the acute byssinotic reaction and *in vitro* responses in various smooth muscle tissues have been observed. Some of these have been mentioned in the introduction to part I of this series of reports. Some recent studies have been directed towards detoxification of raw cotton by heat treatment, **aimed specifically at the reduction in endotoxin content of raw cotton**. In these studies, it was found that, for significant reductions in endotoxin content, temperatures of over 180°C were required; the higher the temperatures, the shorter were the required heat treatment times. Typically, 250-260°C for 19-20 seconds resulted in a one-log reduction in endotoxin content [4]. However, these methods result in an unacceptable reduction in cotton quality.

As well as these practical problems, some doubts have been expressed regarding whether or not endotoxins are as important in the overall syndrome (known as byssinosis), as the recent attention paid to them suggests. For example, the 'Buck' fraction produced a bronchoconstrictor response [in guinea pigs, *in vivo*] significantly greater than the response to CDE at one hour and was of similar magnitude to the response at two hours even though the endotoxin concentration was 200 times lower. This suggests that endotoxins may not be the primary mediators of the acute bronchoconstriction [5]. Moreover, in a study which demonstrated CDE-induced activation of the alternative complement pathway, the primary involvement of

endotoxins was rejected [6]. This latter view was supported by a study which showed both classical and alternative pathway activation of complement in operation [7]. More recently, no effect was found of endotoxin either as a single exposure nor as an adjuvent to the dust extract on exposed cell cultures [8]. Also, heat treatment enhances the cytotoxic potential of [alkali-treated] cotton dust and endotoxin alone cannot be the only cytotoxic agent [8]. In addition, a study of endotoxin levels in farming has reported an "absence of symptoms despite high exposure levels" [9]. Furthermore, an absence of a relationship between organic dust toxic syndrome and very high levels of endotoxin in airborne dust has been described. [10]

In addition to the above citations, other issues caution against oversimplification. Optimism in the role of endotoxins was sparked by reports that good correlations are observed between airborne endotoxin levels and byssinotic symptoms in cotton mills. However, this cannot be considered to be conclusive evidence of a direct functional relationship. In this case, good correlations are also reported between endotoxin content of mill air and the cotton dust content of the air. In fact, the endotoxin content of the mill air has been reported in EU/m^3, inferring a direct association between the endotoxin content and dust levels in mill air. [11,12] Also, not surprisingly, good correlations have been found between the level of cotton dust in mill air and the prevalence of byssinosis. Clearly, given that endotoxin levels correlate well with dust levels and that dust levels correlate well with the incidence of byssinosis, it follows that endotoxin levels would correlate well with the incidence of byssinosis; **as would any other substance that correlates well with dust levels!** This would include endogenous plant constituents as well as most exogenous materials directly associated with cotton processing.

Another problem stems from the non-homogeneity of endotoxins. The commonly used assay methods do not appear to distinguish between different types of endotoxins, yet reports indicate marked chemical and pharmacological variability amongst different bacterial species [13,14].

With these points in mind, let us return to the problem of byssinosis prevention.

There is a general concensus that byssinosis is a syndrome consisting of three phases: (1) acute response (not exclusive to byssinosis), (2) gradual reduction in acute response throughout the working week, the Monday-worst pattern (the main characteristic feature of byssinosis) and (3) chronic reduction in lung function leading to long term airway obstruction (still a contentious issue, it is not exclusive to byssinosis, although histological studies have revealed distinguishing features). It is not unlikely for the phase 3 stage to be a consequence of repeated phase 1 and 2 reactions. Thus, rather than attempting to find the direct cause of the phase 3 response, the answer may be in eliminating the acute response and the discovery of the mechanism of 're-sensitisation' following a short period of absence from the workplace.

As, mentioned above, there is evidence to suggest that the acute response is not a direct function of the endotoxin levels of the dust. Elsewhere in this series of reports (parts I-III and V), evidence has been cited to suggest that, as far as the acute response is concerned, many *in vitro* studies support the *in vivo* evidence. The findings in the present report indicate that heat treatment of raw cotton at temperatures that eliminate or reduce its *in vitro* rat fundus contractor activity (typically 100-120°C) is worthy of consideration given that it may lead to significant decreases in the acute response without appreciable reduction in fibre quality.

On the basis of evidence presented elsewhere (parts II and III), similar experiments with the isotonic guinea pig trachea would be appropriate to discover the extent by which lower temperature heat treatments would affect the mediator release mechanisms observed in that preparation.

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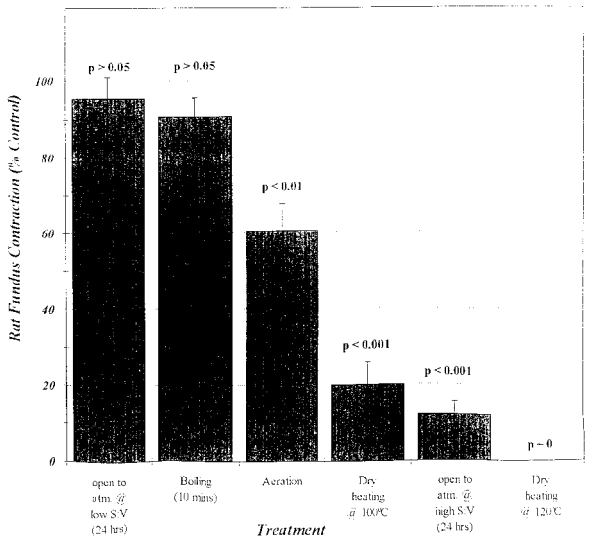

Figure 1. Experiments to Determine the Susceptibility of the Rat Fundus Spasmogenic Agents in CDE to Autoxidation (in solution at room tempertaure) and Heat (in freeze-dried form).

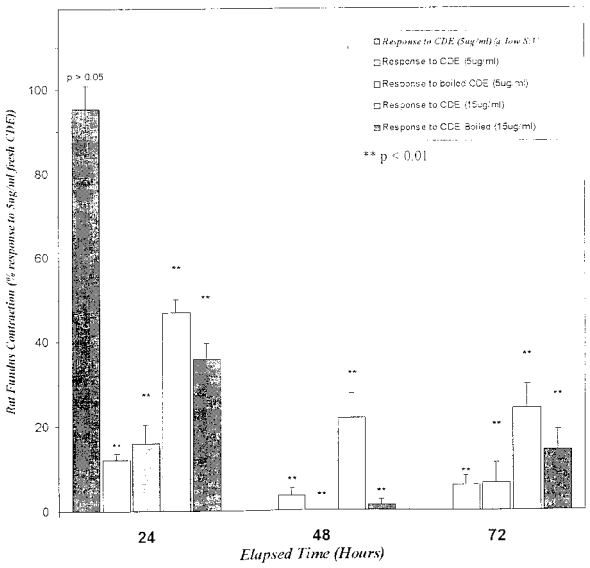

Figure 2. The Effect of Allowing a Small Volume (0.2ml) of CDE Solution (with a high surface to volume ratio) to Stand Open to the Atmosphere for 1, 2 and 3 Days on Its Rat Fundus Contractor Potency.