SMOOTH MUSCLE CONTRACTOR ACTIVITY OF COTTON DUST AQUEOUS EXTRACT *IN VITRO*. V - EFFECTS OF SOLVENT AND CHEMICAL TREATMENTS ON THE RESPONSES OF THE ISOLATED RAT STOMACH FUNDUS.

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Abstract

Studies of the pharmacological activity of cotton dust aqueous extract (CDE), following various solvent and chemical treatments, on the rat stomach fundus (RF) strip revealed that there is more than one RF-contractor agent present. Solvent extraction studies confirm that there is a highly hydrophillic rat fundus contractor agent which is not soluble in absolute ethanol, ether or dry 1,4-dioxan. The major contractor agent was shown to be susceptible to base hydrolysis whilst being relatively stable to boiling in aqueous solution for short periods and to acid hydrolysis. The RF activity of CDE was also found to be stable to iodine and to catalytic low pressure hydrogenation. The major part of the activity could be abolished by selective oxidation of 1,2-diols**.**

Introduction to Part V

V - Effects of Solvent and Chemical Treatments on the Responses of the Isolated Rat Stomach Fundus.

A number of mechanisms has been proposed to explain the bronchoconstriction seen in the acute byssinotic reaction. In addition to the proposed immunological and inflammatory mechanisms, including cell recruitment, hypersensitivity reactions and complement activation, *in vitro* studies have provided evidence for the existence of both direct and indirect smooth muscle contractor agents. Earlier in this series of reports, evidence was presented to suggest some parallels between such *in vitro* studies and *in vivo* findings. Also, our experiments have suggested that there is at least one major and one minor rat fundus contractor agent in CDE and that these are distinct from the agent(s) responsible for the observed contractions in the isotonically measured isolated guinea pig trachea.

Attempts to elucidate the physicochemical nature of the rat stomach fundus spamogenic agnts are presented in this report.

Methods and Results

Unless otherwise stated, the sources of materials were as in Table 1, Part I, of this series of reports.

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A) Thin Layer Chromatography (TLC)

TLC plates (10 x 20 cm) were prepared using fluorescent silica gel 7731 (BDH). Eluted plates were developed in an iodine chamber (5min) before observation under UV light (366 & 254nm). Some substances fluoresced above the background level and others quenched the fluorescence of the plates. This method was used for revealing the degree of separation obtained and identified the most suitable solvent system. CDE and its fractions were separated well with 100% dioxan.

Other plates, prepared in parallel, were not treated with iodine, but used in screening for rat fundus activity. Some of the results obtained are shown in figures 1 and 2.

In earlier experiments we had found that a 95% aqueous dioxan extract of CDE (the S3 fraction), with a mass of around 12% of whole CDE, had a rat fundus contractor potency which was not significantly different from whole CDE (see Part III of this series of reports) This fraction was re-suspended in water to produce a CDE-equivalent concentration of 1g/ml. An aliquot (0.1ml) of this solution was eluted to a solvent front of 14cm. On the basis of fluorescence and fluorescence quenching, 4 major components were identified in the top 10cm of the plate. These were too closely located to be resolvable preparatively. The top (10cm) section (labelled TLCT) was scraped off, extracted with water, and screened for rat fundus activity at a CDE-equivalent concentration of 100μg/ml (1 log scale above the ED_{50} of fresh CDE). The bottom (4cm) section of the plate (TLCB) was similarly treated and screened.

Consistently, TLCT was found to contain the rat fundus contractor agent, although this was less than that of CDE or S3. TLCB was devoid of any activity.

An attempt was made to separate the components of TLCT by solvent extraction. Ether (4ml) was added to 95% aqueous dioxan (4ml) containing S3 (CDE,200mg). The mixture was vortexed and centrifuged (3000xg,15-20min). The supernatant, ES, and the precipitate, EP, were separated, dried in a rotary film evaporator at 80°C, reconstituted in water, and eluted on a TLC plate with 100% dioxan. On the basis of UV observations, this procedure appeared to successfully extract most of the components present in TLCT from those in TLCB. However, neither fraction elicited a response in the rat fundus.

In another approach, improvements to the resolution of the TLCT components were obtained by reducing the polarity of the mobile phase. Preparatively, TLCT was eluted with 50% n-heptane in dioxan. This also appeared successful as 5 distinct, major, resolved bands were observed under UV (254nm). (Later, on closer examination, this section was found to contain many minor constituents, indicating that there were more fluorescence detectable components than

the four which had originally been observed.) The plates were separated into 9 sections, labelled A to I (figure 2). Each of the nine sections, including the non-eluted band, were extracted with 90% aqueous acetone (10ml), dried in a rotary film evaporator at 60°C and reconstituted in water (1ml, fresh CDE≡100mg/ml). Except for a minimal, but reproducible relaxant effect in section B (n=4), none of the other sections exhibited any rat fundus activity.

These results prompted the autoxidation and thermal stability studies reported in Part IV of this series of reports.

B) Solvent Extraction

Table 1 outlines, qualitatively, the results of the preliminary solvent extraction studies.

The activities of ethanol, acidified and basified ether, and propylene glycol extracts on the rat fundus were studied. Saturated ammonium sulphate was used to see if any of the activity components could be salted out. The supernatant of this latter solution was not tested, since the excessive salt concentrations involved would have affected the tissues.

CDE (100mg) was vortexed with solvent (10ml) and centrifuged (3000xg,15-20mins). The precipitate was dried in a Hirsch funnel under a stream of air. The supernatant was blow dried under reduced pressure.

i) Ethanol (fig. 3)

Fractionation of CDE by extraction with absolute ethanol led to 68(±5)% of the response appearing in the precipitate whilst only 8(±3)% of the original response was found in the supernatant. This suggests that, either a single RF contractor agent is partially soluble in absolute ethanol or, more than one such agent is present, a minor one of which is ethanol soluble.

ii) Acidified and Basified Ether Extracts (fig. 3)

CDE solution (100mg/ml, 10ml), prepared with acid (HCl(aq), 0.1M) or base (NaOH(aq), 0.1M) was vigorously shaken with diethyl ether (10ml) in a separating funnel. The ether layer was decanted and vacuum dried. The precipitate was reconstituted in distilled water. A fresh CDE equivalent dose of 5μg/ml was applied to the rat fundus preparation.

Of the total mass of CDE, 3.4% was extracted into the ether layer when the aqueous solution was acidified and, 2.3% after basification. These elicited responses of 3(±3)% and 4(±2)% of control respectively. These are practically insignificant and show that mild acidic or basic conditions do not increase the relative affinity of the rat fundus contractor agents for ether in favour of aqueous solution. It was interesting to note that the characteristic odour of CDE was extracted into ether after basification of the aqueous solution, indicating that these odours are probably due to amines (the same characteristic odour was found in the S3 aqueous dioxan fraction).

C) Propylene Glycol (fig. 3)

Many monosaccharides are soluble in propylene glycol.

In an exploratory study, the contractor activity of the precipitate of propylene glycol treated CDE did not differ significantly from untreated CDE (93(±4)%).

D) Saturated Ammonium Sulphate Solution (fig. 3)

Precipitate of saturated ammonium sulphate solution only produced a response equivalent to 16(±5%) of control (n=4). This may be due to adsorption or complexation of some of the active compounds onto proteins or other salted out components. It is also possible that the minor rat fundus contractor agent was salted out by the ammonium sulphate. In either case, the level of response was higher than could be attributed to experimental errors.

E) Hydrolysis

i) Acid Hydrolysis

CDE solutions (10mg/ml) were prepared with acid (HCl(aq), 0.5M) and boiled (5 min). The solutions were then cooled, neutralized (NaOH, 4M) and administered to the tissue at a fresh CDE-equivalent concentration of 5μg/ml.

Boiling in acid did not have a significant effect on the rat fundus contractile activity of CDE (79(±13)% of control). However, the result was variable (the standard error is large). Therefore, due to the logarithmic nature of the response, if a minor spasmogenic agent were affected, this may not be detected under the current experimental design. Nevertheless, the results do show that it is unlikely for the major contractor agent to be readily susceptible to acid hydrolysis.

ii) Base Hydrolysis

NaOH solution (4M, 0.08ml) was added to solutions of CDE (10mg/ml,1ml). An immediate change of colour was observed on warming (45-50°C). After 5 minutes, the solution was neutralized with HCl and tested on the fundus at a fresh CDE-concentration equivalent of 5μg/ml.

In a second experiment, the basified solutions were boiled (5 mins) before screening.

Warming in alkali led to a drop in response to 64(±3)% of control, whilst boiling in alkali enhanced the loss of activity (reduced to 5.7(±9)% of control; fig. 4).

These results show that the major component of the rat fundus contractile effect of CDE is resistant to relatively mild acid hydrolysis whilst being more susceptible to mild base hydrolysis.

In relation to this experiment, it may be interesting to note that alkali treatment of cotton dust extracts has been reported to decrease its ability to stimulate pulmonary cells

into producing arachidonic acid metabolites from 29pg/10^6 cells to 3.5pg/10^6 cells [1].

F) Low Pressure Hydrogenation

Most hydrogenations with a Pd/C catalyst are carried out in organic solvents. This is often due to the water-insolubility of the compounds to be reduced. Catalytic reductions with Pd/C can, however, be carried out in aqueous solution [2].

CDE (10mg/ml,50ml) was placed in a round bottomed flask (100ml) and shaken (RTP,8 hrs) under hydrogen in the presence of a palladium and carbon catalyst. The amount of hydrogen used was noted (6.7ml). A control experiment was carried out, in the absence of palladium and hydrogen, to allow for adsorption effects of the carbon.

The response on the rat fundus was equivalent to that expected from 0.01 times the same concentration of fresh CDE. The two samples were, therefore, compared at 100 times the original concentration (fig. 5).

A 500μg/ml dose of H_2/Pd/C treated CDE produced a response equivalent to 91(±8)% of a 5μg/ml dose of fresh CDE. When treated with the same amount of activated charcoal, without Pd or H_2, a 500μg/ml dose of this control produced an equivalent response ($92_{(\pm 9)}$% of a 5μg/ml dose of fresh CDE). Thus, the major rat fundus contractor agent of CDE appears not to be susceptible to low pressure hydrogenation and, therefore, does not seem to contain free (non-conjugated) double bonds. This finding is further supported by the results of the iodine experiments (see below).

G) Treatment with Iodine

Iodine (Molecular weight, 254) has a water solubility of 300μg/ml (25°C). Iodine crystals (250mg) were dissolved in ethanol (10ml) (solubility= 200mg/ml). CDE (50mg) was dissolved in water (4.5ml). Ethanolic iodine solution (0.5ml) was added to the CDE solution dropwise whilst shaking (250μg I_2/$mg_{(CDE)}$, = 1mmol I_2/$g_{(CDE)}$).

Treatment of CDE with iodine (240mg/g) produced a response of $93_{(\pm 7)}$% of control (5μg/ml dose of CDE) (fig. 5). This is not statistically different from control, thus supporting the results of the low pressure hydrogenation experiment.

H) Potssium Permanganate Treatment

In 3 separate experiments, $KMnO_4$ (50,100, & 150mg/$g_{(CDE)}$) was added to CDE (10mg/ml) at room temperature and shaken for one minute. These were tested on the rat fundus.

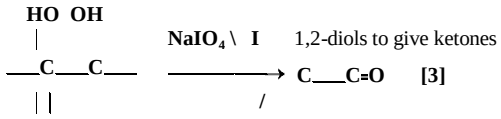
Treatment of CDE with 50, 100, & 150mg/g of $KMnO_4$ reduced CDE-induced responses to $74_{(\pm 2)}$%, $33_{(\pm 3)}$% and $9_{(\pm 2)}$% of a control CDE concentration of 5μg/ml respectively (fig. 6).

A subsequent addition of a dose of 5μg/ml of fresh CDE to the tissues in the presence of $KMnO_4$-treated CDE (150mg/g) led to a cumulative response equivalent to $93_{(\pm 4)}$% of control. This result confirmed that the reduction in response was due to components in CDE being affected by $KMnO_4$ and not the tissue's responsiveness being reduced due to excess $KMnO_4$ or the reaction by-products present.

$KMnO_4$ (1g) was dissolved in acetone (4ml) and NaOH (5ml,0.1M) added. The solution was neutralised with HCl (5ml,0.1M). This solution contained reduced $KMnO_4$ (100mg/ml). This was diluted by a factor of 100 and added (0.15ml) to the physiological bath. Acetone-reduced $KMnO_4$ did not produce any responses in the rat fundus (n=4), indicating that the $KMnO_4$ by-products are not responsible for the observed residual response.

I) Treatment with Sodium Periodate

$NaIO_4$ (molecular weight,243) selectively cleaves:



This reduction takes place readily in cold solution [4]. $NaIO_4$ (25mg) was shaken with CDE (10mg/ml,10ml)(=1mM $NaIO_4$/$g_{(CDE)}$) and heated (40-45°C) for a few seconds. This solution was tested on the fundus.

In a second experiment, 0.3mM $NaIO_4$/$g_{(CDE)}$ was used.

CDE treated with $NaIO_4$ (250mg/g) led to a significant loss of response ($16_{(\pm 5)}$% (n=4) of a 5μg/ml dose-equivalent of CDE), whereas no reduction in response was observed with $NaIO_4$ (85mg/g) ($105_{(\pm 7)}$% of control, n=4) (fig. 7). Since there is a large variety of 1,2-diols (sugars etc.) in CDE, it is conceivable that the oxidations of some of them by $NaIO_4$ are thermodynamically more favourable than the major rat fundus contractor agent. These are, therefore, oxidised first. Any excess $NaIO_4$ would then oxidise the active contractor agent.

Discussion

The TLC results suggest that the rat fundus contractor agent is highly hydrophilic, probably prone to autoxidation at room temperature and that there is a possibility that there is a component that causes relaxation in the rat fundus.

Solvent extraction studies confirm that there is a highly hydrophilic rat fundus contractor agent which is not soluble in absolute ethanol, ether or dry dioxan. These studies, in combination with the dioxan fractionation results (presented in Part III of this series of reports) also suggest that there is at least one minor rat fundus contractor agent

that is soluble in absolute ethanol and acetone but not in ether.

Extraction with saturated ammonium sulphate resulted in a minor, but significant portion of the rat fundus contractor activity to remain in the precipitate. There are a number of possibilities for this result which require further investigation. Adsorption or complexation of some of the major or minor contractor agents onto salted out components may be one explanation. Partial salting out of the major and/or minor contractor agent is another possibility.

At least the major rat fundus contractor agent was shown to be highly susceptible to base hydrolysis whilst being relatively stable to acid hydrolysis. Also, this contractor agent was found not to contain non-conjugated double bonds by virtue of its stability to low pressure hydrogenation and iodine.

The pattern of response of the rat fundus treated with different concentrations of sodium periodate suggests that at least the major component of the CDE-induced rat fundus contractions is due to agent(s) containing 1,2-diols..

Finally, since it is now clear that there is no single "smooth muscle contractor agent" in cotton dust and that there are at least two different rat fundus contractor agents and two different guinea pig trachea contractor agents, all which are likely to invoke different pharmacological mechanisms in their target organs, further *in vitro* studies, with particular emphasis on differentiation of these components may be a prudent step towards breaking down the complex syndrome, byssinosis, into a collection of inter-connected, yet possibly descrete mechanisms.

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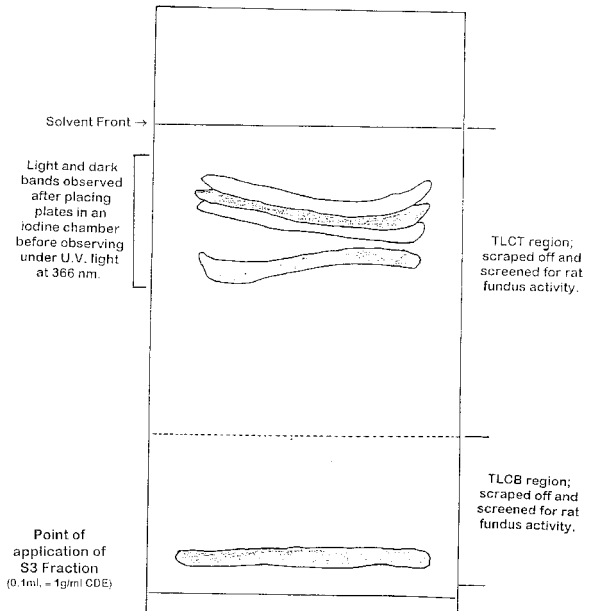

Figure 1. Preparative TLC Plate of the S3 Aqueous Dioxan Fraction [see text] When Eluted with Pure Dioxan.and Observed under UVat 366nm.

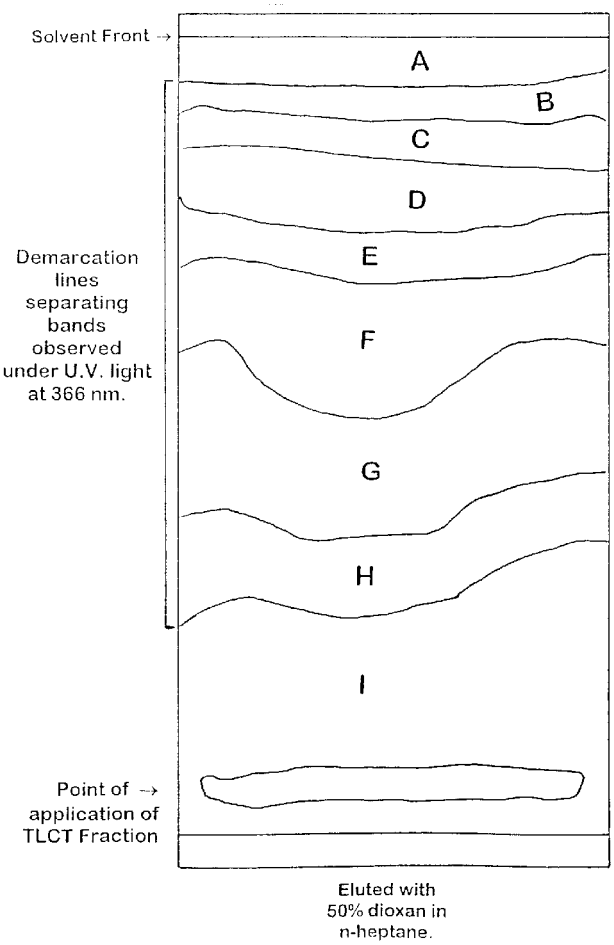

Figure 2. Preparative TLC Plate of the TLCT Fraction [see figure 1] When Eluted with n-Heptane in Dioxan and Observed under UVat 254nm.

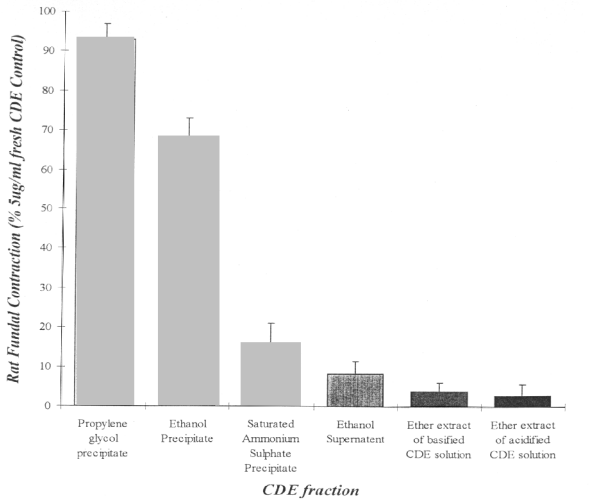

Figure 3. The Effects of Various Solvent Treatments on CDE-induced contractions of the Rat Fundus Relative to a Control CDE concentration of 5μg/ml.

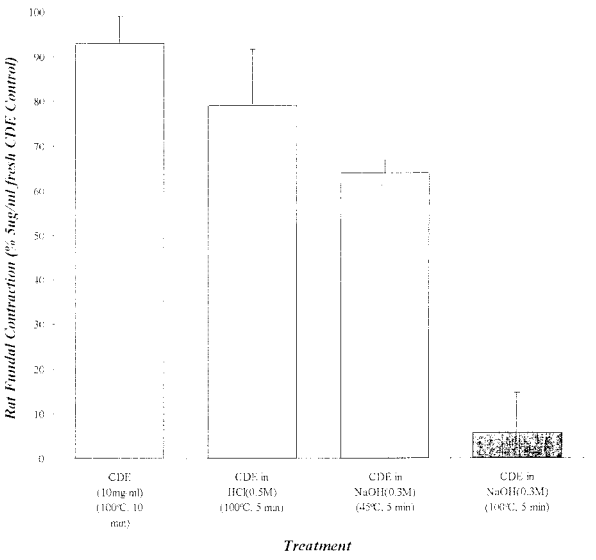

Figure 4. The Effects of Acid and Base hydrolysis on CDE-induced contractions of the Rat Fundus Relative to a Control CDE concentration of 5μg/ml.

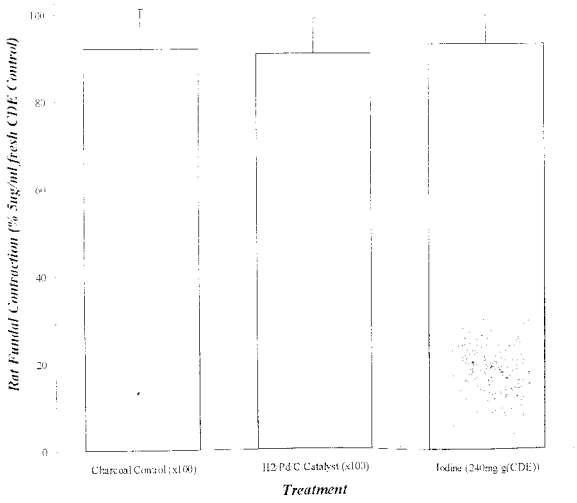

Figure 5. Experiments to Determine Whether or not the Rat Fundus Contractor Agents Are Susceptible to Attack by Treatments that Break Double Bonds.

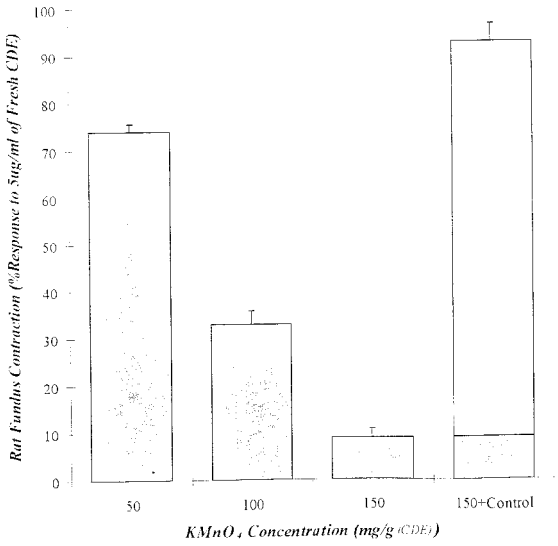

Figure 6. Experiments to Determine Whether or not the Rat Fundus Contractor Agents are Susceptible to Attack by Potassium Permanganate.

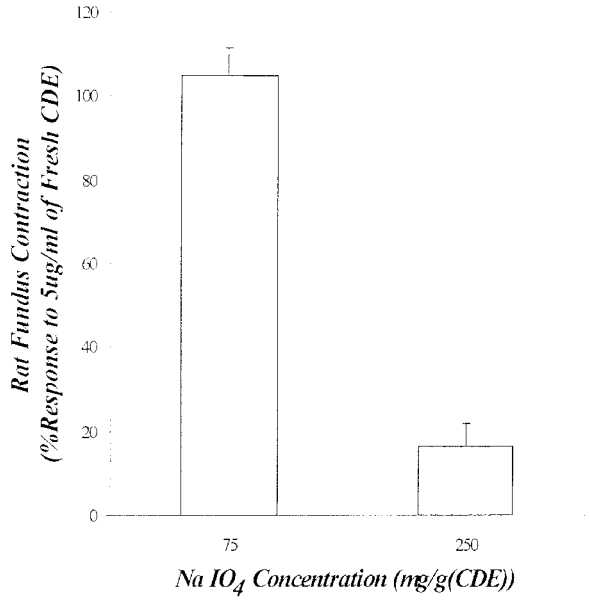

Figure 7. Experiments to Determine Whether or not the Rat Fundus Contractor Agents are Susceptible to Selective Oxidation (of 1,2-Diols) by Sodium Periodate.