X-Ray Diffraction and Infrared Study of the 'Sulphur Granules' of *Actinomyces bovis*

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(Accepted for publication 21 October 1966)

SUMMARY

Bovine actinomycosis exudate was studied to determine the chemical composition and mineral phase(s) of the so-called 'sulphur granules'. The major constituents, CaO and P₂O₅, accounted for 86–89% of the ashed granules. The granules also contained Na, K, Mg (as oxides) and Cl which added up to 10–14%. Although the X-ray diffraction patterns indicated only poorly formed apatite; the infrared spectrum could be interpreted as arising from poorly crystallized apatite and/or a partially dehydrated or hydrolysed octacalcium phosphate either or both mixed with anhydrous dicalcium phosphate. The formation of pyrophosphate (44.5%) in the ignited granules indicated the presence of considerable acid phosphate in the original material.

INTRODUCTION

Because of similarities in the morphology of the organisms and pathological descriptions of actinomycosis (lumpy jaw) in cattle and cervico-facial actinomycosis in man, it was considered for many years that the organisms causing these diseases were the same. Recently, morphological, physiological and biochemical studies (Erikson, 1940; Thompson, 1950; Pine, Howell & Watson, 1960) indicated that the animal disease is caused by *Actinomyces bovis* and the human infections are caused by *A. israelii*, *A. naeslundii* (Thompson & Lovestedt, 1951; Howell, Murphy, Paul & Stephan, 1959) and *Norcardia asteroides* (Burnett & Scherp, 1957).

Although small differences have been found between the animal and human strains, so-called 'sulphur granules' are invariably present in the clinical infection. They can be observed and easily isolated from the draining sinuses; their presence is regarded as a valuable aid in the diagnosis of the disease. The granule is generally a yellowish, hard, spherical body containing a peripheral arrangement of 'clubs' which can be seen upon microscopic examination (Pine *et al.* 1960).

There has been considerable speculation concerning the structure of the granule and the clubs surrounding it. Recent studies (Pine & Overman, 1963) indicated that the granule formed by *Actinomyces bovis* is a mycelial mass cemented together by a polysaccharide+protein complex probably excreted by the organism. The centre of the mass is calcium phosphate according to Pine & Overman (1963), but the mineral phase(s) have not been identified.

Viable *Actinomyces israelii*, *A. naeslundii* and other bacterial species become mineralized, i.e. form apatite when implanted in dialysis bags in the peritoneal cavity of rats (Rizzo, Martin, Scott & Mergenhagen, 1962). The mineral is found intra- and extra-

cellularly, but granules were not observed (Rizzo, Scott & Bladen, 1963). Formation of 'sulphur granules' seems to require the local conditions associated with the chronic infection, because they have been observed only once *in vitro* (Wright, 1905). Our study, therefore, was undertaken to determine the chemical composition and to characterize the mineral phase(s) of the granules.

METHODS

The 'sulphur granules' used for this study were obtained from bovine actinomycosis exudates. Sample 1 (from the National Animal Disease Laboratory, Ames, Iowa) consisted of a pooled exudate fixed in an aqueous solution of 10% (v/v) glycerol and 2% formalin. Sample 2 was removed directly from an infected steer. The identity of the micro-organisms from sample 1 was established by culture and microscopic examination. Sample 2 was considered to be an actinomycosis exudate on the basis of the clinical infection and the morphology of the granules.

Initially, both samples were washed with water at about 40° for 8 hr, centrifuged and dried at 40° in vacuum. To decrease the organic component of the exudate, a portion of sample 1 was ashed by the low-temperature incineration method for ashing micro-organisms described by Thomas (1964). Extraction with organic solvents or strong base was avoided because of the increased possibility of introducing changes in the inorganic phase(s). Portions of the washed and low-temperature incinerated granules from sample 1 were ignited at 400° (50 hr) 500° (24 hr) and 900° (12 hr). Sample 2 was ashed directly at 650° and 900°.

X-ray diffraction and infrared analyses were done on the original water-washed and ashed granules. Diffraction patterns were recorded in a Debye-Scherrer type powder camera (114.59 mm. diameter) using Ni-filtered Cu radiation. Exposure time at 45 kV and 35 mA was 6 hr. Infrared spectra of the samples in KBr pellets were obtained utilizing a double-beam spectrophotometer covering the range 4000–200 cm.⁻¹.

Portions of the water-washed granules were ashed (sample 1 at 500° and sample 2 at 650°) and subsequently analysed for Ca, P, Na, K, Mg, Cl and trace elements. In addition, samples of the low-temperature incinerated portion of sample 1, ignited at 400° and 500°, were analysed for orthophosphate and pyrophosphate. The CO₂ content was estimated from infrared spectra.

RESULTS

Chemical analyses

Chemical analyses of the ashed samples for selected constituents are given in Table 1. The major constituents Ca and P, calculated as the oxides, account for 89% of sample 1 and 86% of sample 2. Except for CO₃, infrared active anions other than ortho- and pyrophosphate were not evident from the infrared spectra of the ashed samples. The Na, K, Mg (as oxides) and Cl percentages totaled accounted for 10% of sample 1 and 14% of sample 2. Approximately 1% of ashed sample 1 was estimated to be carbonate. The Ca:P molar ratios for ashed samples 1 and 2 were 1.45 and 1.21 respectively. The Ca:P molar ratio for sample 1 compares favourably with the 1.40 ratio calculated from the data presented by Pine & Overman (1963). Their ash value was much higher (56.0% compared to 12.0%) apparently caused by the removal of

pus and organic debris by extracting the granules with strong base (Dr L. Pine, personal communication). The low-temperature incinerated granules ignited at 400° and 500° yielded 40·6 and 44·5 % of the phosphorus as pyrophosphate, respectively.

Table 1. *Analyses of ashed sulphur granules*

	Sample 1	Sample 2
Ash	12·0 %	4·0 %
Ca/P molar ratio	1·45	1·21
Pyrophosphate		
(A) 400°	40·6 %	—
(B) 500°	44·5 %	—
Constituent	%	%
CaO	47·50	42·00
P ₂ O ₅	41·49	43·99
Na ₂ O	0·34	0·63
K ₂ O	0·24	0·65
MgO	7·29	9·62
Cl	2·2	3·1
Trace elements*	0·02	0·05
Oxygen equivalent of chlorine	—0·50	—0·70
	98·58	99·34
CO ₂	~1·0	Nil

* Sr, Br, Si, As, Fe, Zn, Cu and Pt summed.

X-ray and infrared analyses of original and low-temperature incinerated granules

The X-ray diffraction patterns of the washed and non-washed granules were characteristic of poorly crystallized apatite. The diffraction lines were weaker and more diffuse than those observed for normal bone (*ASTM publication*, 1960; Carlström & Engström, 1956; Posner, Eanes, Harper & Zipkin, 1963; Frazier, Zipkin & Mills, 1967). The diffraction pattern of the low-temperature incinerated granules remained indistinguishable from poorly crystallized apatite; the primary difference in the diffraction pattern as compared with that of the original was a decrease in background (P. D. Frazier, F. J. Brown, L. S. Rose & B. O. Fowler, in preparation).

Interpretation of the infrared spectra of the original ‘sulphur granules’, with regard to the inorganic phase, was hindered by the organic component (~ 90 %), although poorly defined bands were observed in the proper frequency ranges for apatite.

The infrared spectrum of the inorganic component(s) of the low-temperature incinerated granules consisted of better resolved ν_3 , ν_1 , ν_4 and ν_2 bands of apatite. In addition, the OH stretching band of hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂) was observed at 3570 cm.⁻¹, but was very weak, and the OH librational mode at 631 cm.⁻¹ was barely detectable (Fowler, Moreno & Brown, 1966). A broad shoulder from about 930 to 850 cm.⁻¹ was present. This band could partially arise from anhydrous dicalcium phosphate (CaHPO₄) which has an absorption band at 892 cm.⁻¹ (Fowler *et al.* 1966). Also, octacalcium phosphate (Ca₈H₂(PO₄)₆·5H₂O) has weak absorption bands in this region at 910 and 865 cm.⁻¹ which after partial dehydration, either by heating to 150–200° (Fowler *et al.* 1966) or by hydrolysis (B. O. Fowler, unpublished data) results in a shoulder at about 875–865 cm.⁻¹. In general, the spectrum could be inter-

puted as arising from a poorly crystallized hydroxyapatite and/or a partially dehydrated or hydrolysed octacalcium phosphate, either or both mixed with anhydrous dicalcium phosphate.

X-ray and infrared analyses of ignited granules

The X-ray and infrared patterns of the low-temperature ashed and non-ashed granules ignited at 400° for 50 hr showed poorly crystallized apatite; in addition, the infrared spectrum contained pyrophosphate bands. After continued ignition of the original granules at 400° for 3 weeks, both apatite and γ - $\text{Ca}_2\text{P}_2\text{O}_7$ were detected from the X-ray and infrared patterns. Further ignition of the low-temperature incinerated portion of sample 1 at 500° for 24 hr resulted in the formation of β - $\text{Ca}_2\text{P}_2\text{O}_7$ along with the apatite.

Relative intensities indicated additional X-ray diffraction lines nearly superimposed on the 2.88 and 3.21 Å lines of β - $\text{Ca}_2\text{P}_2\text{O}_7$ and the 2.63 Å line of apatite. These lines could arise from β - $\text{Ca}_3(\text{PO}_4)_2$ or a magnesium substituted β -tricalcium phosphate (Mg content of this sample 4.4 %) which have been shown to form at temperatures as low as 100° in the presence of magnesium (Trautz, Fessenden & Newton, 1954). The portion of ashed sample 1 pre-ignited in the low-temperature incinerator gave an infrared pattern with better resolved pyrophosphate bands as compared with the portion of ashed sample 1 which was not pre-ignited in the low-temperature incinerator. The effects of prior removal of the organic fraction, by this technique, on recrystallization and/or pyrophosphate formation on ignition are at present under investigation.

Sample 2, which was ignited at 650° for 24 hr, formed α - $\text{Ca}_2\text{P}_2\text{O}_7$ and β - $\text{Ca}_3(\text{PO}_4)_2$; the α - $\text{Ca}_2\text{P}_2\text{O}_7$ converted to β - $\text{Ca}_2\text{P}_2\text{O}_7$ after ignition at 900° for 12 hr. The phase transition from β - to α - $\text{Ca}_2\text{P}_2\text{O}_7$ is reported as 1140° (Hill, Faust & Reynolds, 1944). Formation of α - $\text{Ca}_2\text{P}_2\text{O}_7$ below the reported phase transition temperature (1140°) in apatitic calcium phosphates (ignited at 600°) has been reported by Rowles (1964). Sample 1 formed, as expected, β - $\text{Ca}_2\text{P}_2\text{O}_7$ and β - $\text{Ca}_3(\text{PO}_4)_2$ on ignition at 900°.

DISCUSSION

Several investigators have used the formation of pyrophosphate in pyrolysed synthetic calcium phosphates as an index of the presence of acid phosphate in the original material (Gee & Deitz, 1955; Winand, 1961; Herman, Francois & Fabry, 1961; Kühl & Nebergall, 1963; Fowler *et al.* 1966). The pyrophosphate formation in mineralized tissues, upon ignition, has also served as an indication of the presence of acid phosphate in the original material (Herman & Dallemagne, 1961; Francois & Herman, 1961; Dallemagne, 1964).

The low-temperature ashed 'sulphur granules' ignited at 400 and 500° yielded 40.6 and 44.5 % of the phosphorus as pyrophosphate, respectively. These values may not represent maximum conversion because insufficient sample was available to determine the percentages formed with different temperatures and duration of heating. Studies of pyrolyzed synthetic calcium phosphates by Herman *et al.* (1961) and Fowler *et al.* (1966) showed maximum pyrophosphate formation at about 500°.

Probable sources of formation of this pyrophosphate could be CaHPO_4 (Gee & Deitz, 1955), octacalcium phosphate (Brown, Smith, Lehr & Frazier, 1962), and

'tricalcium phosphate hydrate' (Dallemagne, 1964; Herman & Dallemagne, 1961; Francois & Herman, 1961). According to the reactions given by Gee & Deitz (1955) a mixture of hydroxyapatite and CaHPO_4 with the Ca:P molar ratio of our sample (1.45) would be expected to yield 32.3% pyrophosphate; according to Brown *et al.* (1962) a mixture of octacalcium phosphate and hydroxyapatite would also produce 32.3% pyrophosphate.

The percentage of pyrophosphate formed on pyrolysis is well above that predicted on the basis of the two-component model systems listed above; it is also above that predicted assuming, for example, a three-component system containing various proportions of octacalcium phosphate, anhydrous dicalcium phosphate and hydroxyapatite to make a net Ca:P molar ratio of 1.45. However, the actual Ca:P molar ratio of the $\text{CaO} \cdot \text{P}_2\text{O}_5 \cdot \text{H}_2\text{O}$ phases could be less than 1.45 due to the presence of a calcium compound(s) other than phosphate which escaped detection by our methods.

In conclusion, the chemical analyses showed that the inorganic constituents of the 'sulphur granules' examined consisted primarily of calcium phosphate which is in agreement with the work of Pine & Overman (1963). The X-ray and infrared patterns were not sufficiently descriptive to unequivocally identify the calcium phosphate phase(s). The X-ray pattern of the granules indicated poorly crystalized apatite; the infrared spectrum could be interpreted as arising from a poorly crystalized hydroxyapatite and/or a partially dehydrated or hydrolysed octacalcium phosphate, either or both mixed with anhydrous dicalcium phosphate. The formation of 44.5% pyrophosphate in the ignited granules indicated the presence of considerable acid phosphate in the original granules; both octacalcium phosphate and anhydrous dicalcium phosphate are probable sources of the pyrophosphate formed on ignition.

The authors wish to acknowledge Dr A. C. Pier (National Animal Disease Laboratory, Ames, Iowa) for supplying sample 1 of the actinomycosis exudate and thank Dr I. Zipkin (National Institute of Dental Research), Dr W. C. Alford (National Institute of Arthritis and Metabolic Diseases), Mrs B. M. Wallace and Mr H. McDowell (American Dental Association Research Division, National Bureau of Standards) and the Union Carbide Corporation (Oak Ridge, Tennessee) for the chemical analyses.

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