Effective Mummification Compounds Used in Pharaonic Egypt: Reactivity on Bone Alkaline Phosphatase

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In Pharaonic Egypt from the Old Kingdom up to the Ptolemaic Period the deceased were pre-treated in the course of the mummification process using a wealth of embalming components including resins and many different wood tars. GC/MS studies allowed the elucidation of a great number of clearly separated compounds found in the ancient embalming material. Phenols, guaiacols, naphthalenes, monoterpenes, sesquiterpenoids, oxidised diterpene resin acids and triterpenoids were noticed. These results and particularly the detection of an unused embalming material shed some new light on the possible way as to how the different embalming materials might have been prepared and applied. It was striking to see the accordance of the present data with the well-known treatises by Herodotus (490–425 B. C.) and by Pliny the Elder (23/24–79 A. C.). The impact of the historical observations on modern science and in return the dramatic promotion of ancient texts stimulated by the present study is intriguing.

An enzyme, alkaline phosphatase, bound inside mummified bones was a useful tool to reveal the efficacy of the embalming materials. Initial data showed that alkaline phosphatase isolated from embalmed bones from the Old Kingdom and the Ptolemaic Period was significantly more abundant and displayed a higher activity compared to the properties of the corresponding enzyme from non-treated mummified bones.

Additionally, in a model study porcine ribs were pre-treated with four selected embalming compounds – guaiacol, limonene, α-pinene and *p*-cymene – and subsequently air-dried. Among the four selected compounds guaiacol was the most reactive species in the course of the preservation process. The specific activity of bone alkaline phosphatase rose 12-fold compared to that of the control. The enzyme itself remained unharmed as the observed relative molecular mass was surprisingly identical with the contemporary enzyme. It was again striking that the guaiacol derivatives were richly abundant in the unused embalming material mentioned above.

Key words: Alkaline Phosphatase, Cedrium, Guaiacol, Mummified Enzyme, Pliny the Elder

Introduction

Eternal life is an old desire of mankind. The ancient Egyptians developed a way of preserving the bodies of their deceased, in the belief that this would ensure their eternal survival. Especially in Pharaonic Egypt dating from the Old Kingdom (2635–2155 B. C.) up to the Ptolemaic Period (332–30 B. C.) this eternalisation process – mainly for higher class persons consisted of three main steps [1–3]: removing the internal organs, desiccating the remains of the deceased and completing the mummification process with many different embalming materials [4].

The exact mode as to how the inner organs were removed and further processed is not dealt with. Of special interest are the two following steps including desiccation and embalming. In order to understand these procedures the reactivity of some selected compounds used in the ancient mummification processes was examined on contemporary biological tissues and the results compared with those obtained from Egyptian mummies.

The richly abundant and stable bone enzyme alkaline phosphatase was the most appropriate marker to study the reactivity of the employed embalming agents. Unlike the many and fast deteriorating enzymes in aqueous solutions this bone

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enzyme is firmly bound to the bone hydroxyl apatite. Under these conditions it survives thousands of years provided there is no microbial attack. Both structural and functional integrity reflect the efficacy of the preceding mummification process.

The desiccation procedure has been extensively studied in the Tübingen laboratory. One of the latest achievements was the finding that borate was present as an inorganic bactericidal compound both in mummification salts and mummified human bones [5]. In the present study the main interest will be focused on organic components used for embalming the deceased.

The course of the mummification process proceeded not always in the same manner. For example, immediately after the removal of the inner organs as well as the intestines and before the desiccation process was started the body was treated with palm wine, perfumes and spices. The genuine conservation *i. e.* the application of ointments, oils, resins and spices followed after this pre-treatment.

Many additional 'cosmetic' steps were occasionally performed. Eyes, ears, nose and the abdominal incision were sometimes sealed with beeswax and bandages soaked with different resins and glues were frequently applied. This explains the wealth of the many different compounds usually found on mummies.

Those compounds exerting a distinct biochemical and bactericidal reactivity were examined in more detail as they were considered to be responsible for the observed efficient conservation of biological tissues. In this respect beeswax, as is frequently found on mummies [6] was deliberately not studied due to its inert behaviour during the mummification process. The only possible conservation effect might be attributed to its hydrophobicity, which means that water is displaced with the consequence that micro-organisms are unable to survive. Again alkaline bone phosphatase was the most appropriate molecular marker for this task [7].

The aim of the present study was not to elucidate comprehensively and efficiently all possible mummification compounds and the techniques employed in the conservation of the deceased throughout all Pharaonic dynasties. Our interest was focused on the reactivity of some selected embalming compounds isolated from a limited number of samples taken from mummies of Pharaonic Egypt and of different age ranging from the Old Kingdom up to the Ptolemaic period. Prior to the biochemical assays they were examined as to which degree microbial contamination was detectable and whether or not embalming had been carried out. For clarity reasons alkaline phosphatase was monitored in perfectly preserved bone tissues only. In the case of the embalmed mummies the embalming compounds were extracted and characterized. The results were discussed in the light of the treatise of Pliny the Elder in his Natural History on forest trees and their products [8].

Out of the many identified preserving agents a highly reactive group of liquid monoterpenes including α-pinene, limonene and *p*-cymene as well as a phenolic derivative, guaiacol (2-methoxyphenol), were selected. The monoterpenes are detectable in the balsamic resins exuding from coniferous trees whereas guaiacol is detectable during the smouldering or fuming processes using wood of these trees. Fresh porcine ribs were pre-treated with each of these compounds. Upon prolonged drying alkaline phosphatase was isolated and characterized in more detail.

Results and Discussion

Effective preservation of mummified bones in the presence of different embalming components

It was of interest to examine whether or not mummification using a wealth of wood tar and/or resin components would result in an optimal preservation of many a biomolecule. Unearthed bone samples from the Ptolemaic Period and the Old Kingdom, some embalmed and some dried, were examined for alkaline phosphatase activity using the established enzyme assays of earlier work [9, 10]. In fact it could be shown that alkaline phosphatase isolated from embalmed bones was remarkably more stable and displayed a higher enzymic activity compared to the properties of the corresponding enzyme from air dried mummified bones (Table 1).

Quite frequently, embalming using organic tars or other liquid resinous materials resulted in a surface being essentially free of contaminating microorganisms. The sealing effect of the embalming agents on the bone surface ascertained both the

Table 1. Comparison of aged, total extracted soluble protein content and alkaline phosphatase activity of Pharaonic bone samples of different burial conditions. For experimental details see experimental section.

Age		Burial condition	Total protein [*µg/g bone*]	Specific activity [*mU/mg protein*]
Ptolemaic	Female Torso[a], rib and pelvis, 340 ± 170 BC	Embalmed	850 ± 50	120 ± 3
	Female Flexed body[b], vertebra, 192 ± 11 BC (inventory # 17668)	Air dried	390 ± 80	43 ± 2
Old Kingdom	Idu II[c], rib, 2150 ± 50 BC (inventory # 2639)	Embalmed	650 ± 10	50 ± 10
	Vertebra[c], 2250 ± 100 BC (inventory # 3052)	Air dried	215 ± 30	0

[a] Staatliches Museum ägyptischer Kunst, München; [b] Ägyptisches Museum Berlin; [c] Roemer- und Pelizaeus Museum Hildesheim.

safe absorption and the high yield of alkaline phosphatase in the deeper regions of bone mineral. The possible stabilization of the enzyme caused by the different components of the organic embalming material was much too interesting a task to loose sight of. Thus, the organic compounds employed for mummification of some selected mummies originating from the Old Kingdom up to the Ptolemaic age were investigated using GC and GC/MS technology in the Munich laboratory.

In this study four examples of embalming materials were extensively dealt with: Two from the Old Kingdom (~ 2300 B. C.), one from the New Kingdom (~ 1500 B. C.) and one from the Ptolemaic Period (~ 300 B. C.).

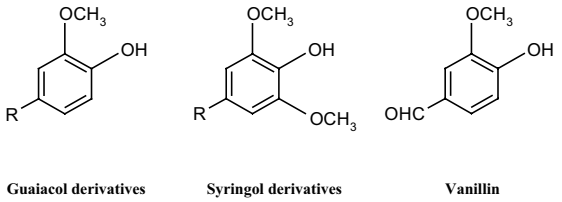
Unused embalming material from the New Kingdom

Due to the great advancements in embalming techniques in the New Kingdom samples from this

Fig. 1. Unused embalming material ('cedrium') entombed together with the mummy *Saankh-kare*, 1500 B. C., cementary field # 26225 at Deir el-Bahari.

period were examined with high priority. However, unlike the usual extraction of embalming resins from mummified tissue [9–11] we were fortunate to obtain a sample of unused embalming material which was entombed together with the mummy 'Saankh-kare', 18th Dynasty, 1500 B. C. cemetery field # 26225 (Metropolitan Museum, New York) at Deir el-Bahari. The brown solid resinous material (Fig. 1) was almost completely dissolved in methanol. The gas chromatogram of the methanolic solution is depicted in two separate chromatograms (Figs. 2 and 3), which contain only liquid and viscous fractions. Surprisingly, no solid fractions could be observed by gas chromatography, *i. e.* no diterpenoid or triterpenoid resin components were detectable.

In Fig. 2 the region of the liquid components and in Fig. 3 the viscous portions are dealt with. There was almost exclusively a mixture of low molecular components. Phenolic components including cresols, xylenols, guaiacols as well as naphthalenes and azulenes were noticed. The phenolic and naphthalene derivatives, most likely, originated from smouldering wood. Of special importance was the recovery of methoxyphenol derivatives (guaiacols, Scheme 1). They are formed when soft coniferous wood is heated. By way of contrast, heating the hard wood of deciduous trees dimethoxyphenols (syringols) are additionally formed [12–15]. The present result, *i. e.* detection of guaiacols without syringols, strongly support the origin of the investigated material to be derived from coniferous wood.



Scheme 1. Structural formulas of (mono-)methoxyphenols (guaiacols), dimethoxyphenols (syringols) and vanillin.

The brown solid resinous material found near the mummy 'Saankh-kare' also contains sesquiterpenoid components normally detected in organic solvent extracted wood from *cedar atlantica* called cedar oil which is composed of junipene, cadalene, cadinatriene, α-curcumene, cuparene etc. Taking into account the former data of the origin of resin-

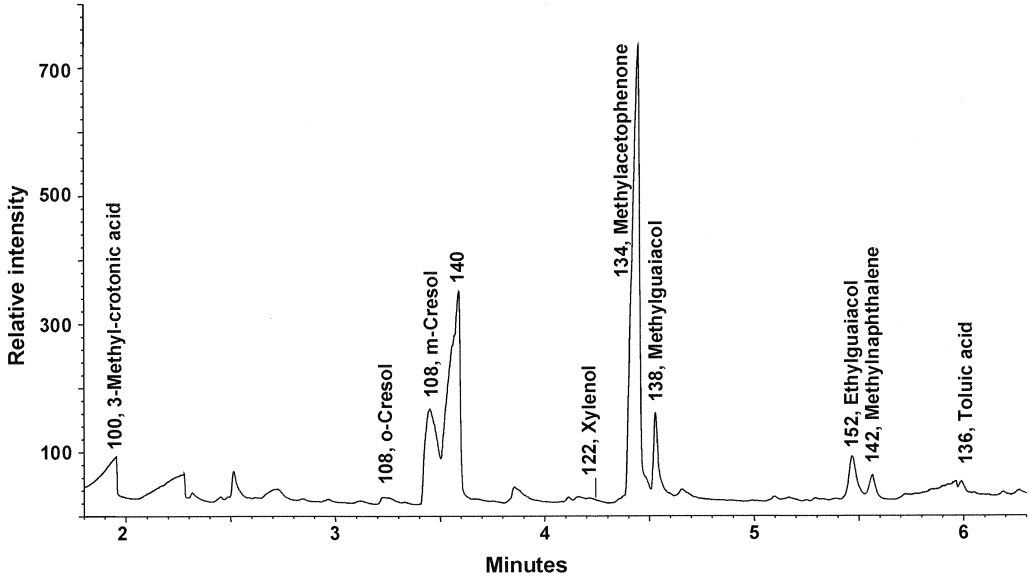


Fig. 2. Gas chromatogram (HP 6890) of the methanolic solution of the unused embalming material. Section containing the liquid fraction.

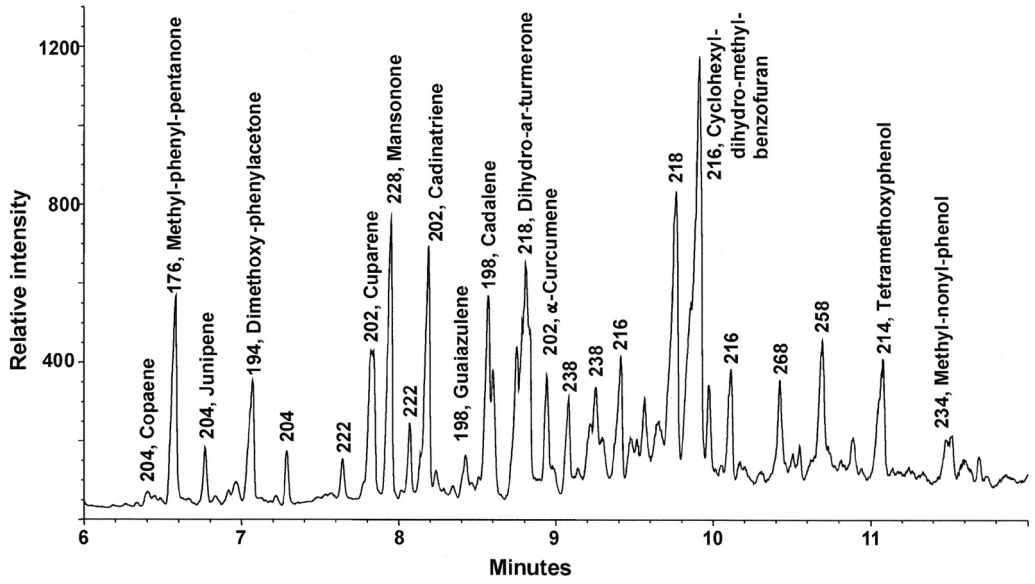


Fig. 3. Gas chromatogram (HP 6890) of the methanolic solution of the unused embalming material. Section containing the viscous fraction.

ous material, in general, attributed to coniferous wood, there was unequivocal evidence that it was produced from a cedar tree. In the 'Natural history' by Pliny the Elder the technology of producing such a product is summarized [[8], XVI, 52]: '*In Europe tar is obtained from the torch-pine by heating it, and is used for coating ships' tackle and many other purposes. The wood of the tree is chopped up and put into ovens and heated by means of a fire packed all round outside. The first liquid that exudes flows like water down a pipe; in Syria this is called "cedar-juice" [Latin: cedrium] [16], and it is so strong that in Egypt it is used for embalming the bodies of the dead*.'

The European 'torch tree' is commonly allocated to the pine tree and the first outflow attributed to pine oil. It might be erroneous to conclude that 'cedrium' produced in Syria might be assigned to some sort of pine oil. This would result in an erratic transposal of (North or West) European conditions into the Syrian territory. Indeed, Pliny the Elder clearly distinguishes between many regionally different trees used in the melting out processes [[8] XVI, 57–59].

Unlike the freely exuding resinous balm from certain trees it has to be emphasized that 'cedrium' is called the liquid tar oil obtained during a melting out or smouldering process of wood from trees enriched with resin. Moreover, it should not be mistaken with cedar oils now obtained by steam distillation or by organic solvent extraction of cedar wood. Particularly some major components found in the unused embalming material like the naphthalenes and phenols formed by a smouldering process when melted out are considered non-specific. They could be attributed to many other coniferous trees. Thus, a direct comparison between the above 'cedrium' and cedar oil shows some differences, *i.e.* additional components deriving from the smouldering process or even missing components. This is not surprising, as genuine cedar oil components contained in the 'cedrium' were subjected to chemical alterations in two different ways. The initial melting out process was followed by an ageing process, which lasted some 3500 years. Originally, 'cedrium' was a viscous but liquid mixture (tar-oil). Eventually, after evaporation of the more volatile portions and partial resinification by building of a high-molecular matrix the remaining residues became solid. This high molecular product is still soluble in alcohols but could not be investigated by GC or GC/

MS. Only the rests of the cedrium-components retained by chance in this solid matrix are analysed by GC and GC/MS and depicted in the Fig. 2 and 3.

Nevertheless, the observed components, particularly the significant portions of guaiacol-derivatives gave strong evidence that there existed a tar oil, which could be used as an embalming solution and eventually as time progressed has dried up.

As a precaution and to avoid erroneous conclusions, another clarification has to be given. In today's terminology different trees that are not cedar are called cedars like the American cedar (*Juniperus virginiana*). Unfortunately, the modern 'oil of cedar' is generally a product of this tree. For this very reason, 'cedrium' should not be compared with modern cedar oils obtained from the American red cedar. It originates from Mediterranean trees like the Lebanon cedar (*Cedrus libani* [17]) or African cedar (*Cedrus atlantica*). In these oils the characteristic components found in American cedar (red cedar) such as cedrol and cedrene are only detectable in small amounts or are even missing.

Cedrol and cedrene are, however, abundant in the Mediterranean juniper trees (*Juniperus oxycedrus*). Juniper berries were often found in tombs of Pharaonic Egypt [17]. They could be detected on the bodies of mummies and even in the hand of a female mummy.

For this very reason Lucas pointed out that "*the 'cedar oil' was probably not from the cedar, but often essential oil of junipers extracted from the berries by soaking them in some ordinary fixed oil,* …" [4]. With this statement Lucas not only ignored the traditional methods used for the production of juniper oils [18], he also ignored that this extract was not a 'strong' one, *i. e.* it has no disinfectant properties. Additionally, the phenolic components identified in our investigations clearly showed that a smouldering product was used as stated by Pliny and not an extract. In an earlier study [19] Lucas even pointed out that the oleaginous wood tar oil called 'cedrium' is mainly a resinous product from juniper tree. Unfortunately, he was at that time not able to realize that for all practical purposes this juniper was not a resin-producing tree [[4] p. 319]! Thus, it is not surprising to realize that, up to now, we could not identify any juniper oils (with high portions of cedrol) or juniper tars (with high portions of cedrene) on Egyptian mummies.

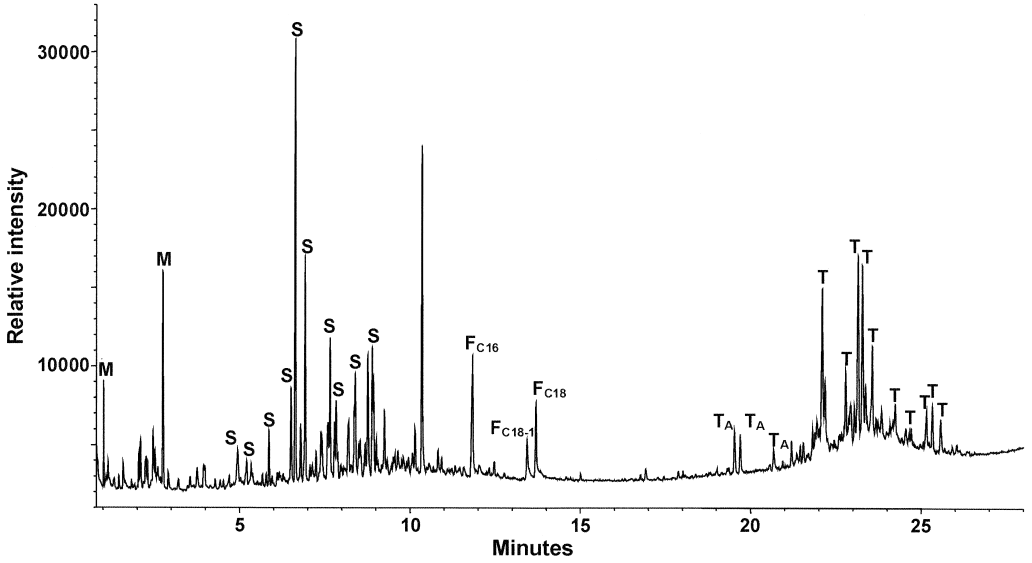


Fig. 4. Gas chromatogram (HP 5890) of the balm materials extracted from mummified skeletal muscle of the Ptolemaic mummy torso (340 ± 170 B. C.). Liquid components appeared during the first 5 min while high viscosity species are eluted up to 10 min. Solid fractions appeared after that retention time. Abbreviations: M = monoterpenes, S = sesquiterpenes, F = fatty acids, T = triterpenoids, T_A = skeletonised triterpenoids.

Embalming resin employed during the Ptolemaic Period

The Ptolemaic embalming resin was successfully extracted from different mummified tissues of a female torso (340 ± 170 BC, estimated by radiocarbon dating [9]), Staatliches Museum Ägyptischer Kunst, München; for experimental details see the experimental section. In the gas chromatogram (Fig. 4) three different chemical classes are detectable.

The liquid portion at retention time (RT) = 1–5 min was followed by a viscous fraction at RT = 5–11 min and a third resinous fraction appeared at RT = 18–28 min. The liquid and viscous fractions are again depicted in two separate chromatograms (Fig. 5 and Fig. 6).

They both show some striking similarities with the embalming solution from the New Kingdom (Figs. 2 and 3). They contain smouldering products, for example, derivatives of phenol, naphthalene and azulene. Some components are lacking and some are additionally present, however, no detectable guaiacol derivatives are found in tissue extracts from Ptolemaic mummies and the number and concentration of naphthalene derivatives are significantly reduced. By way of contrast, constituents of oil of turpentine including α-pinene, limonene and cymene as well as some sesquiterpenoids are additionally found. The observed sesquiterpenoids can be attributed to viscous products prepared from cedar wood. Therefore, we have to consider again a type of cedar wood oil described as 'cedrium' by Pliny the Elder [8].

The resinous portions in Fig. 4 (RT = 18–28 min) consist of pentacyclic triterpenoid components and represent pistachio turpentine. This soft resin exudes freely from the terebinth tree *Pistacia terebinthus* L. [17, 20]. However, a closer look at the resinous portions reveals aromatised and skeletonised pentacyclic products of triterpenoid structure, which belong to a different extraction process. This material was obtained either by melting out the wood of debarked turpentine trees [[8], XVI, 57–59] or by later re-liquefying the above freely exuding pistachio turpentine (= Chios turpentine). Pliny the Elder characterizes such a reliquefied resin 'crapula' [[8], XVI, 54–55]: '*Another mixing process produced "intoxication resin" [Latin: crapula]: raw flower of resin is picked off the tree with a quantity of thin, short chips of the wood, and broken up small in a sieve, and then steeped in*

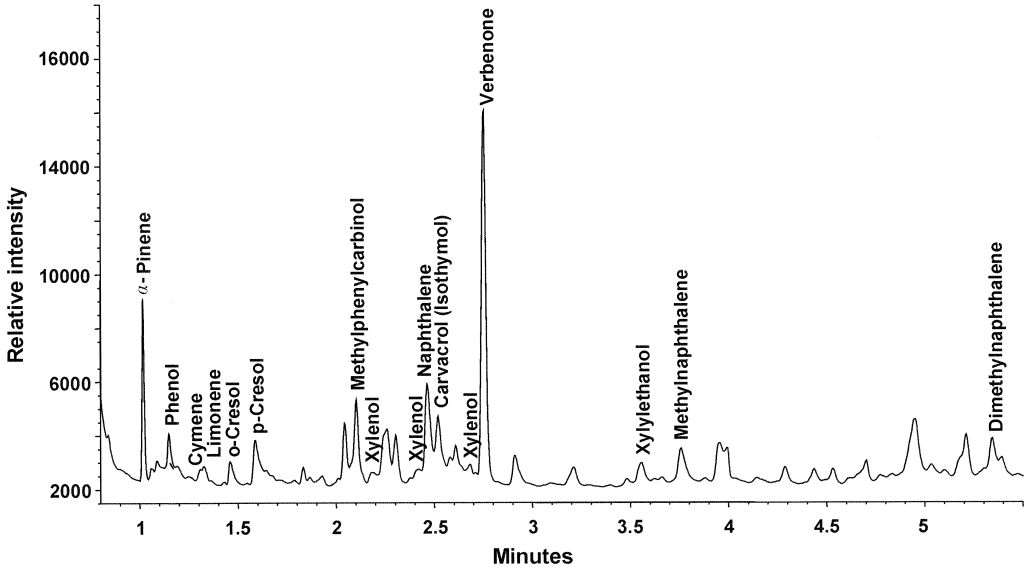


Fig. 5. Gas chromatogram (HP 5890) of the liquid components of the ptolemaic mummy 'tar' (340 ± 170 B. C.). Enlarged section of Fig. 4 mainly containing the monoterpene fraction (essential oils), the aromatic (phenolic) alcohols and the naphthalenes.

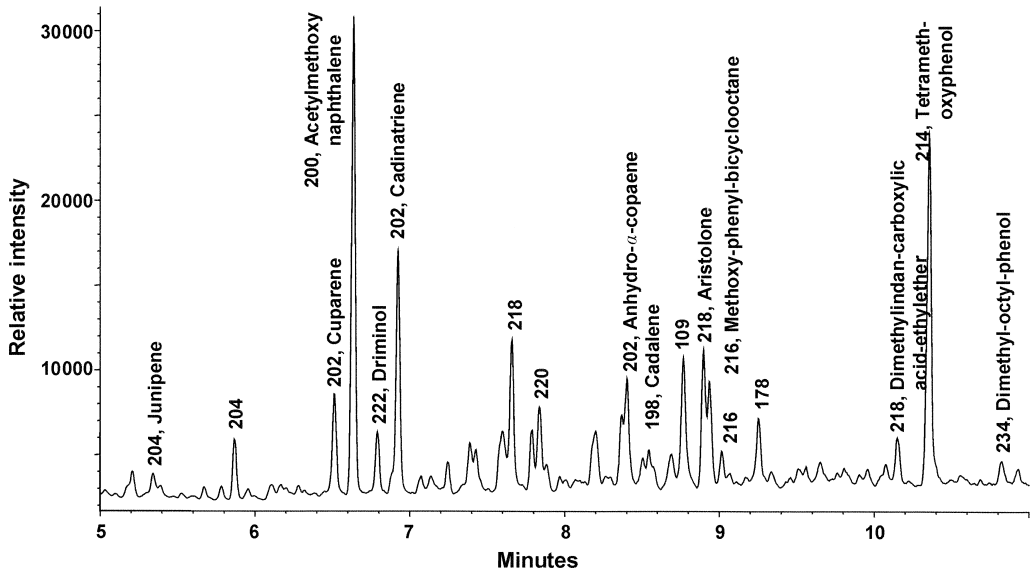


Fig. 6. Gas chromatogram (HP 5890) of the high viscosity species of the ptolemaic mummy 'tar' (340 ± 170 B. C.). Enlarged section of Fig. 4 containing the sesquiterpene fraction.

water heated to boiling. … Others think it pays better to boil it without water over a slow fire for a whole day, and to employ a vessel of white copper, or to boil resin from the turpentine-tree [terebinthinam] in a flat pan on hot ashes, as they prefer this to all the other kinds. The resin of the mastich is rated next.'

Our final conclusion is that the embalming resin (or tar) found in the Ptolemaic mummy consists of two products from different origin: cedrium and 'boiled' pistachio turpentine. Both were applied as liquids but sequentially in different embalming steps.

Resinous embalming materials used in the Old Kingdom

The situation in analysing possible embalming material used in the Old Kingdom was substantially different compared to the studies performed on embalming material of later Pharaonic periods.

Idu II (2150 ± 50 B. C.)

The wealth of wood tar products and sodium ions in the possible defleshed bones of the prominent mummy Idu II (2150 ± 50 B. C., Roemer- und Pelizaeus Museum, Hildesheim) extends the use of embalming already to the Old Kingdom *i. e.* one thousand years earlier than previously thought [11]. Of course, in the case of the mummy of Idu II this conclusion was initially overshadowed by the fact that the mummy was soaked with paraffin immediately after recovery in 1914 [21]. Some results were overlapped in that the two alkanes, pristane (2,6,10,14-tetramethylpentadecane) and phytane (3,7,11,15-tetramethylhexadecane), which are normally present in hydrocarbons of fossil origin (bitumen) became also detectable in the mummy extract. It should be noticed that the employed paraffin likewise was derived from hydrocarbons of fossil origin. Thus, no unequivocal assignment as to where these special alkanes came from was possible.

The detection of components from oil of cedar in the mummy initially led to the erroneous conclusion that this oil was used during the embalming process. Detailed analyses revealed the constancy of its concentration and to the absence of any products normally expected from overheated cedar wood. As the mummy of Idu II was entombed in a coffin of thick-boarded cedar wood it became plausible that some cedar oil diffused into the mummy.

Genuine embalming resins were detectable after sophisticated sample preparation processes. Upon

treatment with chloroform/methanol (7/3, v/v) both paraffin and embalming compounds were extracted in an indistinguishable manner. After evaporation repeated extraction with isooctane became necessary. The residue was dissolved in acidified methanol, *i.e.* a solution of 10% (w/v) anhydrous oxalic acid in methanol and subjected directly to gas chromatography.

The resulting chromatogram (Fig. 7) shows oxidised diterpenoid resin acids and their methyl esters mainly based on dehydroabietic and dehydrodehydroabietic acids. They belong to strongly heated resinous pine wood. A very similar embalming material consisting of oxidised diterpenoid resins was detected in an Egyptian mummy from the Roman period (30 B. C.–4th century A. D.) [22, 23].

The composition of these embalming resins is consistent with the viscous second outflow called 'pitch' which is obtained when resinous pinewood was heated in essentially the same mode as described by Pliny [[8], XVI, 53]: '*The liquor that follows [after the cedrium] is thicker, and now produces pitch; this in its turn is collected in copper cauldrons and thickened by means of vinegar, as making it coagulate, and it has been given the name of Bruttian pitch; it is only useful for casks and similar receptacles, and differs from other pitch by its viscosity and also by its reddish colour and because it is greasier than all the rest. It is made from pitch-resin caused to boil by means of red-hot stones in casks made of strong oak, or, if casks are not available, by piling up a heap of billets, as in the process of making charcoal.*'

The described process is significantly different from the ancient process of wood charring described by Theophrast [24] and from that known in Northern Europe [25, 26]. In a pile resinous wood is directly exposed to a slow fire (exothermic process) and under limited access of air until wood tar is being generated (smouldering process). In the Pliny process above resinous wood is not directly exposed to a slow fire it is rather strongly heated indirectly, in an allothermic process similar to that of a dry distillation in retorts. As a consequence the resulting product is significantly different from a kiln burned tar. Wood tar from the kiln process contains characteristically high concentrations of skeletonized, *i.e.* dehydrogenated, dealkylated and decarboxylated products includ-

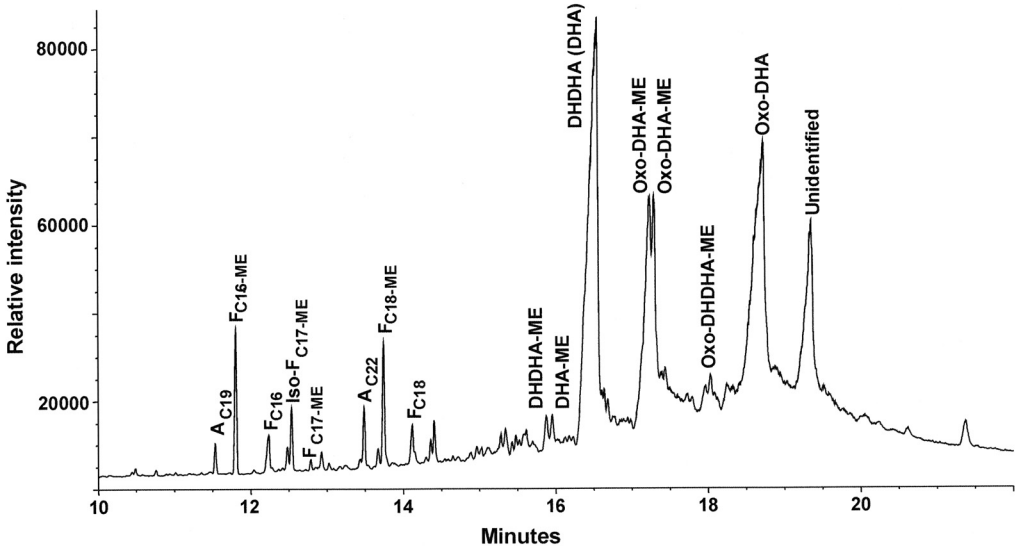


Fig. 7. Gas chromatogram (HP 5890) of the balm material extracted of the mummy of Idu II. Abbreviations: A_{C19} = nonadecane, $F_{C16\text{-}ME}$ = palmitic acid methylester, F_{C16} = palmitic acid, iso-$F_{C17\text{-}ME}$ = iso-heptadecanoic acid methylester, $F_{C17\text{-}Me}$ = heptadecanoic acid methylester, A_{C22} = docosane, $F_{C18\text{-}ME}$ = stearic acid methylester, F_{C18} = stearic acid, DHDHA-ME = dehydrodehydroabietic acid methylester, DHA-ME = dehydroabietic acid methylester, (DHA) = dehydroabietic acid (small amounts), DHDHA = dehydrodehydroabietic acid, oxo-DHA-ME = 7-oxo-dehydroabietic acid methylester (2 isomers), oxo-DHDHA-ME = 7-oxo-dehydrodehydroabietic acid methylester, oxo-DHA = 7-oxo-dehydroabietic acid.

ing retene, tetrahydroretene, abietatriene or norabietatriene [27]. They are completely absent and/or occasionally found only in traces in a process like the second outflow described by Pliny the Elder (Bruttian pitch). Liquid pitch obtained after indirectly heating the resinous wood contains isomerised and dehydrated products including dehydroabietic acid, dehydrodehydroabietic acid (abietatetraenoic acid) and oxidised products like 7-oxo-dehydroabietic acid formed in the presence of some air. These oxidised products would not be stable in an exothermic process.

Furthermore, liquid products appearing in the first outflow, which may be related to different type of 'cedrium', *i.e.* a tar oil not derived from cedar wood, are detectable. The major abundant components include monoterpenes, phenols and azulenes (*i.e.* guaiol). It is attractive to suggest their use in initial embalming (= applying of cedrium) or concomitant with generated pitch. Unfortunately they appear in the same region as the paraffin constituents and are indistinguishable to allow the assignment of the ongoing embalming steps.

Bone fragments from the Junker excavation at Giza (Old Kingdom)

For comparison a second mummy from the Old Kingdom was investigated. Bone fragments, now deposited in the Naturhistorisches Museum at Vienna, Department of Anthropology, were extracted and subjected to GC and GC/MS analyses.

Unfortunately, the bones have been found to be soaked with modern materials used for conservation. A lot of fungicides, mainly consisting of hexachlorobenzene, could be observed. Fortunately, subsequent analysis for possible ancient embalming components were not strongly hampered by these modern products.

Three main groups could be detected: lipids, hydrocarbons and diterpenoids. Beside this, some low molecular materials were identified containing vanillin (4-hydroxy-3-methoxybenzaldehyde) a compound that is very similar to the guaiacols (see Scheme 1) and produced during the same process [28].

The lipid group consisted of a wealth of fatty acids and some oxidised sterols (*e.g.* cholestadienone). The whole material belongs to the organic products naturally contained in human bones.

The second group consists of alkanes and polycyclic aromatic hydrocarbons. Pristane and phytane were again detected together with the alkanes. There is strong evidence that a bituminous material was applied on the surface of the mummy and parts of it have migrated into the bones. However, unlike in other bitumen finds on the mummies surface [29, 30] no skeletonised triterpenoids could be observed in bone extracts. By way of contrast, polycyclic aromatic hydrocarbons like anthracene, phenanthrene, methyl phenanthrene and fluoranthene occur. These special compounds are known from soot and may have precipitated from the fume of the open fire used for re-liquefying the embalming mixture, particularly the resinous components detected in the third group.

The third group consists of diterpenoid resin acids and represents an embalming material comparable to that found in the mummy of Idu II. Its components are listed in Table 2 and brought to face with the components of the mummy of Idu II and of raw pine resins. Both embalming materials are derived from pine resin, probably from *Pinus halepensis* [17]. Nevertheless, they show great differences in their composition.

'Crapula' or 'Bruttian pitch'?

For embalming purposes raw pine resin collected from the trees or 'resin' produced by melting out of resinous wood (liquid pitch) could be used. However, prior to their application on mummies both products must be re-liquefied. In the course of these processes, *i.e.* melting out and re-liquefying, the components of raw pine resin were transformed in different ways.

In principle resin from pinewood consists of three diterpenoid acid types: abietadiene acids (abietic acid, neoabietic acid, laevopimaric acid), abietatriene acids (dehydroabietic acid) and pimaradiene acids (pimaric acid, isopimaric acid[s]). Characteristic representatives are shown in Fig. 8.

If a dried up pine resin has to be re-liquefied by melting ('crapula-type'), *i.e.* heated to about 130 to 170 °C, Sandermann's reaction takes place [31–33]: Abietadiene- and pimaradiene-type resin acids dominating in the raw resins are transformed by heat isomerisation and mild oxidation (dehydrogenation) into one abietatriene-type acid, namely dehydroabietic acid (Fig. 8a, b and c). For

Table 2. Comparison of the diterpenoid components found in bone fragments from the Old Kingdom, the mummy of Idu II (Hildesheim) and from the Junker excavation at Giza (Vienna) with the pine resin components. Relative amounts: +++++ = dominating major component, over 50%; ++++ = major component, over 40%; +++ = minor component, over 20%; ++ = over 5%, + = over 1%, (+) = small portions may occur. Considerable portions of isopimaric acids are present in Mediterranean raw resins, *i. e.* resins from Pinus halepensis. The content of abietic and of dehydroabietic acid in untreated pine resin may occur within a broad range. In the table this is shown in parentheses.

Diterpenoid embalming components found in mummified bones of the Old Kingdom	Idu II (Hildesheim)	Samples of the Junker excavation (Vienna)	Raw pine resins	
			Pinus halepensis	Pinus sylvestris
Abietic acid	–	–	++	++++
Pimaric and isopimaric acid	–	+	++++	+++
Dehydroabietic acid	++	+++++	+(+++)	++++
Dehydrodehydroabietic acid	+++	–	–	–
Methyl dehydroabietate	+	+	(+)	(+)
Methyl dehydrodehydroabietate	+	–	–	–
Oxo-dehydroabietic acid	+++	–	(+)	(+)
Methyl 7-oxo-dehydroabietate	+++	+	–	–
Methyl 7-oxo-dehydrodehydroabietate	+	–	–	–

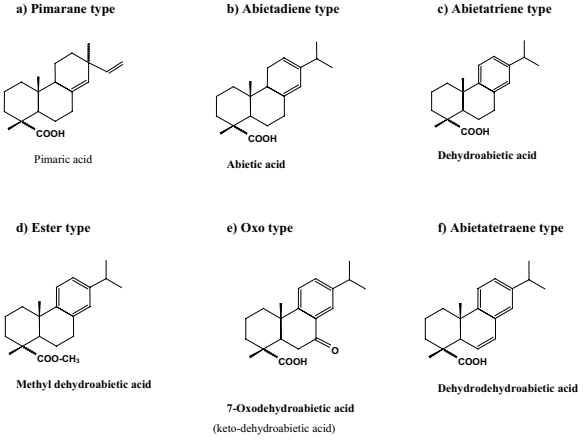


Fig. 8. Structures of characteristic diterpenoid resin acids found in pine resin.

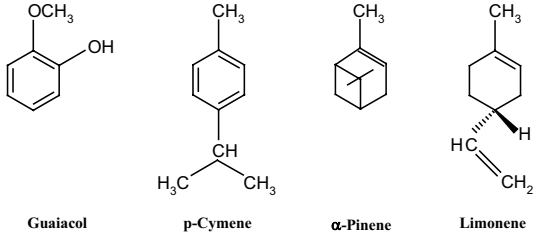
this very reason 'colophony', produced by heating of the balsamic resin and evaporation of the volatile parts, consist mainly of dehydroabietic acid. In the presence of atmospheric di-oxygen small amounts of oxo-compounds, *i.e.* keto-dehydroabietic acid, are also produced. Our conclusion is, that the embalming material in the Vienna bones (Old Kingdom) mainly consisting of dehydroabietic acid (Table 2) belongs to the 'crapula-type' resins.

If the molten resin (liquid pitch) is produced directly from resinous pinewood by a melting-out process ('Bruttian pitch') higher temperatures must be applied. Under theses conditions wood components are decomposed by pyrolytic processes [15, 27, 34]. Phenols, guaiacols and naphthalenes found in the first outflow ('cedrium') and a lot of other components like methanol and acetic acid are released from the lignin parts of the pinewood. Beck's reaction takes its course, *i. e.* methanol reacts with dehydroabietic acid (and other diterpenoid resins) under formation of methyl esters, whereas acetic acid acts as a catalyst [35]. Strongly heating of the resinous wood during the melting out process also leads to an increasing dehydrogenation, *i. e.* formation of abietatetraene-type resins (dehydrodehydroabietic acids). All these new compounds react with air oxygen and a lot of oxo compounds develop (Fig. 7).

Investigations of the dry distillation process (retort made tar) applied to raw pine resin from pinus halepensis show that the amount of oxidised components increases up to a maximum at 350 °C and then decreases to zero at 450 °C [36]. The melting out process of 'pix liquida' described by Pliny resembles the dry distillation using retorts. Therefore, a good impression of the reactions taking their course during the production of 'Bruttian pitch' is obtained. Our conclusion is, that the embalming material in the mummy of Idu II (Old Kingdom) belongs to this type.

Reactivity of organic embalming compounds on alkaline bone phosphatase

Out of the many different identified organic compounds two monoterpenes (α-pinene and limonene), a benzene derivative (*p*-cymene) and a phenolic derivative (guaiacol) were selected to examine their possible reactivity on freshly prepared alkaline phosphatase. α-pinene, limonene and *p*-cymene, all constituents of oil of turpentine from the Aleppo pine (*Pinus halepensis*) [17], were identified in tissue extracts of the Ptolemaic torso (Fig. 5, 340 ± 170 B.C.) (Cut 2) [7]. The phenolic derivative guaiacol was of special interest (Scheme 2). Derivatives of this compound were detected in the 'cedrium' used for embalming the bodies of the dead in Ancient Egypt (Fig. 2).



Scheme 2. Selected homogeneous components used for mummification in Pharaonic Egypt.

The possible reactivity of the above four organic compounds on the molecular architecture and function of bone alkaline phosphatase was examined. Contemporary fresh porcine ribs were thoroughly coated with these embalming compounds and air dried for 35 days at 20 °C. After grinding the bones crude extracts of alkaline phosphatase were prepared in the presence of protease inhibitors employing the established techniques developed earlier [9].

Guaiacol proved to be the most reactive compound to raise the specific enzymic activity of alkaline phosphatase. Compared to an untreated control a 2-fold higher specific alkaline phosphatase activity was seen in the crude extract of guaiacol-treated rib fragments (Table 3). The same enzymic activity was diminished to 50 % in the extracts of *p*-cymene, limonene and α-pinene coated ribs. This reduced activity may be attributed to uncontrolled inhibition of the enzyme in the presence of these three embalming compounds.

Table 3. Specific alkaline phosphatase activity in porcine rib extracts. The bone fragments were coated with guaiacol, *p*-cymene, limonene and α-pinene for 35 days at 22 °C prior to the enzyme assay.

	Crude extract	
	Total protein [*mg/g bone*]	Specific activity [*mU/mg protein*]
Control	9.9	642
Guaiacol	2.1	1504
p-Cymene	13.6	297
Limonene	13.8	308
α-Pinene	9.1	423

Alkaline phosphatase was successfully enriched following the gel filtration on Superdex 200. A control molecular sieve chromatography of the crude extract from an untreated porcine rib yielded a clear separation of the phosphatase enriched proteins appearing at 200 kDalton (Fig. 9). The distinct protein band was overlapped by the enzyme's activity peak in the same relative molecular mass region. Usually at this molecular size alkaline phosphatase migrates in its dimeric form. Significant parts of accompanying high M_r mass proteins appear near 200 kDa which contribute to the observed low specific enzyme activity. The major portions of contaminating proteins below 200 kDalton are clearly separated in these fractions.

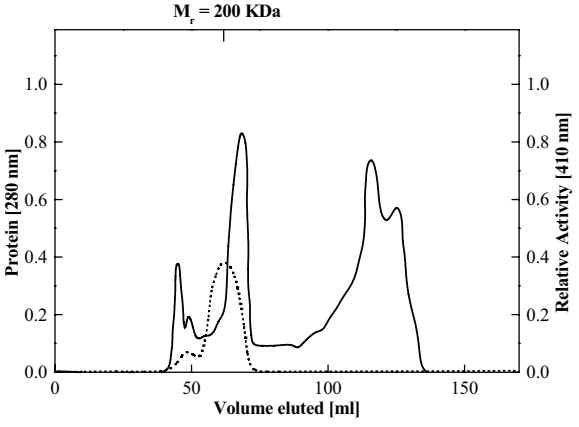


Fig. 9. Gel filtration on a HiLoad 16/60 Superdex 200 prep grade column of an non-treated porcine rib extract. 2,3 mg protein (1,5 U total alkaline phosphatase activity) was applied onto the column and processed in the same manner as stated in the legend to Fig. 10. Protein (–) and alkaline phosphatase activity (·····). The reproducibility of the phosphatase assay was better than ± 5 %.

The elution profiles of all extracts of pre-treated ribs (Fig. 10a–d) were essentially the same compared to that observed for an untreated sample (Fig. 9).

Both activity and protein bands are clearly separated from accompanying non-active proteins. A 7-fold higher content of alkaline bone phosphatase compared to that of the untreated bone was seen in the gel filtrate of the guaiacol sample whereas the phosphatase content in the α-pinene and *p*-cymene pre-treated porcine ribs rose by a factor between two and three only (Table 4). There is a dramatic rise of the specific phosphatase activity up to a factor of twelve in the chromatographed extracts of guaiacol pre-treated bones. This is assigned to the small portion of M_r 200 kDalton proteins being responsible for the observed rise in specific activity (Fig. 10a and Table 4).

A possible alternative explanation for this substantial rise may be attributed to the phenomenon that the enzyme itself remains unharmed in the presence of guaiacol. At the same time proteins other than alkaline phosphatase would react with guaiacol and therefore did not survive the treatment unharmed. This would explain the observed elevated specific activity of the enzyme. α-pinene, limonene and *p*-cymene were less active towards the accompanying proteins leading to a smaller

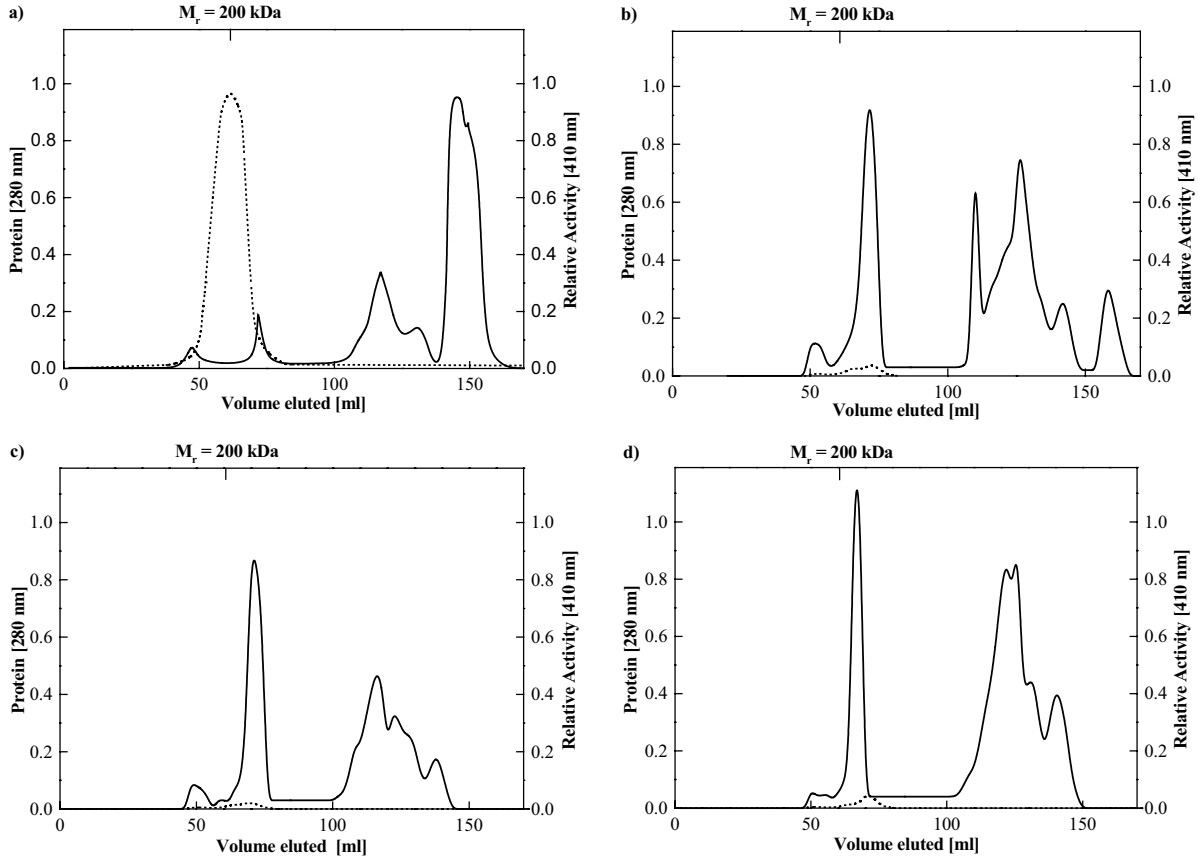


Fig. 10. Protein (–) and alkaline phosphates activity (·····) distribution of a gel filtrated extract of embalmed porcine ribs were chromatographed on a HiLoad 16/60 Superdex 200 prep grade column previously equilibrated with 20 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.1% (v/v) Triton X-100. Elution with the same buffer was controlled at 280 nm. Two ml fractions were collected and assayed for alkaline phosphatase activity at pH 9.6 and 23 °C. The reproducibility of the phosphatase assay was better than ± 5 %.
(**a**) guaiacol (0.8 mg protein, 1.2 U total alkaline phosphatase activity), (**b**) *p*-cymene (3.0 mg protein, 0.9 U total alkaline phosphatase activity), (**c**) limonene (2.4 mg protein, 0.7 U total alkaline phosphatase activity), (**d**) α-pinene (2.6 mg protein, 1.1 U total alkaline phosphatase activity) pre-treated porcine ribs.

Table 4. Total and specific enzymic activity of porcine bone alkaline phosphatase after gel filtration. The stimulating factor compared to the control column is listed in the second column.

	Gel chromatographed porcine rib extract	
	Specific activity [*mU/mg protein*]	Total activity [*mU/g bone*]
Control	1304	40
Guaiacol	15197	288
p-Cymene	1234	83
Limonene	702	44
α-Pinene	707	119

specific activity. A surprisingly unexpected side effect of the guaiacol treatment lies in the fact that this compound might be used as a novel, efficient and unorthodox reagent in the purification process of human alkaline phosphatase. Unlike the successful crystallization of the E. coli enzyme all efforts to crystallize the human enzyme have failed so far. The possible stabilizing effect of these organic mummification compounds on the protein structure of bone alkaline phosphatase was examined by SDS gel electrophoresis (Fig. 11).

The electropherogram of the untreated rib extract revealed a diffuse separation pattern. This separation profile is obvious in both the commercial preparation of the human enzyme and in the extract of untreated porcine bones. A remarkable monodisperse separation band is seen when extracts of guaiacol treated porcine bones where subjected to gel electrophoresis (lane 5 of Fig. 11a). It was interesting to see one single and intensive band attributable to the exclusive prevalence of 57 kDa subunits. This protein band could be attributed to the carbohydrate stripped subunit of alkaline phosphatase. Obviously the carbohydrate envelope of the dimeric protein resists silver staining of the protein portions. Nevertheless, it was striking to see the exclusive presence of just one single band. This supports the phenomenon that guaiacol has a powerful reactivity during the purification process of alkaline phosphatase. This is consistent with the earlier observed separation pattern during the gel filtration of the crude extract obtained from guaiacol coated porcine ribs (Fig. 10a).

Activity staining was performed after SDS-gel electrophoresis. In all cases except in the extract of guaiacol treated porcine ribs diffuse bands were seen below 200 kDa (Fig. 11b). Obviously guaiacol appears to stabilize the enzyme in the presence of SDS in a remarkable manner. The relative molecular mass of the most intensive activity band is clearly higher compared to that detected for all the other examined species (lane 3 of Fig. 11b).

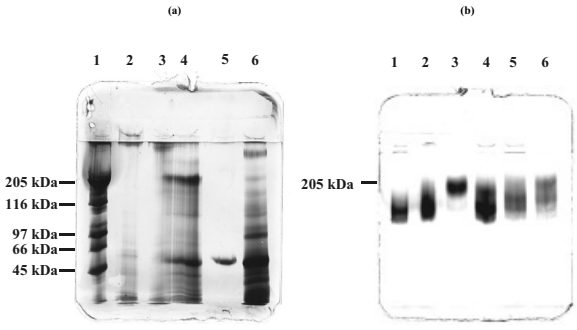


Fig. 11. (**a**) Protein staining of porcine rib extracts after SDS gel electrophoresis. (1) M_r-marker, (2–4), gel-filtrated extract of an untreated porcine rib, (2) 0.1 μg protein, (3) 0.9 μg protein, (4) 2.5 μg protein (5) extract of a guaiacol pre-treated porcine rib (0.5 μg protein), (6) control, alkaline bone phosphatase (Calzyme, 4 μg protein).
(**b**) Activity staining of porcine rib alkaline phosphatase after SDS gel electrophoresis. Each lane was loaded with 350–700 mU bone alkaline phosphatase. (1) human rib extract (700 mU ALP), (2) control, non-treated porcine rib (700 mU), (3) guaiacol (350 mU), (4) *p*-cymene (600 mU), (5) limonene (440 mU), (6) α-pinene (420 mU) pre-treated porcine rib extracts.

Conclusion

The ancient Egyptians have documented daily life in both writing and artwork. Strangely enough, embalming of the deceased is only depicted two times and no authentic written report is known. Written treatises have been summarized by two reputed non-Egyptian authors Herodotus and Pliny the Elder. Pliny [8] states in a more marginal manner while dealing with a different subject the use of 'cedrium'. A more detailed description of different embalming processes was reviewed by Herodotus [40].

Nevertheless, our knowledge as to how the ancient Egyptians had mummified their deceased is limited. It was unknown which types of conserving reagents had been employed to avoid bacterial attack on biological tissues. The wealth of the many different non-disinfectant compounds detected on

mummies aggravated this situation. The abundance of beeswax and bitumen are prominent examples. Although their presence was clearly allocated knowledge on the molecular reactivity as disinfectants is missing. In the case of bitumen its use for conservation cannot be seen either even in ancient texts.

The efficacy of different anti-microbial and conserving compounds was successfully demonstrated by examining both the molecular architecture and functional activity of bone alkaline phosphatase as a characteristic target enzyme. An important basic conclusion could be drawn in that liquid resinous materials were the most reactive species. They were obtained by heating liquefied natural resins and wood tars as well as special oleaginous wood tars. For the first time in 1994 the latter species were successfully assayed concomitantly with reliquefied pistachio turpentine. At that time no detailed attention was placed on their molecular reactivity. Advanced progress was made upon examining an unused sample of oleaginous wood tar, which was entombed together with the mummy of Saankh-kare, 1500 B. C.

Modern analytical approaches using GC and GC/MS allow the detection of many different embalming components. In many cases bio-markers allow even the assignment of the origin of single compounds or families thereof. But in most cases it is hard to tell which products are behind these findings. Attempts to apply every day's knowledge to this question quite frequently led to erroneous conclusions. Therefore, the ancient text of 'Naturalis Historia' by Pliny the Elder promised to be most supportive. Our studies have revealed that the employed resinous embalming materials are derived from heated resinous products.

There was a striking consistency in Pliny's description on 'liquid pitches' and our characterized embalming materials. He emphasized on an oleaginous wood tar called 'cedrium' which was used in Pharaonic Egypt for mummification purposes. The name 'cedrium' allows the conclusion of the application of a wood tar oil derived from cedar wood [16]. Likewise Herodotus [40] describes in a detailed version the use of a 'cedar wood oil' which prompted the investigation of extracts obtained from different cedar woods as well as oils and tars obtained from juniper plants. There was a striking result in that the examined mummy-tar oils actually are derived from melting (out) and/or smouldering processes of cedar wood. Unlike the proposal of Lucas [4, 19] indicating the presence of oils from juniper no such conclusion can be drawn from our results.

At elevated temperatures mummies are especially vulnerable to microbial attack. The tar oils from cedar woods contain components known to be highly biocidal. Phenolic compounds, derivatives of guaiacol, mono- and sesquiterpenes are among the major reactive species. All phenolic compounds including phenols, cresols, xylenols and carvacrols are highly reactive disinfectants. For example, a pronounced anti-worm activity has been reported for carvacrol. All mono- and sesquiterpenes are highly fungistatic. Derivatives of guaiacol found in oleaginous cedar wood tar play an outstanding role during conservation. They are also known as highly efficient reagents during smoke dependent meat conservation [12, 14] and smoke tannage. They cross-react with the keratine layers of the skin to yield a rigid structure. Attributable to this phenomenon guaiacols are detectable in unused wood tar oils but not in extracted embalming agents from mummies.

The assignment of the first application of different embalming materials in the course of the respective Dynasties indicates that from the Old Kingdom onwards highly reactive embalming components were used. In samples from the Old Kingdom likewise traces of tar oils (guaiol and vanillin) were detected. It cannot be excluded that many low relative molecular mass components have evaporated from the very old samples, which could explain the absence of these reagents.

The skill of the embalmers of Pharaonic Egypt has to be admired. Their sharp and fascinating capabilities in observing and preparing the many different embalming materials in an exclusive and empirical manner are of far reaching implications up to our present time. Human bones pre-treated in the described ways contain a 3-fold higher protein concentration as well as an increased alkaline phosphatase activity compared to untreated bones. Among the four selected compounds – *p*-cymene, limonene, α-pinene and guaiacol – the last one was the most reactive species in the course of the preservation process. This again explains the outstanding role of guaiacol. Microbial attack was minimized and there was a strong interaction with

accompanying proteins leading to precipitations. As a consequence alkaline phosphatase from guaiacol coated porcine ribs shows a 12-fold higher specific activity compared to that of the control. The enzyme itself remained unharmed as the observed relative molecular mass was surprisingly identical with the contemporary enzyme.

Experimental Section

Materials

Bone alkaline phosphatase (Lot 418, 0.5 Units/mg) was obtained from Calzyme, San Luis Obispo, CA, USA. A Superdex-200 prep grade HiLoad™ 16/60 gel filtration column was obtained from Amersham Bioscience, Uppsala/Sweden. Guaiacol, *p*-cymene, naphthyl acid phosphate and Fast Blue BB were purchased from Sigma-Aldrich, Taufkirchen/Germany. Limonene and α-pinene were of analytical grade quality obtained from Merck, Darmstadt/Germany. If not otherwise stated all reagents were of the highest purity available.

Sampling of the embalming material

Ancient embalming components were extracted at 23 °C for two hours from muscle and bone tissues employing a mixture of chloroform/methanol (7/3, v/v). One gram of finely ground mummified tissue was suspended in 100 ml of solvent. After centrifugation, the clear supernatant was concentrated to yield 0.5 ml. Aliquots of 0.1 ml concentrate were diluted each with 2 ml of methanol, chloroform and acidified methanol (= 10 % w/v, solution of anhydrous oxalic acid in methanol). The resulting solutions were directly injected in the gas chromatograph (GC) or in the combined gas chromatograph/mass spectrometer (GC/MS). To minimize possible loss of volatile compounds no advanced Soxhlet extraction was performed. For the very same reason complete evaporation of the extraction solvent was avoided to guarantee the recovering of phenols, guaiacols and monoterpenes. Prior derivatisation was omitted in the first injection step. In a later state derivatisation was performed using methylation and silylation.

Analysis conditions for GC and GC/MS analyses

Investigation of embalming materials in the Munich laboratory covered a period of ten years. During this time gas chromatographic measurements were performed on two different GC-systems, a HP5890 series II and a HP6890 system, both from Hewlett-Packard (now Agilent).

Due to these advances in separation techniques some parameters (oven temperature, carrier gas flow rates and injection modes) of both systems were not identical. As a consequence the resulting chromatograms of both systems could not be compared directly by their retention time.

Separation columns

In both GC systems 15-meter fused silica capillary columns (J&W, type DB5-ht) were used for separation. The inner diameter was 0.32 mm and the thickness of the liquid phase (phenyl-dimethylsiloxane film) was 0.1 μm. The separation columns had to be changed several times in the course of the entire analytical performance. Unfortunately, the lengths of these columns varied between 14.5 and 15.5 meters. Owing to theses differences shifting of retention times may occasionally occur which again do not allow direct comparisons of gas chromatograms resulting from different columns.

GC conditions for HP 5890 (see Figs. 5, 6, 7 and 8)

Injection was carried out in split mode at 250 °C. For higher boiling resins, *i. e.* triterpenoids, injections temperature was 270 °C. Helium 5.0 (additionally purified) served as carrier gas. Column pressure was at 48 kPa. The initial oven temperature was at 80 °C for two minutes. Heating was raised with a heating rate of 10 K/min until 360 °C. The detection temperature (FID) was at 370 °C.

GC conditions for HP 6890 (see Figs. 2 and 3)

Injection was carried out in splitless mode and injection temperature was again 250 °C for low-boiling resins and 270 °C for high-boiling resins. The helium (5.0) flow rate remained constant at 1.7 ml/min. The initial oven temperature was maintained at 55 °C for one minute. Heating was started with a rate of 11 K/min until 150 °C and continued with a rate of 10 K/min until 360 °C was reached.

GC/MS conditions

The GC/MS analyses were performed using a gas chromatograph of the HP 5890 series II, in conjunction with a Hewlett Packard quadrupole mass spectrometer type 5989B (MS Engine, EI-mode). GC/MS-analysis provides each GC peak with a corresponding characteristic mass spectrum, which serves to accurate identification of each component of the embalming material.

Preparation methods for GC and GC/MS analyses

There are three essential conditions for a successful gas chromatographic analysis: First, the substances under investigation must be soluble in organic solvents; secondly, they must be volatile in the heated injection port and thirdly, they must be soluble in the stationary phase of the separation column.

There is a large variety of selective columns in order to meet the last prerequisite. Unfortunately, there is no suitable column for the pinewood resins, tars or pitches usually present in mummy tar. This is mainly due to the strong varying polarities of the many compounds found in these materials. In particular, the strongly polar diterpene resin acids have an unfavourable effect. They are hardly soluble or even insoluble in the stationary phases of conventional columns. On the other hand, they tend to be adsorbed on active sites of the injection port and inside the column. Broad and badly resolved peaks are produced which often show strong tailing-off effects. In general, the gas chromatograms of diterpenoid resin acids show both low efficiency and poor separation.

These problems may be reduced (but not solved) by increasing the velocity of the carrier gas. In the most widely used method of sample preparation, however, resins, tars or pitches are methylated prior to analysis by GC in order to convert the highly polar free resin acids into their more volatile methyl esters. These gave better resolutions but by far no ideal separation in the chromatograms. Methylation, however, destroys useful information, because the esters produced by this preparations step are indistinguishable from any methyl esters that may have been present in the original sample. One may separate by base extraction the samples into acid and neutral fractions. Unfortunately, strong acids (*e.g.* hydrochloric acid) must be used to release the diterpene resin acids for subsequent methylation. These mineral acids as well as the base, could initiate some isomerization processes with diterpenoid components as was shown by Sandermann [31]. More important is, however, that volatile fractions, which have survived" by chance in the original samples, are completely lost by these techniques, *i.e.* direct methylation or separation into acid and neutral fractions.

For this very reason, a different technique was used. We have developed a general preparation scheme for the analysis of old tars and pitches. It consists first of a step-by-step extraction with solvents as shown in Table 5. This table shows that all natural resins, wood tars and wood pitches in question are soluble in methanol. The methanol extracts are injected directly in underivatised form into the GC as well as in derivatised (methylated) form.

Table 5. Extraction steps used for the analysis of embalming materials.

Solvent	Extracted fraction
1. Isooctane	Waxes, Plasticers
2. Methanol	Natural resins, Pine wood tars and pitches, Fossil resins (partial)
3. Acetone	Pitches from deciduous trees (birch bark pitch)
4. Chloroform	Fossil pitches (bituminous products)

Conventional columns for the above reasons give only unsatisfactory resolutions and poor separations for underivatised diterpene resin acids. Therefore, a modified technique usually employed during lipid analysis was applied. Analyses are conducted on a nearly non-polar and very stable separation column, namely a DB5-ht fused silica column (J&W). This is a weak polar column, which in principle is not suitable for the strong polar diterpene resin acids. For this very reason, we selectively change the polarity of the stationary phase during the GC-run. This is done by adding at least 10% (w/v) of anhydrous oxalic acid to the methanol extract. After the injection of this acidified solution, oxalic acid – if not decomposed in the heated injection port – is moving immediately after the solvent through the column. It occupies all active sites, so that the polar resin acids could no longer be adsorbed at these positions. At the same time, the polarity of the stationary phase is at least partially shifted into a polar range. The polar resin acids are then able to interact with the stationary phase of the column. Attributable to these contacts of the resin acids with the stationary phase a satisfactory resolution is accomplished.

Embalming of porcine ribs

Mimetical embalming experiments were performed under standardized methods. Muscle tissue was removed from porcine ribs using a scalpel. *Periosteum* (bone skin) was removed by thorough scratching the bone surface. The ribs were coated employing a marten hairbrush soaked with guaiacol, *p*-cymene, limonene and α-pinene, respec-

tively. All treated samples were kept for 35 days in a sealed glass container at 22 °C.

Preparation of bone Zn_2Mg alkaline phosphatase

Dry porcine rib fragments (5–10 g) were finely ground in a porcelain mortar and pistil. The powder was suspended in 3–4 ml 20 mM Tris acetate (pH 7.4) containing 1 mM magnesium acetate, 0.3 % (v/v) Triton X-100 and protease inhibitors (100 mM 6-aminohexanoic acid, 0.5 mM phenylmethylsulphonylfluoride, 5 mM N-ethylmaleimide) per g tissue under slight agitation for 20 h at 4 °C. After centrifugation at 18,000 g the supernatant was concentrated by ultrafiltration through a YM-10 membrane (M_r cut-off 10 kD) and separated by gel chromatography on a HiLoad 16/60 Superdex 200 prep grade column (Pharmacia) previously equilibrated and eluted with 20 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.1 % (v/v) Triton X-100. Two millilitre fractions were collected each and assayed for both protein concentration and alkaline phosphatase activity.

Alkaline phosphatase activity

The alkaline phosphatase activity assay in aqueous solution was based on the increase of *p*-nitrophenol formation as a result of *p*-nitrophenyl phosphate hydrolysis catalysed by alkaline phosphatase. One ml of the assay volume contained 50 μl enzyme solution in elution buffer and 950 μl substrate buffer (1.8 mM p-nitrophenyl phosphate, 0.5 mM magnesium acetate, 1 M diethanolamine, pH 9.6). The rate of p-nitrophenol formation was recorded on a Beckman DU 7400 spectrometer at 410 nm for 10 min against a blank containing the solvent buffer at 20 °C. An absorption coefficient of $\varepsilon_{410} = 1.84 \times 10^4\ M^{-1}\ cm^{-1}$ was used.

Relative molecular mass (M_r) determination by SDS-gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.55% (w/v) sodium dodecyl sulphate (SDS) was performed in a Pharmacia Phast System flat bed unit according to the procedures described in the manual [37]. A Phast Gel Homogenous 7.5 with a stacking gel (5 % (w/v)) and a separation gel (7.5 % (w/v)) was used. Electrophoresis conditions were 10 mA constant current and 300 V. After electrophoresis the protein bands were visualized by silver staining [38].

For enzymic activity staining after SDS-electrophoresis polyacrylamide gel electrophoresis in the presence of 0.55% (w/v) SDS was carried out at 4 °C in a Pharmacia Phast System flat bed unit according to the procedures described above. Prior to the assay the gel was gently washed 3 times in 0.38 M Tris acetate (pH 9.6), 0.5 mM magnesium acetate and 1 mM zinc acetate for 30 min at 4 °C. Enzymic activity was determined by incubating the gel in 1 mg of α-naphthyl acid phosphate per ml in the same buffer for 30 min followed by the addition of 1.33 mg Fast Blue BB per ml in the above buffer to develop the activity bands [39]. The staining was carried out in the dark at 23 °C for 2–12 h.

Acknowledgements

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