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A CHEMICAL INVESTIGATION OF THE SHROUD OF TURIN[1]

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ABSTRACT

Michrochemical testing of materials recovered on "sticky" tape samples taken from the Shroud of Turin was undertaken. The Shroud is a linen cloth, bearing the image of what appears to be a crucified man with the classical stigmata of Christ's crucifixion. The presence of whole blood was established by detection of heme derivatives, bile pigments, and proteins. Although iron in several forms is found over the whole cloth its distribution is shown to be accounted for by natural processes rather than as an added pigment. There is no chemical evidence for the application of any pigments, stains, or dyes on the cloth to produce the image found thereon. The chemical differences between image and non-image areas of the cloth indicate that the image was produced by some dehydrative oxidative process of the cellulose structure of the linen to yield a conjugated carbonyl group as the chromophore. However, a detailed mechanism for the production of this image, accounting for all of its properties, remains undetermined.

KEY WORDS: Shroud of Turin, microchemical testing, textiles, blood, protein, linen, cellulose, pigments.

RÉSUMÉ

On a soumis à des tests microchimiques les matériaux récupérés sur des rubans "adhésifs" d'échantillons prélevés du suaire de Turin. Le suaire est de lin et porte l'image de ce que semble être un crucifié et reproduit les signes classiques associés au crucifiement du Christ. On a établi la présence du sang entier par la détection de dérivés du sang, de bilirubine et de protéines. Bien qu'on retrouve du fer sous diverses formes partout sur le suaire, sa distribution provient de réactions chimiques naturelles plutôt que d'un pigment rajouté. Il n'existe aucune preuve chimique de ce que l'image ait été appliquée sur le suaire au moyen de pigments, de taches ou de teintures. Les différences chimiques que l'on trouve entre le tissu imprimé de l'image et le tissu que ne l'est pas démontrent que l'image a été produite par un processus de déshydratation et d'oxydation quelconque de la composition cellulosique du lin dont le résultat est une substance carbonyle combinée comme le groupement chromophore. Toutefois, le mécanisme détaillé qui a produit cette image et qui expliquerait toutes ses propriétés reste toujours un mystère.

MOTS CLÉ: Suaire de Turin, test microchimique, textiles, sang, protéine, lin, cellulose, pigments.

Since 1977 an American scientific team has been extensively investigating the Shroud of Turin(1-3). This 4.36 × 1.10 m. linen cloth, now kept as a relic at the Cathedral of St. John in Turin, Italy, has had a long and controversial history(4-7), as it is alleged to be the authentic burial shroud of Jesus Christ. It bears both a complete head-to-head, front and back, straw yellow colored "reversed" image of what appears to be a crucified man with "blood-colored" wounds and scourge marks in accordance with Biblical description in the four Gospels(1-7). The Shroud first came to

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historical attention in 1356 in France and was often displayed. Subsequently, it was kept in a silver box. A fire in 1532 melted portions of this box and molten silver burned its way through the folded cloth leaving scorched areas which were later repaired(1-7). The fire was extinguished with water which penetrated the holes in the casket, making large water stains on the cloth. Refer to Figure 1 for these details.

In 1898 Secundo Pia observed(1-7) that the image is "reversed" in that a photographic negative gives a positive image. When these and later photographs of the image were examined by a VP-8 image analyzer(8,9), startling three dimensional images of a man were obtained. These are the graphic results of a one-to-one mapping function relating image density to cloth-body distance(9). The method for creating this reversed image with such striking three dimensional characteristics is unknown(8-16).

A prior investigator(17-19) has published a microscopic evaluation of the samples from the Shroud. This study claims that the body image is due to an iron oxide earth pigment bound with an age yellowed animal protein binder that had been painted onto the cloth(17,18). The blood marks are attributed to a mixture of iron oxide pigments and vermillion in this same binder(19). In light of our chemical findings we disagree with these conclusions.

We have carried out a complete analysis of the fibrils and particles found on the Shroud samples. The fibrils and particles seen are of several types (cf. Tables 1 and 2). There are (a) the clear to pale yellow background fibrils (non-image) of the cloth which bear no image or blood stains. There are (b) yellow fibrils (body image) which are responsible for the body image and, also, (c) golden yellow coated fibrils adjacent to heavy blood flows. (d) "Blood" stained fibrils (blood-image) and a variety of variously colored particles (cf. Table 2) are also seen. (e) Elongated dark scorch marks from the fire in 1532 frame the entire length of the images thus providing scorched fibrils and (f) extensive well-defined water stains incurred in extinguishing this fire partially overlap the body image itself providing "water stained" samples. (g) Linen patches were used to repair the larger scorch areas, and backing clothes have since been sewn to the entire length of the cloth to further strengthen and protect the relic, providing various types of other adventitious fibrils. See Figure 1 for a photographic reproduction of the Shroud.

While scientific testing could readily establish if this artifact is a "forgery", e.g. a painting, it should be noted that it cannot establish unequivocally that it is "authentic". The best that such studies can do is to frame and test hypotheses that would demonstrate that it is definitely a forgery or that the results are not inconsistent with a position accepting it as authentic should one so choose to believe. Several of the group's investigations, mainly physical, have already been reported(8-16). However, questions such as the chemical nature of the blood and the image, are also susceptible to direct chemical testing. The purpose of this paper is to report such an investigation.

EXPERIMENTAL

Using a special Mylar tape with a proprietary toluene soluble hydrocarbon polymer adhesive (supplied by 3M Corp.), Rogers removed specimens from the surface of the cloth (fibrils and particles) from identified locations by a "sticky tape" sampling technique(10) for chemical analysis.

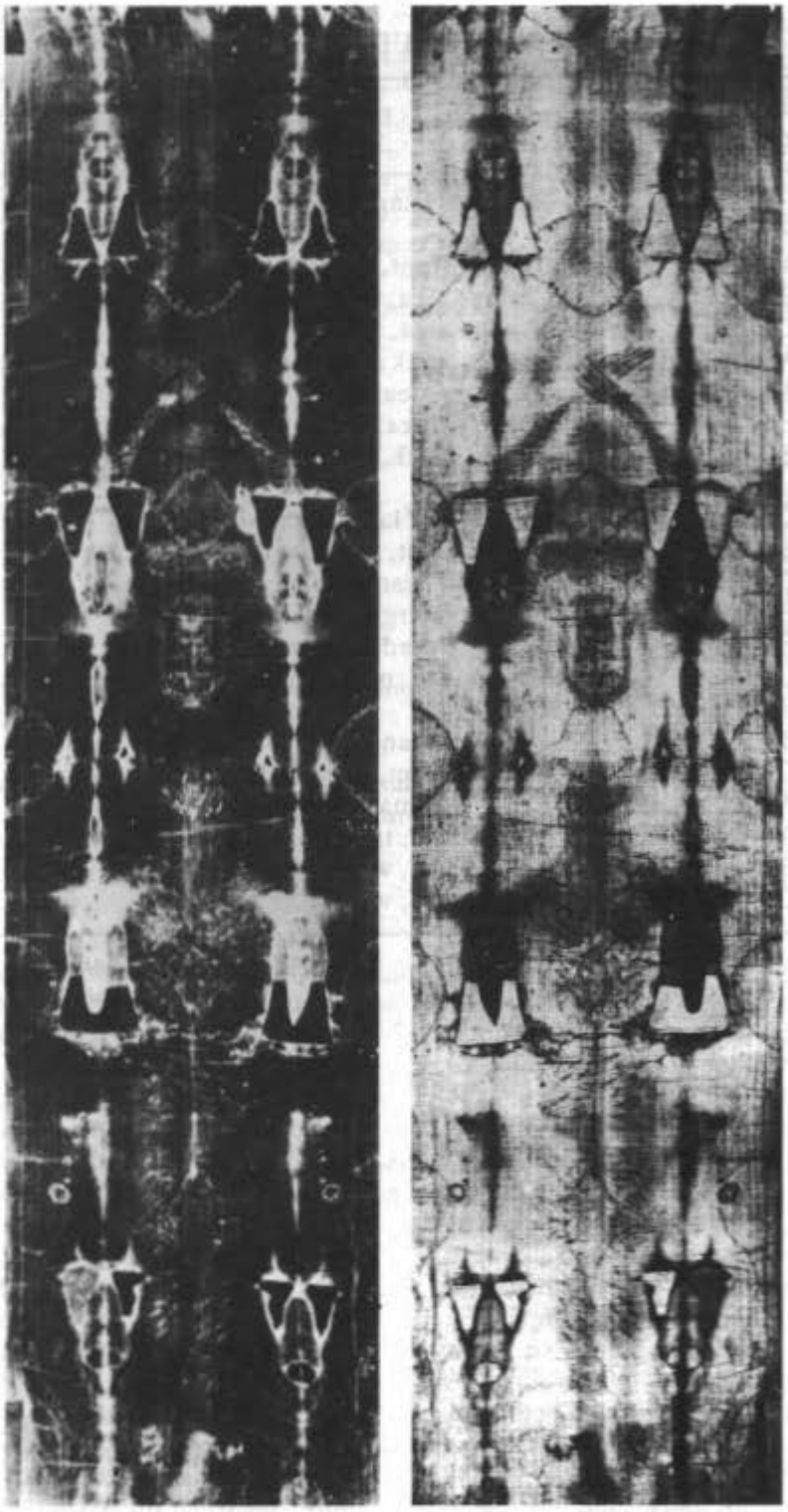

Figure 1. Negative and positive photographs of the Shroud of Turin. Note features described in the text.

Table 1

AREAS OF THE SHROUD OF TURIN SAMPLED

Identification Code	Designation
1IB	Scorch area, back (image figure)
1FH	Patch cloth
2AF	Knee non-image, front, water stain margin
2BF	Knee body image area, front, water stain margin
2CF	Knee body image area, front, inside water stain
3AB	Body image area, back, adjacent ''lance'' wound
3AF	Finger body image area, front
3EF	Wrist blood image area, front
3BB	Body image area, back, water stain margin adjacent blood
3CB	Blood image, back, ''lance'' blood flow
3BF	Non-image area, front, adjacent hand image
3CF	Scorch area, front, near hand image
3FB	Blood-water stain margin, back
4F	Backing cloth, exposed on front corner
4CB	Scourge blood image, middle back
5BH	Backing cloth
6BF	Blood image, front, lance area
6DF	Body image area, front, near lance area
6AF	Blood-scorch image margin
9AF	Head non-image area, inside water stain
9BF	Head non-image area, water stain margin
9CF	Head non-image area, water stain margin

Each tape was about 5 cm^2 in area and 36 such samples were taken. The identification code and Shroud location of the samples that we received for study are given in Table 1. These tape samples were later affixed to microscope cover glasses by a prior investigator(17) and we received them in this form. Of the 22 samples received, 6 were blood image samples, 2 were body image adjacent to blood marks, 2 were body image (only) samples, 3 were water stain/body image samples, 1 was non-image, 4 were water stain/non-image, and, also, 2 scorch, 1 patch, and 2 backing cloth samples. 3BB is a water stain/body image sample adjacent to blood and has been counted twice in the above designations.

Prior to chemical testing, the tape samples were examined microscopically. A Zeiss Photomicroscope III, (both transmission and reflection mode), a Bausch and Lomb model LI polarizing microscope, Leitz Ortholux, and a Nikon PFM phase contrast microscope were used to locate and characterize the classes of objects of interest. Tungsten sources and xenon arcs were employed. The Mylar tape was found to be optically anisotropic with its optic axes aligned with the edges of the tape and is therefore a ''crystalline'' form of this polyethylene terephthalate polymer (glass transition point = 80° C). Therefore, polarization studies were carried out on specimens only after their removal from the tape and adhering

Table 2

CLASSES OF SAMPLE OBJECTS TESTED

Designation	Predominant Locations	Comments
1) Very pale fibrils	Backing cloth, patch cloth samples	Appear very smooth surfaced under phase contrast
2) Pale yellow fibrils	Non-image areas	Surfaces appear slightly corroded under phase contrast
3) Yellow fibrils	Body image (non-blood) areas	Surfaces appear corroded under phase contrast
4) Dark fibrils	Scorch areas	Surfaces appear very corroded under phase contrast, color varies from light to dark brown
5) Red coated fibrils	Blood areas	Coating varies from smooth to fractured to particulate appearance, color varies from red to orange, coating not birefringent or pleochroic
6) Golden yellow coated fibrils	Margins of blood areas	Except for color, same general characteristics as previous, 5)
7) Birefringent red particulate coated fibrils	Water stain margins and blood scorch areas	Color varies from deep red to dark brown; particulates birefringent and pleochroic; phase shows some of them inside the hollow core or lumen of the fibril
8) Shards	Blood areas and areas adjacent blood	Fractured ''replica'' coatings from red coated fibrils, not birefringent or pleochroic, mostly red colored, but olive brown near scorch areas
9) Orange globs	Blood areas and areas adjacent blood	Color varies from red-orange to orange-yellow, not birefringent or pleochroic
10) Brown globs	Blood-scorch margins	Appear to be scorched versions of the previous, but with small, dark particulates embedded therein
11) Black particulates	Scorch areas	Irregular, opaque particles

adhesive to prevent misinterpretation due to the optical properties of the tape itself.

The microscopic survey revealed that there were a large variety of fibrils and particulates present in the samples along with occasional assorted "incidental" debris found in limited quantities such as insect parts, pollen, spores, wax, modern synthetic fibrils, red and blue silk, wool, and flair tip pen dye marks (due to a previous investigator(17)). The red and blue silk fibrils appear to be from backing cloths and borders and are present in almost every sample. This illustrates that there has been transposition of materials from one area of the cloth to another, as it was folded and unfolded numerous times through the centuries (as first noted by John Jackson). Therefore, we have arbitrarily set a minimum threshold of 15 specimens of a particular set of characteristics to constitute a class of fibrils and particles typical of a specific location on the cloth. These classes, their "locations", and microscopic characteristics are given in Table 2. The fibrils range in color from pale yellow to dark brown. Some are coated, others uncoated. The particles range in color from yellow-orange through red to black. Some of the red particles are birefringent, while others are not.

To prepare a specimen for testing, the portion of tape containing it was excised by scalpel from the sample. It was then washed free of the tweezer-held tape with toluene into a spot plate well. The adhesive was then removed by repeated washings with toluene. The wash toluene was removed by micropipet or by sorption into small pointed strips of filter paper while the specimen was held in place with a glass needle. Final removal of the adhesive could be tested with short wave UV irradiation, under which the adhesive gives a bluish-white fluorescence. All of the above operations were carried out under a Bausch and Lomb Stereozoom binocular microscope. White spot plates were used for colorimetric tests, black spot plates for fluorimetric tests, and transparent well slides for those tests where it was desired to examine the results under the microscopes cited above at magnifications higher than 30 X.

The majority of the linen fibrils ranged from 10-15 μm in diameter. They were from 40 μm to several mm in length. The silk fibrils were longer and some were almost one cm in length. Most of the red particulates ranged from submicron to about 3 μm, and the birefringent red particulates from 0.7 μm to about 1 μm diameter. These particulates were therefore too small for accurate quantitive determination of their optical parameters by the standard methods(20-23). Highly variable results were obtained even for refractive index measurements by immersion methods. However, when a red non-birefringent coated fibril and a birefringent red particulate coated fibril were compared side by side immersed in benzene (index of refraction = 1.50), the Becke line movements were in opposite directions. Therefore, the refractive index of the non-birefringent red particles is less than 1.5, while it is greater than 1.5 for the birefringent red particles, indicating that they are quite different materials. As shown below, the birefringent materials are Fe_2O_3 while the non-birefringent are proteinaceous and therefore the refractive index characteristics seen are those that might be expected.

The globs vary in size from about 5 μm to 50 μm in linear dimensions. The opaque black particles vary from about 25 μm to 100 μm in linear dimensions. Therefore, assuming densities between

approximately 1 (protein and cellulose) to 3 gm/cm^3 (iron oxides), we see that the masses of individual specimens range from a few nanograms for the smallest objects up to micrograms for the largest.

Once separated, the specimens were tested by standard microchemical techniques(24-27). When necessary, digestions were carried out with a heat lamp. Chamot and Mason(24) have indicated that the sensitivity of many microchemical tests can be enhanced by saturating a sorbent fiber in the reagent and introducing it into the test solution of the unknown as the color change remains "concentrated" on the test surface. We found the reverse procedure, i.e. a fibril unknown introduced into concentrated test reagent solution, also enhanced sensitivity. All tests of unknowns were run side by side with controls; a blank reagent control, a known "Spanish linen" fibril control (see below), or a solution set at the lowest level of positive detection. All chemicals used were reagent grade. (Most test reagents were obtained from G. Frederick Smith, inorganics from Fisher Scientific, organics from either Aldrich or Eastman Kodak, and specialty biochemicals from Sigma.)

A number of specific 1 cm^2 linen controls were prepared from a 300 year old Spanish linen sample used previously in our testing(14). These controls are listed in Table 3. Fibrils of this Spanish linen were also used as the "known" controls in the manner referred to above for tests for specific ions, functional groups, or substances. Samples of Coptic (circa 350 A.D.) and Pharonic (circa 1,500 B.C.) burial linens supplied to us by Professor G. Riggi of Turin were also used as controls.

It had been considered that the linen of the Shroud might have been treated with extracts of *Saponaria officinalis*(28). Specimens of this plant were identified(29) and gathered locally just prior to blooming and extracted with 1% NaOH, and the extracts were then used to prepare Spanish linen Saponaria treated controls. It was observed that samples of such Saponaria treated cloth and, also, fibrils from it, after being twice rinsed with distilled water immediately turn deep brown on treatment with concentrated sulfuric acid, thereby providing a simple test for such Saponaria treatment.

In the process of mordanting and/or weighting textiles various metal oxides, e.g., Cr, Sn, Fe, Al, etc., are precipated and affixed onto the cloth(30,31). Thus, an earlier method of producing "mineral khaki" was by impregnating linen or cotton cloth with solutions of iron salts, precipitating iron hydroxides onto and into the fibers with alkali, and then dehydrating, producing iron oxide particulates "fixed" to the cloth(32). We therefore also prepared such iron oxide "khakied" Spanish linen controls to test various postulates and to compare with Shroud specimens.

RESULTS

Proteins and heme derivatives — We have previously reported(14) spectroscopic and chemical tests on the "shards" (replica casts which had broken off "blood" coated fibrils) and the non-birefringent red coated fibrils, indicating the presence of blood materials on the Shroud. This previous chemical test consisted of dissolving the "blood" material in 97% hydrazine, while simultaneously reducing any presumptive ferric porphyrins present, splitting out the ferrous iron with concentrated formic acid, and then fluorimetrically detecting any porphyrins by their characteristic red fluores-

Table 3

SPECIFIC SPANISH LINEN CONTROLS

Treatment	Comments
1) a) Untreated	Appears similar to Shroud pale yellow fibrils on microscopic examination
b) Above, heated at 100°C for 1 hour	Show very light scorching, structures of fibrils appear more yellow and more corroded under phase contrast
c) Above, heated at 250°C for 1/2 hour	Scorched, microscopic appearance similar to Shroud scorch fibrils
2) a) Saturated with 0.1% gelatin solution	Appearance similar to Shroud golden yellow coated fibrils; fibrils frequently seen cemented together
b) Above, heated at 100°C for 1 hour	Less scorched than untreated fibril
c) Above, heated at 250°C for 1/2 hour	Less scorched than untreated fibril
3) a) Saturated with sonerated 0.1% gelatin and 0.1% Fe_2O_3 (jeweler's rouge, approximately 1 μm diameter)	Fe_2O_3 particles frequently clumped as well as adherent to fibrils, resembles birefringent red particulate Shroud fibrils more closely than the non-birefringent red coated fibrils under phase contrast; no internal particles seen in the lumen of the fibrils
b) Above, heated at 100°C for 1 hour	Clumps tend to break apart, leaving separate fine Fe_2O_3 particles
c) Above, heated at 250°C for 1/2 hour	Fe_2O_3 particles tend to darken to browner shade
4) a) Saturated with whole blood (2 year old human)	Closely resembles Shroud red coated fibrils, except redder colors
b) Above, heated at 100°C for 1 hour	More closely resembles Shroud red coated fibrils microscopically in color and polarization properties
c) Above, heated at 250°C for 1/2 hour	Scorched, some areas similar to Shroud brown globs, others slightly resemble the birefringent red particulate coated fibrils in microscopic properties
5) a) Saturated with Saponaria extract for 1 hour, then rinsed twice with distilled water	Resembles Shroud pale yellow fibrils, shows pale yellow-green fluorescence under short wave UV
b) Above, heated at 100°C for 1 hour	Scorched, more heavily than untreated, now shows pale orange fluorescence under short wave UV
c) Above, heated at 250°C for 1 hour	Heavily scorched, charred in places, fluorescence about same as previous sample

Table 3 (continued)

SPECIFIC SPANISH LINEN CONTROLS

Treatment	Comments
6) a) Khaki (cf. text), air dried	Visually similar to red coated fibrils, but birefringent and pleochroic; some particles seen inside lumen
b) Above, heated at 100°C for 1 hour	Closely resembles Shroud birefringent red particulate coated fibrils in all microscopic properties
c) Above, heated at 250°C for 1/2 hour	Scorches, particles darken to very dark brown

cence excited under long wave UV. Controls showed this test was sensitive to about one ng of methemoglobin for the dark-adapted eye. We have since confirmed a similar positive result on the larger, redder orange globs. However, none of the brown globs reacted, as might be expected if they represent partially charred blood materials. If the shards are barely covered, i.e., microspotted with a film of hydrazine, they slowly dissolve and give a characteristic pink hemochromagen-like color(33). Further, if the shards are similarly treated with 1 M NH_4OH solution saturated with KCN, a characteristic cyanmethemoglobin type color(33) slowly develops on the surface. Similar hemochromagen and cyanmethemoglobin results can be obtained on the red coated fibrils, but not on any of the brown globs as again would be expected for partially charred hemeprotein materials. These results demonstrate, in our opinion, that the shards and red coated fibrils contain heme derivatives, thus corroborating our earlier results in concluding that the "blood" marks were in fact composed of blood.

In addition, we tested for heme breakdown products, i.e., bile pigments such as bilirubin, with Erlich's reagent(34,35) using the method of Jendrassik(36). For these purposes we employed the commercially available American Monitor "525" bilirubin test kit. Using the same microspotting technique as described above, characteristic blue azobilirubin colors could be positively detected in reflected light on the surfaces of the olive colored shards, the orange globs, and, also, weakly on the more orange colored red coated fibrils, The test color was sensitive to acid, turning a paler purple, and was discharged by 10 minutes of short wave UV light as is characteristic of this color test(35). In the previous spectral work(14) it was noted that a peak does appear both in the whole cloth reflection studies and in microspectrophotometry of the tape samples at about 450 nm. This is quite typical of bilidienes(33,37) and similar bile pigment structures, thus further indicating their presence on the Shroud itself in the "blood" image areas by both chemical test and spectroscopy.

There are a wide variety of tests available for proteins. Hydrolysis and detection of the formed amino acids by reaction with ninhydrin is quite sensitive(38). However, we observed that the Ca^{+2} and Fe^{+3} levels found on the Shroud interfere with the color reaction (Ruhmann's purple) when this method was tested with controls. Amido black is frequently used as a general histological protein stain(39). It has been

specifically used to detect the presence of proteins in some paint media(40) and was employed as such by McCrone(18). However, we found that it also strongly stained pure cellulose (viscose rayon), and even more strongly stained the heat treated and scorched Spanish linen controls. The stain was not removed from these controls by treatment with proteases (trypsin, chymotrypsin, carboxypeptidase and lysozyme at pH 8.4). However, control Spanish linen fibrils impregnated with protein, stained with amido black and then treated with proteases returned the fibril color to the unstained condition within 20 minutes. These two tests show that amido black staining is not due to trace protein impurities in the linen, but due to the cellulose itself accepting the dye. We therefore felt that amido black was an unreliable test for protein outside the blood areas as it reacts similarly to other basic dyes, e.g. methylene blue, in staining surface oxidized cellulosic materials. The Coomassie Brilliant Blues have also been used in a similar fashion to amido black(41). In our tests they behaved in the same way as amido black and they therefore were rejected for similar reasons. These dyes stain strongly as they react with the cellulosic carboxyl groups present (see below).

As neither of these dyes are metachromatic, i.e. do not change color on reaction, but only adsorb onto the sample surface, we used Bromothymol Blue(42,43) which does show a color change on interaction with protein. However, this proved only sensitive to the 1.0 μg level in control tests. Similarly, the standard Biuret-Lowry test(44) also, only proved sensitive to the 0.1 μg level in our control test conditions. As Bromcresol Green is also metachromatic and considered selective(45) for albumin (cf. e.g., Albustix), we tested it and found that it could detect to the 0.1 μg level for albumin under these conditions. The best suited protein reagent tested was fluorescamine(46). While specific for primary amino groups, it has been reported to detect one ng of protein(46) by the generation of a characteristic green fluorescence under long wave UV. Control tests confirmed that it would do so under our test conditions.

Positive Bromcresol Green tests indicating albumin could be obtained for the larger, deeper yellow orange globs and also for the golden yellow (''serum'') coated fibrils. Thus, this indicates that blood constituents other than hemeproteins are present in the blood areas. Similar albumin positive tests were also found in areas adjacent to the blood, e.g., the lance wound area. Elsewhere, expressed ''serum'', apparently due to clot retraction, is noted as might be expected if the wounds truly represent clotted images as proposed by Barbet(5).

Positive fluorescamine tests were obtained on both the red and golden yellow coated fibrils, on the shards, and on both the orange and brown globs. The fluorescamine tests were definitely negative on all fibrils away from blood areas. This specifically includes yellow (body image) fibrils and the birefringent red particulate coated fibrils (these are found primarily in the water stain margins.). Thus, protein is only found associated with ''blood'' areas and is definitely not present as a ''pigment binder'' in the body image areas above the nanogram level. Therefore, age-yellowed protein materials cannot account for the image as has been suggested(18).

One further direct and specific test was carried out for proteins, namely, the effect of proteolytic enzymes. For these purposes a fresh concentrated

mixture of trypsin, chymotrypsin, carboxypeptidase and lysozyme in pH 8.4 buffer was employed. Within a half hour this solution completely "dissolved" the non-birefrigent red particulate coated fibril coatings, leaving no particulate residues. This further indicates that these particulates are blood and not Fe_2O_3 impregnated protein binder. This protease treatment also removes the golden yellow coating from the golden yellow fibrils, corroborating their identification as "serum" coated fibrils. Interestingly, fibrils freed of their coatings using this technique closely resemble the non-image fibrils when viewed under phase contrast. The protease solution also dissolved the shards, the orange globs, and the brown globs (leaving the small dark embedded particulates, probably carbonized material, as residue). Proteases had absolutely no effect on the yellow (body) image or pale yellow non-image fibrils of the Shroud. Protease treatment had no effect on the birefrigent red particulates coating fibrils. This further confirms that these birefrigent red particulates are definitely different from those in the blood areas and, also, demonstrates that they are not cemented to their fibrils with a protein binder. Further, in our opinion, the totality of these tests does confirm the blood image areas as blood. The question as to whether or not the blood is human blood is best left to future immunologic testing.

A summary of the protein tests employable is shown in Table 4. Table 5 summarizes the tests indicating the presence of blood on the Shroud.

Tests for metallic species — The methods employed in testing for specific metal ions(27,47-50) are given in Table 6. While an individual test may be equivocal, the tests taken in combination enable positive identification of the presence of major elements above a level that could produce a colored image evident to the eye. It should also be noted that while all the particulates present are soluble in aqua regia, none is soluble in 8N KOH. Thus the presence of such alkali soluble pigments as realgar (AsS), orpiment (As_2S_3) and litharge (PbO) are immediately excluded on the basis of solubility.

The uncoated fibrils (non-image, image, and water stain) all give relatively strong positive tests only for Ca and Fe, except for those from the inside of the water stains which give somewhat weaker, though still definite positive reactions. Thus, we find no evidence for any other metallic species on the body image fibrils at a level that would provide color evident to the eye.

It should be noted that no acidic digestion is needed to obtain the above iron tests, demonstrating that this is some form of cellulosic bound iron on the fibril surface. Such cellulosic bound iron is probably a chelated covalent form held by the aldehyde and carboxylic groups of the partially oxidized cellulose structure (see below, section on prepartion of flax). The orange and brown globs also give weak tests for iron, but only after digestion with aqua regia as would be expected for blood derived materials (a strong oxidant is required to liberate the iron from heme(14)). The red shards, which contain more hemeprotein, when similarly digested give stronger tests than the globs, but weaker than the fibrils, as would be expected since their color comes from the porphyrin moiety and not their iron content which is relatively small(14,33). The birefrigent red coated fibrils give the strongest test of all. Thus, fibrils themselves contain more iron than the isolated blood particulates showing that a great deal of cellulosic bound iron is present on

the Shroud. Positive iron tests can also be obtained on the birefrigent red particulates by treatment with cold concentrated HCl in which they slowly dissolve. This indicates that the birefrigent red particulates are not heme iron which would require a strong oxidant to liberate its iron. A consistent explanation for the intrepretation of all these results is that while non-birefrigent red particulates are heme containing materials, the birefrigent red particles are Fe_2O_3 by both chemical and microscopic tests(21,22). Digestion of about 50 birefrigent red coated fibrils concomitantly, with aqua regia and subsequent testing specifically for trace Mn, Co, Ni, and Al established that such impurities could only be present at a level of less than 1%. Chemically and microscopically, therefore, these birefirgent red particles appear to be Fe_2O_3 and are quite pure by the chemical tests employed.

Table 4
TESTS EMPLOYABLE FOR THE DETECTION OF PROTEINS

Reagent	Comments
Ninhydrin	Interferences under our conditions preclude use of this test
Amido Black	Also stains cellulosic carboxyls, therefore not specific enough
Coomassie Brilliant Blue	Same caveat as for Amido Black
Bromthymol Blue	Metachromatic, but not sensitive enough under these conditions
Biuret-Lowry	Same caveat as for Bromthymol Blue, but selective for peptides
Bromcresol Green	Metachromatic, only relatively sensitive, but selective for albumin
Fluorescamine	Fluorimetric test, very sensitive, specific for primary amines
Proteases	Specific for proteins only

Table 5
TESTS CONFIRMING THE PRESENCE OF WHOLE BLOOD ON THE SHROUD

1) High Fe in blood areas by X-ray fluorescence
2) Indicative reflection spectra
3) Indicative microspectrophotometric transmission spectra
4) Chemical generation of characteristic porphyrin fluorescence
5) Positive hemochromagen tests
6) Positive cyanmethemoglobin tests
7) Positive detectionn of bile pigments
8) Positive demonstration of protein
9) Positive indication of albumin specifically
10) Protease tests, leaving no residues
11) Microscopic appearance as compared with appropriate controls
12) Forensic judgement of the appearance of the various wound and blood marks

Table 6
TESTS EMPLOYED FOR DETECTION OF METALLIC SPECIES

Element	Method	(Reference), page
Fe	1) Mg Bathophenathroline	(34), 924; (47)
	b) 3 — (4-phenyl-2 pyridyl)-5 phenyl-1,2,4 — triazine disulfonic acid, disodium salt (PPTS)	(48)
Ca	a) o — cresolpthalein complexone	(49)
	b) glyoxal bis (2-hydroxyanil)	(327), 534-535
Cu	a) Neocuproine	(50)
	b) Cuprizone	(50)
Mn	Ag+ and catalyzed acid persulfate	(27), 173
Ni (Pd)	a) dimethyl glyoxime	(27), 149-153
(Co)	b) Nioxime	(27), 149-153
Co	ammonium thiocyanate — fluoride	(27), 146-147
Al	a) Aluminon	(27), 191
	b) alizarin	(287), 185-188
Zn (Hg)	a) dithizone	(27), 178-180
	b) Zincon	(27), 178-180
Ag (Hg)	p-dimethylaminobenzalrhodanine	(27), 59-61
Hg (Cd)	a) diphenyl carbazone	(27), 64-65
	b) dithizone	(27), 71-72
Pb	a) dithizone	(27), 74-75
	b) sodium rhodizonate	(27), 73-74
As	stannous chloride	'27), 99-100
Sb	rhodamine B	(27), 105-107
Sn	phosphomolybdate	(27), 108-109
Cr	diphenylcarbazone — persulfate	(27), 170

Thus we find three types of iron on the Shroud:

a) a cellulose bound chelated form
b) heme bound forms
c) iron oxide (Fe_2O_3)

The predominant form is the cellulose bound form. We have been able to identify Fe_2O_3 primarily in the water stain margins and charred blood areas indicating that it only constitutes a very small percentage of the total iron forms found on the Shroud.

The black particles from the scorch areas after aqua regia digestion give strong positive tests only for Ag. Control tests establish, though, that traces of Hg, Pb, or Cd could be present as impurities below the level of 1%. No other trace metals are indicated by the tests, however. Silver was not seen by X-ray fluorescence in the scorch areas or other areas of the Shroud(15), indicating that it could only be present in quantities below the level of M-series detection, i.e., trace amounts only. This is in agreement with our findings, therefore, as we see it only as an "occasional" specimen and only in scorch areas.

During the microscopic survey of the slides, we noted one large prismatic hexagonal object (150 × 50 μm) on the edge of the tape but in the adhesive on tape sample 6BF. This fractured and we obtained one large (130 × 100 × 10 μm) deep crimson rhombohedral

shaped plate. When excised and digested with aqua regia, this object gave a very strong positive test for Hg and tested negatively for other metals. On the basis of the optical appearance(51) and the chemical behavior, this can be identified as "cinnabar" (HgS). Fragments of this particle were found in the same area along a definite track, the exact width of the base of the prismatic hexagonal object. This is clearly an "accidental" artifact as we have seen nothing like it on any other slides, nor have any other red particulates even from this same tape away from this track given a positive test for Hg.

The results of these metal tests are in agreement with the general conclusions reached by X-ray fluorescence(13) which found only in Ca, Fe and Sr on the Shroud above trace levels. However, the chemical testing allows us to be much more specific in the assignment of actual structures of the metallic compounds present. These results do not support the hypothesis that the image is painted with inorganic pigments.

Tests for organic species and functional groups — The methods employed in testing for specific organic structures and functional groups of possible interest(14,34,42,52-56) are given in Table 7. These tests were performed on the uncoated fibrils: body image, non-image and scorch fibrils. With the exception of positive aldehyde and cellulosic carboxyl tests, all other species tested for tested nega-

Table 7
TESTS EMPLOYED FOR THE DETECTION OF ORGANIC STRUCTURES AND FUNCTIONAL GROUPS

Species	Method	(Reference), page
phenols	a) nitrous acid	(42), 179-181
	b) ferric ion	(34), 1147-1148
riboflavin	a) UV fluorescence, H_2O_2 quenched	(34), 559
steroids	a) Lieberman — Burchard	(34), 507-514
	b) Salkowski	(34), 507-514
indoles	a) glutaconic aldehyde	(42), 382-383
	b) nitrosonaphthol, nitrous acid	(34), 819-821
lignin (allyls)	phloroglucinol — HCl	(52); (42), 169-170
starch	iodine — iodide	(13), 239
porphyrins	hydrazine — formic acid	(14)
pyrroles	p-dimethylaminobenzaldehyde	(42), 381-382
creatinine	alkaline picrate	(34), 996-998
uric acid	phosphotungstic acid	(34), 999-1002
urea dferivatives	phenylhydrazine	(42), 390
amines, primary	a) ninhydrin	(38)
	b) fluorescamine	(46)
nitro derivatives	a) diphenylamine	(42), 295
	b) fast blue salt B	(42), 296
aldehydes	Schiff funchin — sulfurous acid	(42), 195-196
carboxyl, cellulosic	a) methylene blue	(54)
	b) toluidine blue O	(56)
Saponaria extract	sulfuric acid	cf. text

tively. Thus we see no evidence for stains or dyes on the body image fibrils at levels that would be evident to the eye. The fibrils all stain strongly with the Schiff reagent and with methylene blue or toluidine blue O(54,56). Note that the amido black and Coomassie Blue reactions mentioned above occur, as they also represent the binding of such basic dyes to these cellulosic carboxyls(56) in a manner similar to the methylene blue reaction. This further confirms the presence of such cellulosic carboxyl groups. However, if tested side by side the fibrils can be seen to form a progression with pale yellow fibrils (non-image) staining the weakest, the dark fibrils (scorch) staining the strongest, and the yellow fibrils (body image) giving an intermediate reaction. This is the same order as seen in the progressively corroded appearance of their surfaces under observation by phase contrast microscopy (cf., Table 2). It will be shown below that these results can be accounted for by dehydrative oxidation of the cellulosic structure(53).

It should be noted that although all of the other organic tests are negative, this does not preclude the possibility that some of these substances may have resided on the cloth in the past and been "lost" over time through oxidation, degradation, etc. For example, the possible presence of fats or oils was checked with the standard Hanus' and Wij's(57,58) iodine addition reagents (IBr, ICl). It was found that dilute solutions of such addition reagents were not discharged by the yellow fibrils, showing that unsaturated fatty acids are not now present on the fibrils. This does not preclude their possible past occurrence and loss through slow peroxidation. This would also apply to trace amounts of Saponaria. This simply demonstrates that positive tests in some cases would have been more meaningful than the negative tests.

All known organic dyes and/or stains, both natural and synthetic, act as either acid-base indicators or redox indicators, or can be extracted by some solvent(59-63). Therefore, such behavior also constitutes a basis for testing. The "color" of the yellow body image fibrils is neither altered nor extracted by methanol, ethanol, benzene, toluene, acetone, carbon tetrachloride, chloroform, pyridine, ethyl acetate, dimethyl formamide, cyclohexane, ether, morpholine, dioxane, or water. Further, the color is unaffected by either concentrated HCl, concentrated NH_4OH, or 8N K0H.

Concentrated H_2SO_4 does not extract any color from the yellow image fibrils. However, it readily did so for any of the dyed synthetics found as incidentals on the Shroud and from the brown Coptic and Pharonic controls. This test has been used extensively in studying ancient dyed textiles(64). It does, however, deepen the yellow color of image fibrils over about a half hour. In fact, we found that we could prepare "yellow" fibrils identical in all chemical and microscopic characteristics to those found on the Shroud by treatment of the Spanish linen fibrils with concentrated H_2SO_4 for half an hour. The extraction test solvents employed are summarized in Table 8.

Neutral Superoxyl (30% H_2O_2) does not bleach the yellow color from the Shroud fibrils, even in the presence of UV irradiation, nor do weak reductants such as ascorbate. However, hydrazine bleaches the yellow body image fibril color very slowly and diimide(65,66), a very powerful reducing agent, bleaches it instantly. Alkaline peroxide, a very powerful oxidizing agent, similarly bleaches(28) the yel-

Table 8
SOLVENTS EMPLOYED IN DYE EXTRACTION TESTS

methanol	carbon tetrachloride	cyclohexane
ethanol	chloroform	ether
benzene	pyridine	morpholine
toluene	ethyl acetate	dioxane
acetone	dimethylforamide	water
concentrated HCl	concentrated NH_4OH	30% H_2O_2
concentrated H_2SO_4	8N KOH	hydrazine

low body image fibril color quickly. Thus the solvent, redox, and acid-base tests are also consistent with the lack of any applied stains or dyes, but are consistent with the chemistry of carbony groups as a chromphore.

These results are like the metal tests, consistent with previously reported spectroscopic studies on the Shroud(11-13) which also found no evidence for the presence of dyes or stains. However, again, the chemical studies permit us to be more specific in postulating chromophores, which appear to be integral to the cellulosic structure itself.

DISCUSSION

The heme derivative and protein work reported here confirms our earlier study(14). However, the indication of bile pigments and serum type proteins, i.e., albumin, allows one to form the opinion that it is, in fact, whole blood on the cloth and not just heme protein. Further, the observations that non-heme proteins can be detected in border areas adjacent to blood images supports Barbet's contention(5) that these blood images generally represent clotted blood and not free blood flows. These conclusions are further supported by the UV photographic studies(67) which show "serum" halo effects about the scourge marks and at the margins of blood clots. That such a chemically and anatomically correct representation could have been produced by any means other than direct contact of the cloth with a wounded human body is difficult to conceive. The detail in the "scourge" marks as revealed in the fluorescence photography also supports this suggestion of intimate contact(67).

The presence of several occasional particles of Ag species only in the scorch areas can be readily accounted for. As noted above, the silver casket in which the Shroud was kept at the time of the 1532 fire became hot enough to melt partially(4). In fact, the scorches are attributed to burns from this molten silver falling on the folded cloth, forming the scorch pattern now observed. We can safely assume that these silver artifacts are simply residue from this historically recorded event.

While the detection of "cinnabar" might be construed as evidence of the presence of paint pigments, e.g. vermillion, we note that only a single instance of this material has been detected in any of our samples and we therefore consider it incidental. Further, the X-ray fluorescence studies(15) do not show Hg in the body image areas. It has already been observed that the Shroud has been copied by artists on several occasions, e.g. Reubens and Van Dyke(6,7,18). Therefore, the presence of such occasional "accidental" artifacts indicating artists pigments(18) is not unexpected. To conclude that any of the images were produced by the applica-

tion of such pigments, they must be shown to be predominantly present and in the appropriate locations. This cinnabar particle was detected in a blood area and more than sufficient evidence has now been presented to establish that these images were formed by real blood and are therefore not composed of "vermillion". Our microchemical tests do not reveal Hg to be present in these areas generally at levels that could be evident to the eye.

We found the calcium to be uniformly distributed over the tape-sampled Shroud areas, while the iron, though also ubiquitous, was enhanced in the blood image areas and concentrated in the margins of the water stains. This is consistent with the X-ray study(15) which showed a uniform concentration of the iron in the image and non-image areas, but enhancement in the areas of blood marks. X-ray fluorescence studies of the water stain margins also show Fe is concentrated here. Further, the margins are clearly discernable in the X-ray photoimage of the Shroud, although the body image itself is not.

As noted above the chemistry reveals that three types of iron are present: heme bound iron in blood materials, cellulose bound chelated iron found ubiquitously and more or less uniformly over the whole Shroud, and iron oxide particulates mainly, but not exclusively, concentrated in the water stain margins and in some of the scorched areas especially adjacent to blood.

The enhancement of observed total iron and heme iron in the "blood" image areas and the presence of Fe_2O_3 in the scorch areas (especially near "blood") are readily explained. The charring of blood to iron oxide was first observed (but not identified as such) by Boyle in 1684(68).

However, the cellulose bound iron and the water stain margin Fe_2O_3 must now be accounted for. It should be recalled that both of these types of iron were quite pure, definitely free of trace Mn, Co, Ni, and Al to below the level of 1%. The geochemistry of iron(69,70) is such that to find an iron earth pigment as pure as this would be most unusual unless pure hematite were used. In fact, even fairly recent European artist's iron pigments contain either Mn, Ni, Co, or Al above the 1% level(69,71). However, artists of the middle ages reportedly did prepare and use quite pure iron oxide pigments (72), although they undoubtedly had typical contaminants below their ability to detect them. Professor L. Riggi has examined Shroud particles with electron microprobe and finds strong iron signals but not the expected impurity signals for minerologically derived material. However, when he examines specimens of new and old "Venetian" red, the expected contaminants of other elements such as Mn, Co, Ni and Al are clearly seen. While the possibility exists that such "pigment" iron might account for some of the Fe_2O_3 observations, it cannot account for cellulose bound iron. Fortunately, simpler and more reasonable explanations than accepting a painting process exist.

In the process of converting flax to linen, the material was "retted", i.e. fermented, a practice which required submerging the material in natural bodies of water for extended periods of time. This process was employed up until quite recent times(30,31,73,74). During this process the natural ion exchange properties of cellulose operate and two ions found commonly in natural waters that most strongly bind in this way are Ca and Fe, with the former being more strongly bound(75,76), as reflected in the rela-

tive concentrations seen in the X-ray and present studies. Iron found deposited on linen this way is quite common and offers a very simple explanation of our finding for the cellulose bound iron not only on the Shroud linen but on the samples of Spanish, Coptic and Pharonic linens. In fact, linen makers are specifically enjoined against using ferruginous waters for retting as it will stain the cloth(30,73,74). The same argument accounts for the calcium and, also, for the Sr(75,76) seen in the X-ray studies(15) and is therefore self-consistent.

At the time of the extinguishing of the 1532 fire(4), it is reasonable to conceive of this cellulose bound iron "chromatographing" to the water stain margins either as "free" iron or bound to the low molecular weight water soluble degraded cellulosics present, where it could precipitate as $Fe(OH)_3$. By a process similar to the "khaki" process we have described above, it could then produce in time the birefringent red particulate coated fibrils seen. Alternatively, the water used in extinguishing the fire could have been high in its iron content and would produce the same result by a similar migration process. We observed that the Spanish linen "khaki" controls are microscopically and chemically identical to the birefringent red particulate coated fibrils. This includes the fact that some of these particles can be seen in the lumen or core of the fibril as seen on the Shroud. Since many of these internal particles on the Shroud are found between intact joints of the linen fibril, it is difficult to conceive of any way they could have gotten there except by a precipitation process similar to the "khaki" process which we have postulated.

In view of the range of our chemical testing for metal pigments and organic stains and dyes, we found no evidence for the application of any such known materials(59-64) on this cloth. Whatever the image is due to, it would appear most unlikely that it has been painted as we normally speak of such a process.

It has been observed that cellulose can be oxidized in two ways(77,78), one producing a "reducing" cellulose (with both aldehyde and carboxyl groups formed) and the other producing a "methylene blue" type cellulose (with carboxyl groups formed exclusively). The latter type is produced by alkaline oxidizing conditions or by ionizing radiation. The former is produced by acid oxidizing conditions (cf. above H_2SO_4 production of a "pseudo-shroud" fibril) or by pyrolytic conditions. This last process is favored by dehydrative conditions and produces a variety of anhydro and unsaturated cellulose derivatives(79). Therefore, it is seen that the image fibrils are simply more dehydratively oxidized than the non-image fibrils, but less than the scorch fibrils. This provides a consistent explanation of our observations.

It would appear that some type of conjugated carbonyl structure is the most likely chromophore. Its R-bands (315-350 nm) would provide a wave length maximum and extinction (80) of the type observed in the UV spectroscopic studies(11,13) and would also be consistent with the IR observations(12). This would suggest some type of low temperature heating process or a high temperature of extremely short duration (insufficient to produce carbonization) as a mechanism for production of the image. For example, near ambient condition processes involving surface contact with foreign substances (e.g. perspiration, olive oil) acting as local catalysts have reproduced many but not all of the observed characteristics of the Shroud body im-

age(11). In particular, while giving the proper chemical and spectroscopic behavior, they do not yield the three dimensional quality required. Thus, we cannot conceive of a single simple mechanism that will also be consistent with the physical and image analysis(9) studies.

As McCrone's studies(17-19) demonstrated the presence of protein, iron oxide, and "vermillion" on the sample tapes, he has concluded the Shroud is a painting, the artist employing the protein as a pigment binder to render the body image with iron oxide and the blood marks with a mixture of iron oxide and vermillion. Although we have also identified these same species on the sample tapes, we have drawn the opposite conclusion. To establish the validity of a painting hypothesis it is necessary, but not sufficient, to identify such materials. One must also demonstrate that they are present in sufficient quantities and in such locations as to account for what is seen. Further, it must be shown that their presence cannot be more simply accounted for by other processes. Still, further, one's conclusions must be in accord with other studies; specifically, in this instance, the physical and image analysis investigations.

Our studies differ in several important regards from McCrone's. As he has not distinguished that there are two types of red particulates present (birefringent and non-birefringent), he has not further distinguished between body image and blood samples, nor has he recognized that there are in fact three types of iron compounds present. His protein test, i.e., amido black, as we have demonstrated is not specific, nor as sensitive or as selective as the tests we have employed. He has demonstrated the presence of Hg by electron microprobe.

We have in our opinion confirmed that the "blood" is in fact real blood (cf. Table 5) and this is in agreement with spectroscopic(11-15) and forensic(5) studies. We have shown that the bulk of the iron present on the Shroud is in the cellulosic bound form and shown that it is readily accounted for by a well understood natural process, viz., retting, that the linen of the Shroud must have been subjected to, and that this conclusion is supported by the X-ray data(15). We find the iron in the blood areas behaves as heme bound iron. We find iron oxide concentrated in the water stain margins and it is not bound there by a protein. We have demonstrated that iron is present in all the old linens we have tested and retting is a reasonable explanation for its occurrence there. Our conclusions are not only self-consistent, but agree with the X-ray(15) and spectroscopic(11) studies which have shown that "iron-oxide" does not correlate with the visually observed image and cannot account for it.

We have further shown that the body image, in fact, is not produced by any pigments, stains, or dyes and is specifically not accounted for by "age yellowed" protein. Protein is present only in blood and in the halo area around some blood. The image arises from dehydratively oxidized cellulose and can be accounted for, but a specific mechanism cannot be provided (see below). This conclusion is consistent with previous work(9-16), in particular the microscopic observations(16), that show there is no evidence of cementation of the body image fibrils to one another, no capillarity or penetration of the color below the top surface fibrils on the crowns of the fibers of the weave, no evidence of brush marks, etc.

We have also seen vermillion (in a "blood" area, though a different sam-

ple from that where McCrone identified it). We feel that it is more easily explained as evidence that artists have copied the Shroud (an historically verifiable fact(6,7,18)) and not that an artist has rendered it (a severely disputed historical fact(7)). In this regard it is interesting to note that the elements other than Hg detected by McCrone's analysis, viz., Na, Mg, Al, Si, P, S, Cl, K, Ca, Fe and Cu, are in fact all found in whole blood(81). However, it would be a most peculiar minerological assemblage that would provide these elements and not the expected iron earth pigment impurities, i.e. Mn, Co, and Ni. His "particle medium agglomerates" where he has detected these elements are the same as our globs which we have shown contain only low amounts of heme bound iron and no visually detectable amounts of Hg. In this regard it should be noted here that the "red color" seen arises from the porphyrin rings present and not the iron contained therein(14,33). In fact, insertation of iron into a porphyrin lowers its intrinsic extinction. Therefore, the "iron" we see in the globs is at the lower limits of our detection, which would place the "mercury" that McCrone sees at trace levels far below the limits that would provide a visually detectable color evident to the eye. This is entirely consistent with contamination due to the artists who have copied the Shroud.

Finally, any applied pigment is incapable of rendering all of the image characteristics found on this cloth. It is highly improbable that any 14th century artist would produce a "reversed" image or could encode the degree of three dimensional, computer readable information(9) found in this image and leave no other surviving historical evidence of his evident genius.

It is remarkable how closely all these results were predicted by Rogers prior to the actual investigation of the Shroud(82). Until further studies are made, the explanation of the image on this intriguing and controversial relic remains a mystery.

Using a Kevex ISI 100B Energy Dispersive Spectrometer, we have examined 16 different globs and fibrils from blood image, body image, and non-image tape samples. The fibrils all show strong Ca and Fe signals. The globs all show Na, Mg, Al, Si, P, S, Cl, K, Ca, and Fe. Some also show Cu and Zn. Fibrils and globs from the cinnabar "track" area on 6BF also show Hg. Most importantly, there is no Co, Mn, or Ni detected anywhere and the Hg is only detectable in "track" samples. Similar results were obtained by J. Jackson and W. Ercoline in their SEM studies. These results and the conclusions to be drawn therefrom are identical with those from the microchemistry.

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