A QUANTITATIVE IMMUNOLOGICAL STUDY OF PLASMA PROTEINS IN BLOOD STAINS.

by

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SUMMARY.

An electroimmunodiffusion technique, which enables precise measurements of the concentrations of some plasma proteins in blood stain extracts to be made, has been developed. It is possible that if a number of plasma proteins, with high discriminating power were found to be stable in blood stains, and were measured by this technique, that the information so obtained could be of value for the forensic examination of blood stains. The "Tandem" antigen-antibody crossed electrophoretic technique has also been examined, and its application to this problem assessed.

Nine plasma proteins have been examined, and some of them found to be stable in blood stains. Additional immunological techniques have been employed in an attempt to establish the nature of the changes which the unstable proteins underwent.

Seven of the proteins were quantitated in samples provided in a number of "blind trials", in order to test the feasibility of a plasma profiling system for matching blood stains.

Finally, the advantages and disadvantages of a plasma profiling system over existing methods of blood stain analysis have been discussed.

CONTENTS.

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1. INTRODUCTION.

Man was described by Hippocrates as an "infinitely variable organism". Since this early observation, research into the uniqueness of the individual has been pursued, since it has proved to be of great importance in a number of disciplines. Sir William Osler, recognising the relevance of this phenomenon to the medical profession, reported that it was "more important to know what sort of patient has a disease, than what sort of disease a patient has". This observation has been of great importance in recent years, in connection with blood transfusion and transplant surgery, where close "typing" of donor and recipient is often a prerequisite of a successful operation.

In the forensic field, the differences between individuals are exploited in order to identify evidence from the scene of a crime with the criminal responsible. Fingerprints are known to be unique to the individual and prints left by a criminal provide useful evidence for the forensic scientist. Collection of the relevant information from the scene of the crime, and cross-matching it with the fingerprints of the suspects is a relatively easy procedure. Blood is often found at

the scenes of crimes; a burglar may cut himself on glass while entering a building, or blood from the victim of an attack may be spilt. It would be of great value if a system, analogous to the fingerprint system, could be employed on blood stains. Collection of data from the scene of the crime, and then cross-matching this with either fresh blood from the suspects, or with other stains, perhaps from the assailant's clothing, would not be as straightforward as fingerprint-matching. Attributes must be present in the blood which are as unique to the individual as are his fingerprints, they must be stable in stains, and, ideally, be as stable within the individual as his fingerprints. Some of these requirements are met by the parameters currently used for blood stain characterisation.

Existing methods for the forensic examination of blood stains depend on the identification of the types of a number of genetically-controlled polymorphisms. These include the red-cell antigen systems, ABO, MN and Rhesus systems, which can be detected in blood stains after many years, and also some polymorphic enzyme systems. A blood stain extract would normally be subjected to a number of these tests, and classified according to each attribute. The probability of another blood stain having the same classification as the first can be calculated from the known population split according to Jones (1972). For example, two stains are to be classified using the haptoglobin, ABO and serum cholinesterase systems. The haptoglobin system has four classes, with a proportion within the population as below,

$$p_1 = 0.139, \quad p_2 = 0.526, \quad p_3 = 0.320, \quad p_4 = 0.015$$

The probability of matching the two stains according to this system is

$$PM_1 = \sum_{i=1}^{4} pi^2 = 0.3986$$

Calculating the probability of a match with the other two systems similarly, for the ABO system $PM_2 = 0.3423$, and for the serum cholinesterase system, $PM_3 = 0.8248$. Combining the results from all three

systems, the probability of matching the two stains can be calculated from the equation,

$$\text{Probability of match, PM} = \prod_{j=1}^{3} (PM)_j = 0.1125$$

Therefore, there is a probability of 0.8875 that discrimination between the two stains is possible using these tests.

Although this apprcach to blood stain characterisation is useful, it has limitations. Williams (1973) has calculated that a discrimination of 1:100 would be possible in the United Kingdom if a blood sample was found to have the most common classification for all the attributes which can now be studied. Improvement on this figure would obviously be obtained if any of the rarer groups were found. However, few of the regional forensic laboratories are able to perform all of the necessary tests on a blood sample. Different techniques have to be applied for the assessment of different parameters, and this may not be possible, either through inadequate facilities for the techniques or through insufficient sample. Many of the tests have been improved, so that smaller samples are required (Culliford, 1971), but the question arises of which tests to perform to give maximum discriminating power from a given volume of sample (Smalldon and Moffatt, 1973). Also, especially when older stains are being examined, the results of polymorph-typing may be ambiguous and lead to false classification.

The concentrations of the plasma proteins in the blood are believed to be unique to the individual. It may be possible to devise a method for the characterisation of blood by measuring the amounts of a number of these. Hill and Treverrow (1942) studied albumin, globulin and fibrinogen levels in over five hundred individuals and found that wide inter-individual variations occurred. Electrophoretic studies of the plasma proteins by Hallman et al. (1952), Young and Webber (1953), and Bernfeld et al. (1953) have supported the view of Hill and Treverrow. More recently, quantitation of some plasma proteins by, for example,

more precise immunological techniques, has been used to show the variations in levels of the plasma proteins which might be expected within the individual (Freeman, 1969, Mondorf and Kollmar, 1969, Sweetin and Thomson, 1973), and within the population (Gill et al., 1971, Lyngbye and Krøll, 1971, Clarke and Freeman, 1968, Clarke, Freeman and Pryse-Phillips, 1970, Storiko, 1968, Weeke and Krasilnikoff, 1972). Unlike the polymorph systems, which contain a number of discrete sub-groups, the plasma proteins are a group of proteins whose concentrations in the blood vary continuously within the population. Culliford (1973) has discussed the use of continuously variable parameters in blood stain identification. He envisages the construction of a profile of a number of parameters which would be unique to an individual, and therefore analogous with the fingerprint system. Although this approach has limitations, some of which are discussed by King (1974), it seems possible that the method may offer certain advantages over existing techniques. Some information must be known before the discriminating power for a series of continuously variable parameters can be calculated. A population survey should be available, and also the expected intra-person variation should be known. It is important that intra-person variation is less than inter-person variation. King (1974) points out that should the comparison of two blood samples, shed at different times, be made on the basis of a number of continuously variable parameters, it is important to know the intra-individual variations for those parameters in the donors of the samples. Without this knowledge, it is impossible to state whether any significance can be attached to the differences reported between the samples.

Biochemical profiling of serum is routinely performed in clinical laboratories, where up to eighteen constituents of serum are studied. King (1974) has evaluated the suitability of the parameters used in the clinical examination of serum for the purpose of discriminating between blood stains. He used data from Cotlove et al.(1970)

which included information concerning personal and population variations in a number of serum constituents, and used an extension of the equation derived by Smalldon and Moffatt (1973) to calculate the discriminating power for one continuously variable attribute. Smalldon and Moffatt (1973) showed that the probability of matching two samples by measuring an attribute, x_i, which has a distribution function in the population of $f(x_i)$, can be calculated from the equation

$$PM_i = 2E_i \int_{-\infty}^{+\infty} \left[f(x_i)\right]^2 . dx_i \qquad \ldots(1)$$

where E_i is the error in measuring x_i. Assuming that a plasma protein has a normal distribution within the population, equation (1) for that protein becomes

$$PM_i = \frac{E_i}{\sigma_i} \cdot \frac{1}{\sqrt{\pi}} \qquad \ldots(2)$$

where σ_i = population standard deviation

$\pi = 3.142$

E_i = error due to intra-person variation and analytical error.

King (1974) points out that the term σ_i in equation (2) is not only dependent on the inter-person variation but also on the variation in protein level within the individual and on the analytical error. Correction can be made for this. At the 99% confidence limit, 99% of the results will be within $\pm 2.58\sigma$ of the mean. If $\overline{\sigma_p}$ represents the mean personal standard deviation, then the term E_i in equation (2) becomes $2.58\overline{\sigma_p}$. Thus, substituting this in the equation above gives,

$$PM = \frac{2.58\overline{\sigma_p}}{\sigma_g} \cdot \frac{1}{\sqrt{\pi}} \qquad \ldots(3)$$

where σ_g = standard deviation for the population. It can be seen from equation (3) that the probability of matching samples is reduced when the ratio $\frac{\overline{\sigma_p}}{\sigma_g}$ is reduced. Thus, for maximum discriminating power in

blood stain indentification, parameters should be chosen which have the lowest value of $\overline{\sigma_p}/\sigma_g$. For any discrimination at the 99% confidence limit, the value of $\overline{\sigma_p}/\sigma_g$ should be 0.69 or less. Only two of the serum constituents studied by King (1974) have values of $\overline{\sigma_p}/\sigma_g$ of less than 0.69, so it can be seen that the parameters measured for clinical examination of serum would not be of value in blood stain characterisation.

Examination of the data in the literature concerning personal and population variation in the plasma protein levels suggests that these may be of greater use in blood classification than the biochemical constituents of serum referred to above. Population data have generally been presented in two forms, where either the population has been regarded as a whole (Gill et al.,1971, Weeke and Krasilnikoff, 1972, Clarke et al.,1970) or where the population has been divided into sub-groups, depending on age and sex, to emphasise the differences in plasma protein levels with respect to these (Lyngbye and Krøll, 1971, Clarke and Freeman, 1968). Intra-person variations are not so well documented, but some data from Freeman (1969) and from Mondorf and Kollmar (1969) are available. In examining the data in the literature, several problems arise concerning the calculation of the discriminating power. The formula derived by King (1974) applies only to parameters which are distributed normally within the population, and within the individual. While the intra-person variation in the plasma proteins appears to be normally distributed, from the information of Freeman (1969), it is not easy to tell whether any of the plasma proteins are normally distributed within the population, because of the lack of data. Gill et al. (1971) have reported values from twentyfour individuals anly, Mondorf and Kollmar (1969) from seventeen, and elsewhere population data have not included the plasma protein levels of the individuals in the population studied. Clarke and Freeman (1968), although they present their population data in groups according to age and sex, do suggest that the

plasma protein levels of the ten proteins they studied were approximately normally distributed within the population. Weeke and Krasilnikoff (1972) however, believed that in some cases the distribution of the logarithms of the protein concentrations followed the normal curve more closely than the numerical values. This treatment was applied to the results for orosomucoid, α_1-lipoprotein, haptoglobin, ceruloplasmin, haemopexin, β_1-lipoprotein, transferrin and immunoglobulins G, A and M. It has already been stated that the data presented in population studies do not generally include the individual figures obtained for each subject, so it is difficult to illiustrate the effect of taking logarithms on the proximity of the distribution to the Gaussian curve. Therefore, it is proposed that the distributions are treated as normal for the purpose of estimating the discriminating power. Although the values calculated may not be accurate, they will be a guide in determining which proteins are capable of giving maximum discriminating power, and therefore best suited to this study. The data presented by Clarke and Freeman (1968) and by Lyngbye and Krøll (1971) cannot be used in this way, since the population has been studied in subgroups, divided according to age and sex. However, their results have been of use since they illustrate how the population variations arise, indicating the differences in concentration of a number of plasma proteins between children and adults, and between males and females.

Another problem contributing to the difficulty of calculating the discriminating power arises because of the different ways in which results are expressed. Where the two-dimensional Laurell technique has been used, a pooled normal serum is often used as a standard, and the amount of specific proteins related to the amount of the protein in the standard (Clarke and Freeman, 1968, Freeman, 1969). Because of this, the amount of data in the literature which can be used to calculate the discriminating power is limited. Suitable data have been provided by Mondorf and Kollmar (1969). They examined the levels of transferrin,

ceruloplasmin, β_1A-globulin and immunoglobulins G, A and M in a group of seventeen healthy men and women between the ages of twenty-four and forty-eight over a five-month period. They used the Mancini technique of single radial immunodiffusion for protein quantitation (Mancini et al., 1965). They concluded that for the plasma proteins studied, the inter-person difference was greater than the intra-person variation. Their results are represented in Table 1.I.

Table 1.I.

Intra- and Inter-person Variation in Some Plasma Proteins, Reported by Mondorf and Kollmar (1969).

Protein	Mean Individual Standard Deviation ($\overline{\sigma_p}$) /mg per 100cm^3	Population Standard Deviation (σ_g) /mg per 100cm^3
α_1-Antitrypsin	22.0	27.1
Ceruloplasmin	4.75	33.1
Transferrin	28.7	28.4
β_1A-Globulin	6.7	19.3
IgG	155.4	251.88
IgA	33.5	78.2
IgM	25.9	84.2

Further data on intra-person variation are provided by Freeman (1969). He collected data from a single subject, measuring thirteen proteins on fourteen sampling days over a period of two years, by the two-dimensional Laurell technique. This gave a precision of approximately 10%. The amounts of the specific proteins present in each sample were reported on arbitrary units and also in relation to the amount of Gc-protein. Six of the sampling days fell within sixteen days, and the results obtained from these six samples were treated as a separate subgroup. The results are represented in Tables 1.II.a and 1.II.b. It can be seen that improved results are obtained when the

Table 1. II.a.

Intra-person variation in a number of plasma proteins measured, in arbitrary units, over (i) a two-year period and (ii) a 16-day period.

Protein	(i) Two-year period m	s.d.	c.v.	(ii) 16-day period m	s.d.	c.v.
Orosomucoid	79	12	15	72	11	15
α_1-Lipoprotein	136	18	13	128	18	14
α_1-Antitrypsin	165	35	21	180	19	11
Gc-Protein	127	24	19	126	20	16
α_2-Macroglobulin	139	32	23	131	26	20
Ceruloplasmin	217	60	28	225	50	22
Haptoglobin	109	22	20	102	17	17
Haemopexin	122	23	19	117	19	16
Transferrin	135	26	19	131	21	16
β-Lipoprotein	131	45	34	112	23	21
β_1A/C-Globulin	92	18	19	87	15	17

Table 1. II.b.

Intra-person variation of a number of plasma proteins, the concentrations being compared with that of Gc-protein over (i) a two-year period and (ii) over a 16-day period.

Protein	(i) Two-year period m	s.d.	c.v.	(ii) 16-day period m	s.d.	c.v.
Orosomucoid	0.60	0.40	6	0.59	0.03	5
α_1-Lipoprotein	1.04	0.10	10	1.02	0.06	6
α_1-Antitrypsin	1.26	0.07	6	1.30	0.08	6
α_2-Macroglobulin	1.10	0.17	16	1.04	0.07	7
Ceruloplasmin	1.70	0.30	18	1.80	0.31	17
Haptoglobin	0.86	0.09	11	0.81	0.06	7
Haemopexin	0.96	0.07	7	0.93	0.06	6
Transferrin	1.08	0.04	10	1.04	0.03	3
β-Lipoprotein	1.05	0.40	36	0.89	0.07	8
β_1A/C-Globulin	0.74	0.19	25	0.69	0.09	13

Table 1.III.

Population Variations in a Number of Plasma Proteins.

Protein	Population Coefficient of Variation	Reference*
α_1-Antitrypsin	22.1	1
	21.6	1
	21.3	2
	15.8	3
	14.1	4
Ceruloplasmin	29.6	1
	29.6	1
	18.3	3
	25.0	4
Transferrin	13.6	3
	11.3	4
β_1A-Globulin	27.0	2
	22.4	3
	10.0	4
IgG	22.6	1
	22.6	1
	9.2**	2
	21.2	4
IgA	32.3	1
	32.9	1
	9.5**	2
	34.1	4
IgM	41.3	1
	43.0	1
	12.0**	2
	51.8	4
Orosomucoid	26.0	1
	21.6	1
	5.5**	2
Haptoglobin	42.2	1
	42.0	1
	9.3**	2
	44.0	3

* References:
1 Gill et al. (1971)
2 Weeke and Krasilnikoff (1972)
3 Clarke et al. (1970)
4 Mondorf and Kollmar (1969)

** Results from Weeke and Krasilnikoff, where logarithms of the concentrations were used to calculate the population standard deviations.

figures for the individual proteins are related to the Gc-protein concentration. This may result from the Gc-protein acting as an internal standard, making corrections for changes in sample volume and antiserum changes. It may also be acting as a standard in the blood, making allowances for changes in blood volume. The coefficients of variation for the results obtained during the sixteen day period are lower than those obtained over two years. This is not only a reflection of the reduced variation in the protein levels within the subject over the shorter period, but also a result of greater precision in the technique. Over a period of two years, differences between batches of antiserum and gel will both tend to reduce the precision.

The data in the literature in which populations consisting of more than seventeen people have been studied indicate that the figures reported by Mondorf and Kollmar (1969) may underestimate the extent of the population variations for a number of proteins. The values of σ_p/σ_g obtained by Mondorf and Kollmar (1969) would therefore tend to give pessimistic values for the discriminating power calculated. Population coefficients of variation calculated from other authors (Ganrot and Schersten, 1967, Clarke, Freeman and Pryse-Phillips, 1970, Gill et al., 1971, Weeke and Krasilnikoff, 1972) are reported in Table 1.III, for comparison with the results obtained by Mondorf and Kollmar (1969).

The results of Mondorf and Kollmar (1969) suggest that the mean personal variation is generally lower than the mean population variation in the plasma protein levels. Calculation of the ratio $\overline{\sigma_p}/\sigma_g$ will give some indication of the suitability of the proteins for use in the characterisation of blood stains. As stated earlier, for discrimination to be possible between two stains at the 99% confidence limit, using one continuously variable attribute, the value of $\overline{\sigma_p}/\sigma_g$ should be less than 0.69, and ideally as small as possible. Table 1.IV shows the values of $\overline{\sigma_p}/\sigma_g$ which have been calculated from the data in the literature. It must be borne in mind that the distributions of the proteins within the population may not be normal, and the discriminating

power calculated may not be accurate, both because of this factor, and because the results of only a single survey have been used.

Table 1.IV.

Ratios of Intra- to Inter-individual Variations for Some Plasma Proteins.

Protein	$\overline{\sigma_p}/\sigma_g$
α_1-Antitrypsin	0.81
Ceruloplasmin	0.14
Transferrin	1.01
β_1A-Globulin	0.35
IgG	0.62
IgA	0.43
IgM	0.31
Albumin	0.89

Inspection of the values of $\overline{\sigma_p}/\sigma_g$ in Table 1.IV indicates that some of the plasma proteins examined are not suitable for inclusion into a profiling system for blood differentiation. However, some of those proteins which have a personal to population variation ratio of greater than 0.69 may be of value. Examination of the results of Clarke and Freeman (1968) and Lyngbye and Krøll (1971), shows that for some of the proteins their distribution within the population may be such that an investigation into their behaviour in blood stains may be justified. α_2-Macroglobulin, for example, is present at significantly different levels in men and women, and also between adults and children, and so might provide useful information in blood stain characterisation. Other proteins also show significant differences in level between males and females (Weeke and Krasilnikoff, 1972, Lyngbye and Krøll, 1971) at certain ages. For example, the levels of albumin, α_1-antitrypsin, ceruloplasmin, α_2-macroglobulin, haemopexin and IgM appear to be significantly different between men and women, when the student's t-test is

applied.

Thus, a review of the literature can assist in suggesting the most useful proteins to study, but a final decision cannot rest entirely on the discriminating power calculated according to King (1974). However, the way in which the discriminating power increases with the number of parameters studied can be illustrated. Assuming that a number of plasma proteins have a mean personal to population ratio of 0.4, then the discriminating power for one protein alone is 0.42 (from $D.P. = 1 - \frac{2.58\overline{\sigma_p}}{\sigma_g} \cdot \frac{1}{\sqrt{\pi}}$). If two proteins are examined, the discriminating power is 0.66, for three proteins 0.80, for four 0.89, for five 0.93 and for six proteins 0.96. Therefore if six proteins were found with a discriminating power of 0.42 each, there would be sufficient information to discriminate between stains. Discriminating power would be reduced, however, if the levels of any of the proteins were correlated. Phillips and Blackmore (1971) examined the data presented by Clarke and Freeman (1968) to determine whether the proteins they studied were truly independent parameters. The correlation coefficients they calculated suggested that there may be some correlation between certain pairs of proteins. However, the greatest coefficient reported was 0.56 for the correlation between α_1-antitrypsin and ceruloplasmin. Smalldon and Moffatt (1973) have examined the effect of correlation between two normally distributed attributes on the discriminating power. When no correlation exists between two attributes, each of which has a mean personal variation to population variation ratio of 0.4, then the discriminating power, calculated from the equation

$$D.P. = 1 - \left(\frac{2.58\overline{\sigma_{p1}}}{\sigma_{g1}} \cdot \frac{2.58\overline{\sigma}_{p2}}{\sigma_{g2}} \cdot \frac{1}{\pi} \right)$$

is 0.66. $\overline{\sigma}_{p1}$ and $\overline{\sigma}_{p2}$ are the mean personal variations in attributes 1 and 2, and σ_{g1} and σ_{g2} are the corresponding population standard deviations. If the same two attributes are found to be correlated, with

a correlation coefficient, r_{12}, then the discriminating power must be calculated according to the equation derived by Smalldon and Moffatt (1973),

$$D.P. = 1 - \left[\left(\frac{2.58\overline{\sigma}_{p1}}{\sigma_{g1}} \cdot \frac{2.58\overline{\sigma}_{p2}}{\sigma_{g2}} \cdot \frac{1}{\pi}\right) \frac{1}{\sqrt{1 - r_{12}^2}}\right]$$

which reduces the discriminating power to 0.59. Thus, even with the highest correlation coefficient recorded, the discriminating power is not greatly affected for two attributes. When more parameters are as strongly correlated as this, discriminating power is more seriously reduced. However, since no other pair of proteins is as closely correlated as ceruloplasmin and α_1-antitrypsin, the choice of proteins to study need not be affected by this factor.

In 1962, Laudel et al.reported that certain differences between individuals could be recognised when their sera were examined by immunoelectrophoresis on cellulose acetate membranes. Their technique of assessing the individual proteins was crude, consisting of a comparison of the lengths and positions of the precipitin arcs by means of a grid system. More recently the techniques available for protein quantitation have improved considerably. Russell (1965) attempted to quantitate the serum proteins, in a similar way to Laudel et al., by measuring the curvature of the precipitin lines of an immunoelectrophoretic separation. A number of precise quantitative techniques have been developed (Axelsen and Bock, 1972) and replace, to some extent, the earlier techniques of protein quantitation by immunoprecipitation (Kabat and Mayer, 1969). The Mancini technique of single radial immunodiffusion (Mancini, Carbonara and Heremans, 1965) claims a precison of approximately 7%, and while this has been superseded by the 'Rocket' technique of electroimmunodiffusion (Laurell, 1966) in precision (less than 5%), the method is still widely used for protein quantitation, (Vergani et al.,1967, Scolari et al.,1968, Störiko, 1968, Mondorf and

Kollmar, 1969, Gill et al.,1971, Lanzerotti and Gullino, 1972). In clinical studies, the Mancini technique has an adequate precision to facilitate the observation of gross changes in the level of certain proteins, which may be associated with a particular disorder. In the present study, however, precision must be as high as possible, in order to maintain the maximum discriminating power. An analytical error of 10% in the method would severely reduce the discriminating power for a particular protein. A precision of 5% would not reduce the discriminating power significantly, but a precision of 2-3% was more acceptable in the present work.

One of the most precise immunological methods for plasma protein quantitation is the Laurell 'rocket' technique. The method was introduced by Laurell in 1966, following the development of the two-dimensional immunoelectrophoretic technique by Laurell (1965) from a principle suggested by Ressler (1960b). Ressler suggested that following an electrophoretic separation of a protein mixture in one dimension, the proteins should be subjected to electrophoresis again but at right angles to the first dimension and into a bed of antibodies. This resulted in the formation of precipitin arcs, one for each protein, by a technique that was more rapid than the diffusion method of immunoelectrophoresis introduced by Grabar and Williams (1953). Clarke and Freeman (1968) later modified the technique to enable quantitative measurements to be made. The two-dimensional technique has been assessed for its suitability in blood stain examination (Whitehead et al.,1970, Beauchamp et al.,1971). It was found that the technique, superficially very useful since it would be possible to construct a complete protein profile in a single run, would require improvement before it could be applied to the present work. The inter-plate precision of 10% was too great for reproducibility of results. Therefore, the 'rocket' technique with a reproducibility of 5% or less seemed better suited for use in the present work.

The choice of proteins to be included in the profile was

governed by a number of factors. It was pointed out at the beginning of the chapter that the parameters studied should be stable. The stability of the plasma proteins within the individual, and the effect on the discriminating power has already been discussed. The stability of plasma proteins in blood stains has apparently not been examined. Saint-Paul et al.(1972) have examined the effect of storage at $4^{o}C$ on the plasma proteins in the blood taken from a corpse, and the storage of serum proteins under different conditions has been examined (Weeke, 1972a, Finlayson and Armstrong, 1972, Sweetin and Thomson, 1973), but no direct evidence of the behaviour of plasma proteins in dried blood stains is available. Therefore one of the aims of this study was to discover whether any of the plasma proteins could be extracted quantitatively from blood stains of different ages, and thus be used in the construction of a unique profile for the donor of the stains. The technique used was the Laurell 'rocket' technique, and the choice of proteins rested mainly on the suitability of the plasma protein to quantitation by this method, and the availability of monospecific antisera and a suitable standard for the proteins. Although the immunoglobulins may provide excellent discriminating power (Table 1.IV), they cannot be quantitated by the 'rocket' technique unless they are modified first by carbamylation (Weeke, 1968), or unless the antibodies in the gel are carbamylated (Bjerrum et al.,1973). Carbamylation lowers the pI of the molecules and results in a difference in electrophoretic mobility between antigen and antibody which is necessary for precipitation to occur. With the risk of impaired quantitation during this procedure, the immunoglobulins were not included in the list of proteins to be studied.

The final list of proteins to be examined included prealbumin, albumin, orosomucoid, α_1-antitrypsin, haptoglobin, haemopexin, α_2-macroglobulin, transferrin and β_1C/A globulin. Some of the properties of these proteins are listed in Table 1.V.

Since the two-dimensional immunoelectrophoretic technique of

Table 1.V.

Some Properties of the Proteins Studied in the Present Work.

Protein	Molecular Weight	pI	No. of polypeptide chains	% Carbohydrate
Prealbumin	54,980	4.7	4	0.50
Albumin	69,000	4.9	1	0.08
Orosomucoid	44,100	2.7	1	41.40
α_1-Antitrypsin	51,000	4.0	1	12.40
Haemopexin	80,000	---	-	22.60
Haptoglobin	100,000	4.1	3	19.30
Transferrin	90,000	5.9	1	5.87
α_2-Macroglobulin	820,000	5.4	4/10?	8.40
β_1C/A-Globulin	---	---	-	3.03

Laurell appeared to be a convenient method for obtaining a plasma protein profile, apart from the problem of lack of precision, the present work included a reappraisal of the technique, modified to give lower coefficients of variation. This was done by reducing the number of proteins quantitated by using a mixture of monospecific antisera in the second dimension gel, as suggested by Ganrot (1972), and by using carbamylated transferrin as an internal standard to reduce sampling errors (Weeke,1970a). Tandem-crossed immunoelectrophoresis (Krøll, 1973) was also used to compare two samples on a single immunoelectrophoretic plate, with a simple mixture of antisera in the second-dimension gel. This method is discussed in chapter 2.

Great care must be taken when the results of quantitative immunological techniques are being assessed (Bock and Axelsen, 1973). When two non-identical proteins are present in a mixture which is subjected to the 'rocket' technique, or the two-dimensional Laurell technique, two separate precipitates will be formed, assuming that antibodies to each antigen are present. When two proteins which are partially identical, i.e. with some, but not all antibody binding sites identical, one or two precipitates may be formed, depending on the closeness of the relationship between the two antigens, and also on the relative amounts of the two species. When one precipitate occurs, it cannot be assumed, therefore, that a single antigen is present, and so it is important to know whether any change in the identity of the protein has occurred. It is especially important in this study since the proteins in blood stains may undergo partial denaturation on drying which may not be reversed on extraction of the proteins into a liquid. Therefore the extracted proteins may not be immunologically identical with the native protein, and the results of the 'rocket' technique should be compared with those from techniques which can indentify differences in the identity of proteins. The Ouchterlony test (Ouchterlony, 1967) has been used to detect such changes in identity. Classic

immunoelectrophoresis (Grabar and Williams, 1953) was also performed to detect changes in the electrophoretic mobility of the proteins concerned, and the quantitative immunoprecipitation technique of Heidelberger and Kendal (1929) was carried out on the albumin extracted from blood stains of different ages, to determine whether any change had taken place in the antibody-binding capacity of this protein.

Finally, a number of blind trials have been carried out on whole blood, and stains of different ages to determine whether stains from the same person, prepared at different times, could be matched by the profiling system derived. In this way the suitability of a plasma protein profile for the identification of an individual was assessed.

2. Experimental Techniques.

2.1. Antigen Quantitation by Means of Immunological Methods.

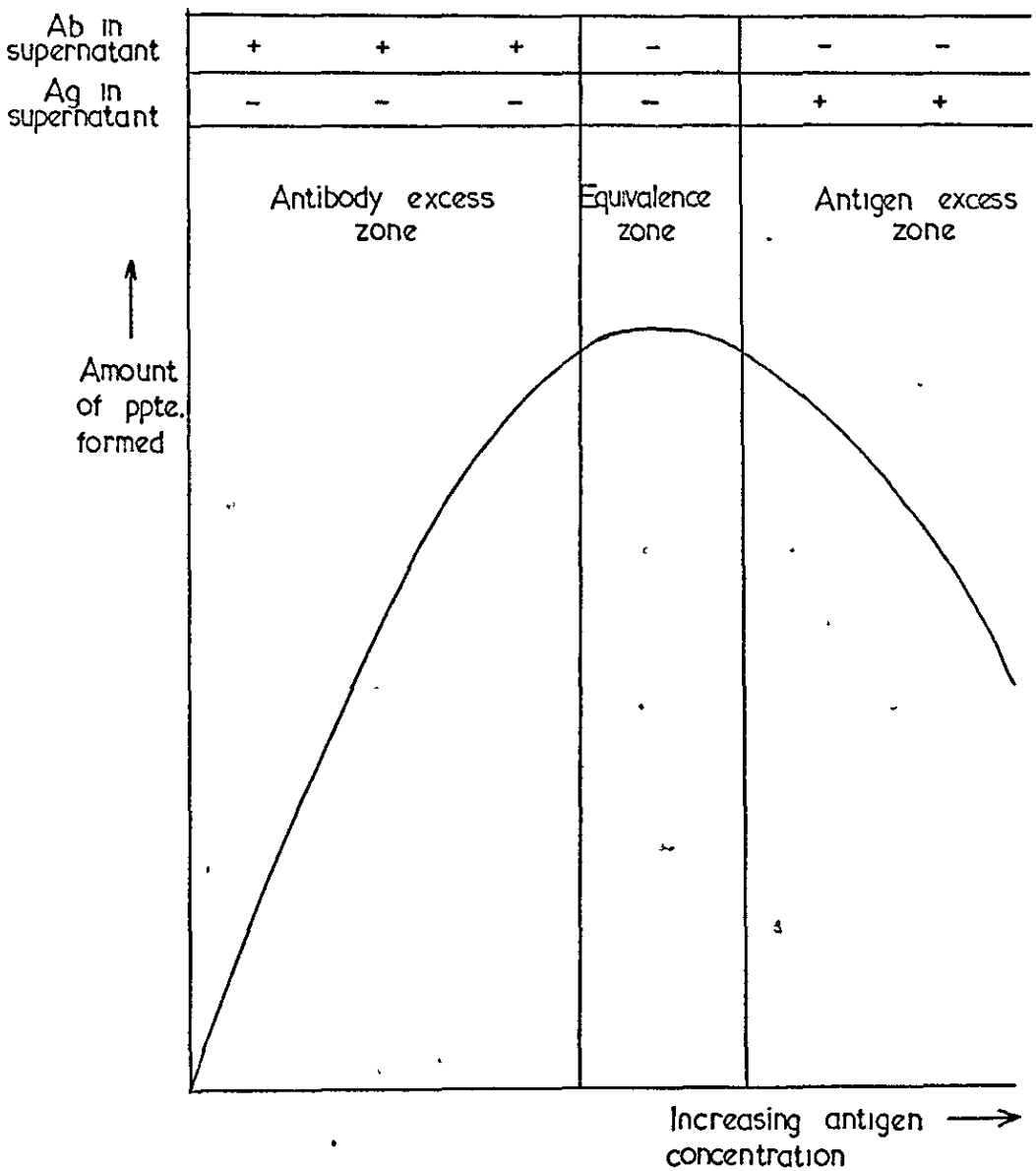
The term "antigen" is used to describe a substance which, when injected into an appropriate animal can bring about the production of antibodies, and which has the ability to react specifically with the antibodies so produced. The antigen may be natural or synthetic, and protein, lipoprotein, glycoprotein or polysaccharide in nature. The antibodies produced are a heterogeneous mixture of proteins, which are believed to be secreted by plasma cells, following the injection of antigen. Reaction between the antibodies and the antigen used to elicit their production can take place in vivo or in vitro. The specificity of the reaction is believed to depend on the interaction of sites on the antigen surface with specific areas of the antibody molecule. The heterogeneity of the antibody population arises from the fact that the different antibodies recognise different sites on the surface of the antigen. These sites may involve, in the case of proteins, only three or four amino acids, and the spatial arrangement of these is important

in determining the specificity of the reaction. The strength of binding in between antigen and antibody, which occurs mainly through ionic and van der Waal's forces, depends on the closeness of alignment between the two species.

The reaction between antigen and antibody can be observed by means of the precipitin reaction. This was first described by Kraus in 1897, following the observation of a precipitate formed by the reaction of a bacterial antigen, Pasturella pestis with antibody raised against it. Arrhenius in 1907 attempted to describe the reaction mathematically by applying the law of Mass Action, but a better understanding of antigen-antibody interaction did not appear until Heidelberger and Kendall (1929) described a generalised form of the precipitation reaction. By using a purified capsular polysaccharide of pneumococcus, which is devoid of nitrogen, the antigen-antibody reaction could be followed by the presence of nitrogen in the precipitate. They examined a series of tubes containing a constant amount of antibody, and an increasing concentration of antigen, and estimated the nitrogen, derived from the antibody, in the precipitate formed in each tube. Their results are indicated in Figure 2.(i). At low antigen concentrations, the amount of precipitate formed increases as the amount of antigen present increases, and free antibody can be detected in the supernatant. This is the antibody excess region. Addition of more antigen results in the formation of more precipitate, until a point is reached where no free antigen or antibody is detectable in the supernatant. This is the equivalence zone. Beyond this, excess antigen is present, and the amount of precipitate present decreases as the concentration of antigen increases. The precipitation reaction depends on the antigen being multivalent, and the antibody at least bivalent. At the equivalence point, Marrack (1938) proposed that optimal proportions of antigen and antibody were present, and a stable antigen-antibody lattice was formed. With antigen in excess, solubilisation of the lattice occurs as a result of the free antigen competing for sites on the antibody in the precipitate.

Figure 2(1)

The quantitative precipitin reaction.



Early observations of the precipitation reaction between antigen and antibody were made with the reagents in liquid media. More recently, semi-solid gels have been used. Many qualitative techniques exist whereby the reaction is observed in a gel through the precipitate formed at the equivalence point (Kwapinski, 1965). Quantitative techniques have been developed, which depend on the fact that maximum precipitation will only occur at the point where antigen and antibody are at the equivalence ratio. The Mancini technique of single radial immunodiffusion illustrates this principle. Antigen is placed in a well in a gel containing a suitable concentration of antibodies to that antigen. The antigen molecules will diffuse radially into the gel, at a rate dependent on the temperature, the initial concentration of the antigen and the antigen diffusion coefficient. As it diffuses, the antigen will encounter antibody, but precipitation will only be possible where the antigen has diffused furthest, and is sufficiently dilute to form an insoluble precipitate with antibody. However, as more antigen diffuses towards the precipitate, this will dissolve in the antigen excess, and a precipitate will form further from the application well. Eventually, precipitation will be complete when all the antigen has been exhausted by reaction with antibody, and the diameter of the precipitin ring will be related to the amount of antigen originally present in the sample. (If standard solutions, covering a suitable range of antigen concentrations are applied to the same plate, a calibration graph can be drawn, and the amount of antigen in the sample calculated). It can be seen that if the antiserum in the gel is not sufficiently concentrated, the antigen may not completely precipitate, since it will always be in excess. Conversely, if there is too much antiserum, quantitation may be impaired since the antigen only diffuses a short distance before it is completely precipitated by antibody.

In quantitative immunological techniques, the fundamental fact is that a certain amount of antigen, under optimum conditions, will react with a specific amount of antibody. If conditions are sub-optimal,

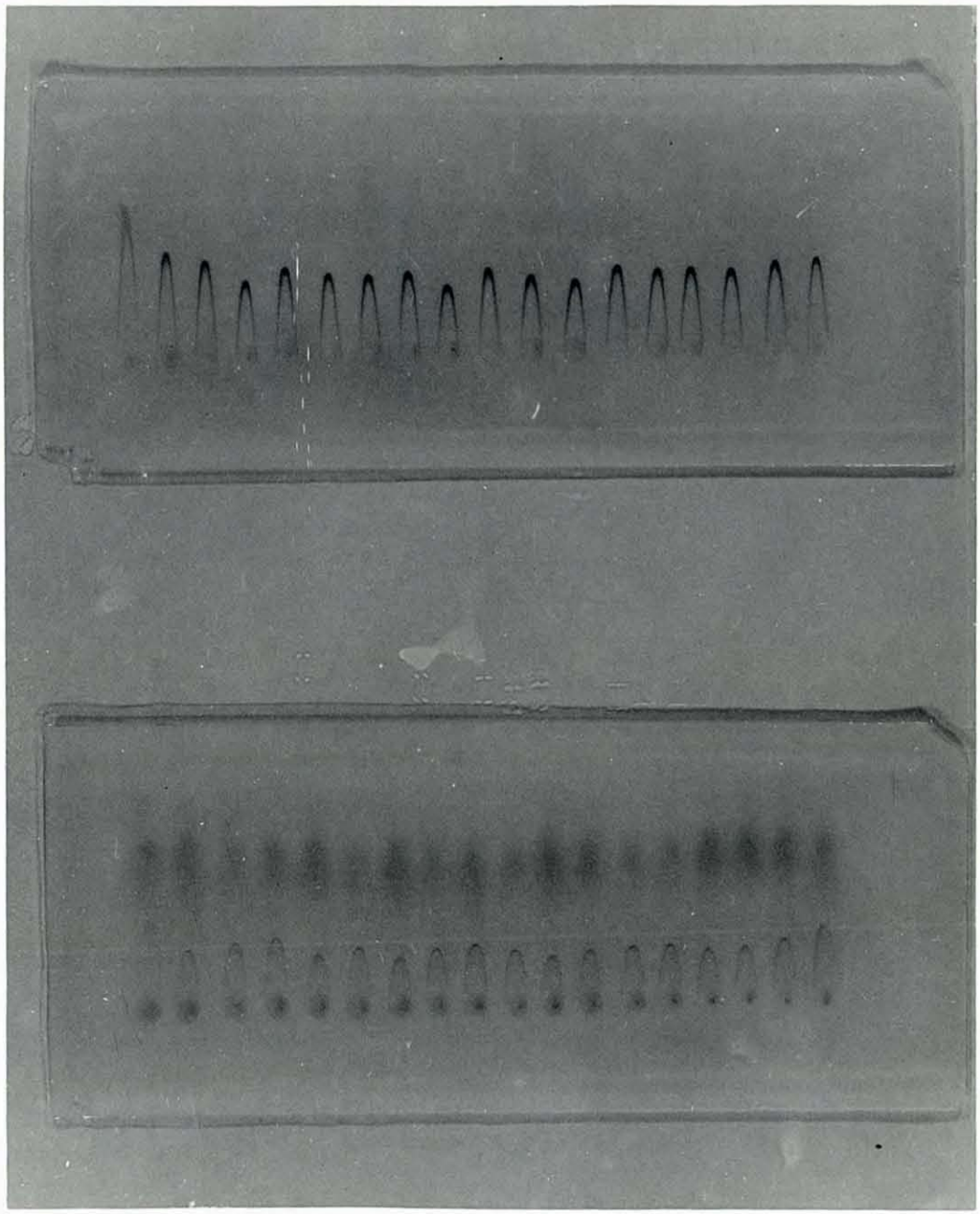
either a precipitate may not form , or a precipitate is formed, but cannot be regarded as a true measure of the amount of protein present.

2.2. Antigen Quantitation by Means of Electroimmunodiffusion and Antigen -Antibody Crossed Electrophoresis.

Libich in 1959 observed that the difference in electrophoretic mobility between antibodies and the plasma proteins could be used to effect a fast precipitation reaction between them in an agar gel, by the application of an electric field. Antigen and antibody were placed in adjacent wells, and reacted after migration towards one another, the antigen moving as a result of its negative charge towards the anode, and the antibodies moving in the opposite direction due to electro-endosmosis. Quantitation of antigens was attempted by Libich (1959), by measuring the position of the precipitates formed.

Ressler (1960a) developed a technique which enabled immunologically distinct proteins, indistinguishable by electrophoresis alone, to be identified. Plasma proteins were forced, in an electric field, into a stabilised buffer containing antiserum, such that precipitin lines developed, one for each protein. In the same year, Ressler (1960b) described a technique for the analysis of the individual proteins in a complex mixture. This involved the electrophoretic separation of the protein mixture in starch gel, followed by a second electrophoretic migration of the separated proteins into a stabilised buffer solution containing anti-human serum. The resultant immuno-electropherograms demonstrated a number of overlapping precipitation lines, each of which corresponded to a specific protein. Both of the techniques described by Ressler have been subsequently modified. The former technique is now known as "electroimmunodiffusion" (Merrill et al., 1967), or the "Rocket" technique (Figure 2(ii)). Laurell (1965) developed the two-dimensional technique, which he referred to as "antigen-antibody crossed electrophoresis" (Figure 2(iii)). In their present forms the techniques

Figure 2(ii)



(a)

(b)

Albumin (a), and transferrin (b) quantitated by rocket immunoelectrophoresis on Cellogel.

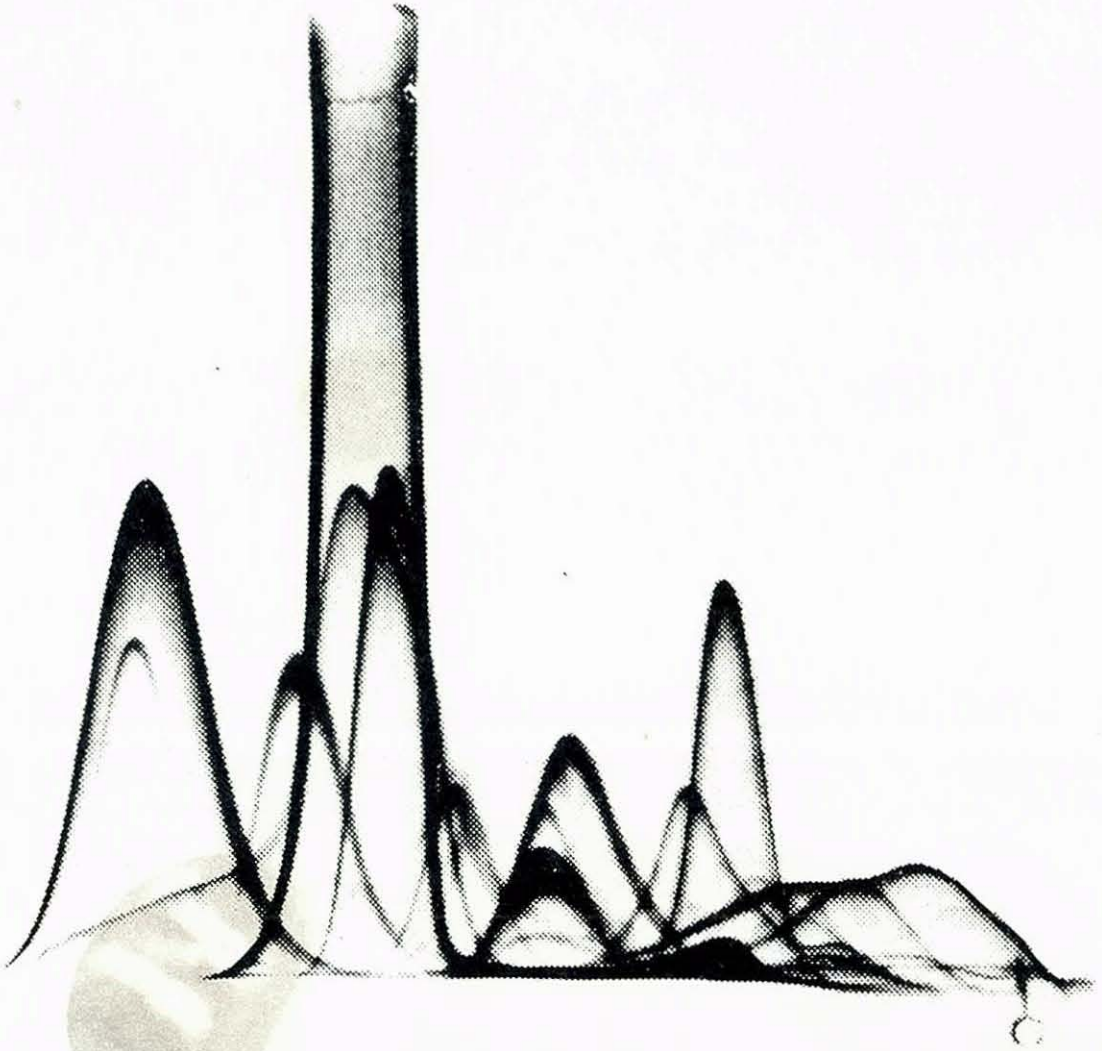


Figure 2(iii) Antigen-antibody crossed electrophoresis: Serum sample examined in agarose gel, with anti-whole human serum in the second dimension gel.

enable relatively precise quantitative data to be obtained for plasma proteins.

The mode of precipitate formation is similar in both techniques. Antigen molecules are driven, by the the force of the applied electric field, into a gel bed containing antibodies, which contains a buffer whose pH is such that the antibodies are uncharged, or only slightly charged. Usually a pH of 8.6 is chosen, which results in a very low antibody mobility. At this pH, most plasma proteins are negatively charged, and tend to move towards the anode. Antigen and antibody concentrations are chosen such that the antigen is originally in excess with respect to the antiserum, so that no precipitation occurs between them. As the antigen molecules migrate, both through diffusion from the position of sample application and as a result of the applied electric field, they become diluted to such an extent that precipitation is possible. At the "cone" of the "rocket"-shaped precipitate that is formed, there is a constant influx of antigen which dissolves the precipitate, and a stable antigen-antibody complex reforms nearer the anode. Thus the precipitation front is constantly displaced forwards, until its final position is reached when all the antigen is consumed in the formation of a stable precipitate. The precipitin line marks the equivalence point, and the interior of the "rocket" corresponds to an area of antigen excess. Once precipitation is complete, there should be no further migration of the "rocket". However, further movement may be possible if the antigen is of low molecular weight (e.g. orosomucoid, Laurell, 1972).

The position of the precipitation front at the end of the migration can be used as a measure of the amount of antigen present. In electroimmunodiffusion techniques, the peak height is compared with the peak heights obtained from a series of standard antigen solutions to estimate the antigen concentration. Height measurements are not sufficient to quantitate the individual proteins studied by antigen-

antibody crossed electrophoresis. Separation of the proteins according to their electrophoretic mobility in the first dimension migration results in spreading of the protein bands. This leads to broadening of the bases of the precipitin lines, and thus area measurements must be used if the proteins are to be quantitated satisfactorily.

Clarke and Freeman (1966) showed that the area under each precipitin peak was proportional to the concentration of the protein giving rise to it, and inversely proportional to the concentration of the antibody specific for that antigen. In the electroimmunodiffusion techniques, a monospecific antiserum is usually used, and a single antigen quantitated at one time (although it has been shown that two antigens may be quantitated simultaneously , Laurell, 1972). Thus, a single antiserum concentration is used, and a large "rocket" corresponds to a high concentration of antigen, while a small "rocket" suggests the presence of smaller quantities of antigen. If electrophoresis continues until precipitation is complete, then a linear relationship exists between peak height and antigen concentration. In the antigen-antibody crossed electrophoretic techniques, a large number of antigen-antibody systems may be examined. In the multi-valent antiserum used in the second dimension gel, the concentrations of the antibodies to individual proteins will be different. Therefore, a precipitate of large area does not necessarily imply the presence of a large amount of antigen, since the concentration of antibody that is specific for that protein may be low. Standardisation of this method may be achieved in several ways. Clarke and Freeman (1968) compared the area obtained for a particular protein peak with the precipitate area obtained for the same protein in a pooled normal serum, which acted as a reference serum. Arvan and Shaw (1973) have used a commercially-available reference serum in a similar manner, while other workers have related the area obtained for specific proteins to those obtained for a reference standard, such as carbamylated transferrin or acetylated albumin, which is added to the sample in known concentration.

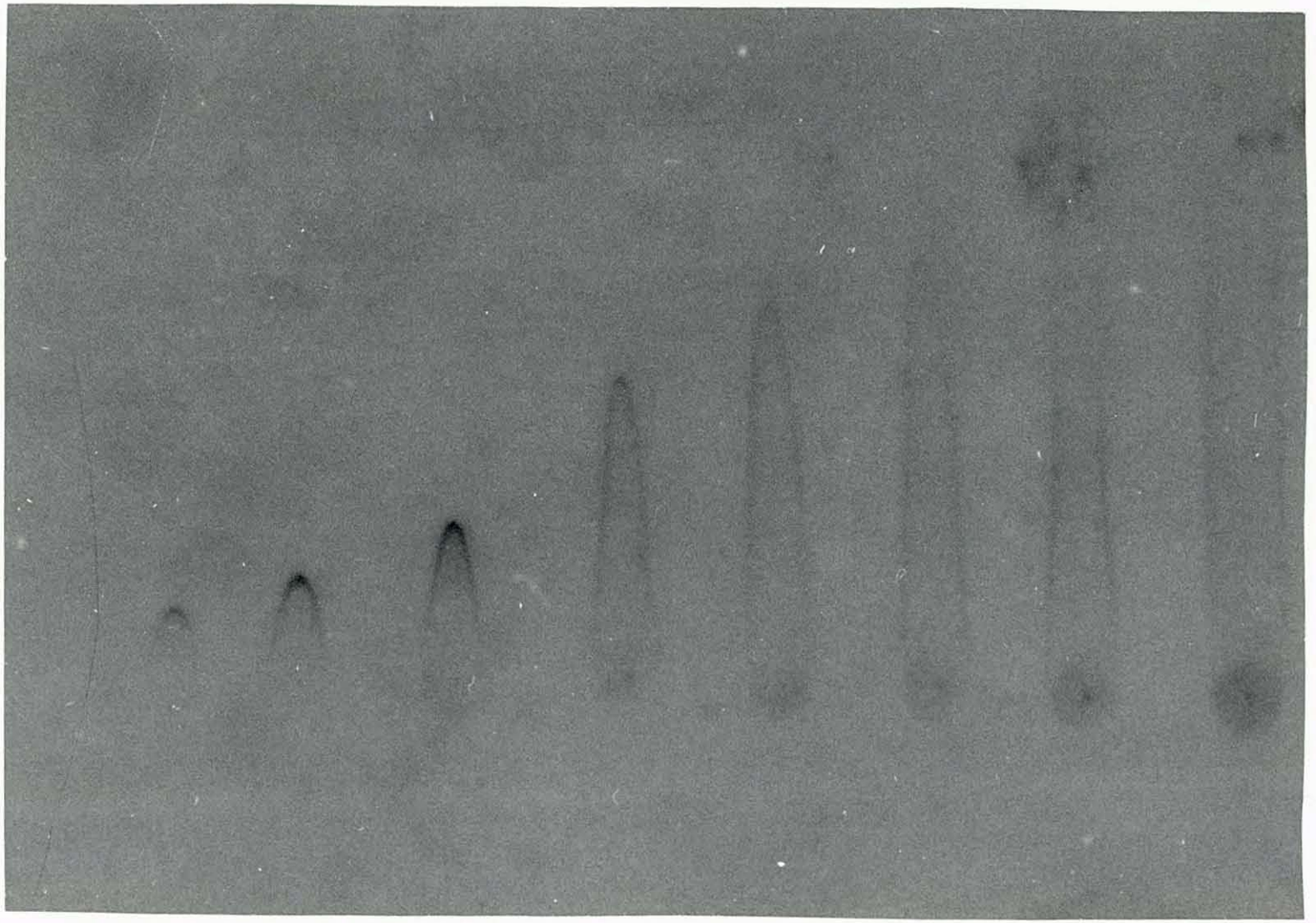
As for the Mancini technique of single radial immunodiffusion, the incorrect choice of antigen to antibody ratio may lead to impaired quantitation. The presence of large quantities of antiserum will result in small precipitate areas, which cannot be measured accurately. If insufficient antiserum is present, complete precipitation may not be possible, since the antigen will always be in excess. If a large excess of antigen is present, the lateral boundaries of the precipitate will diverge, and quantitation will not be possible (see Figure 2(iv)). If large amounts of both antigen and antibody are present, but in the correct proportion, the resultant precipitin lines will be heavy. Adequate quantitation would be possible with smaller amounts of each, but in the same ratio. If insufficient quantities of both antigen and antibody are used, a faint precipitate will result, which may make quantitation difficult, even after staining.

Therefore, in order to quantitate specific proteins by means of antigen-antibody crossed electrophoresis using a multivalent antiserum in the second dimension gel, a concentration of antiserum should be chosen which gives precipitates of suitable size for those proteins of interest. For protein quantitation by electroimmunodiffusion, an antiserum concentration should be selected which permits standardisation over an appropriate range of antigen concentration.

2.3. Electroimmunodiffusion - "Rocket" Technique.

2.3.1. A Survey of Current Methods and Applications of Electroimmunodiffusion Techniques.

The technique described by Laurell (1966) was performed using agarose gel as the carrier medium. Similar techniques have been used in a wide range of applications: for the routine determination of proteins in serum (Scolari, Picard and Heremans, 1968, Krøll, Jensen and Lyngbye, 1970, Weeke, 1972c), as a diagnostic tool for clinical specimens



INCREASING ANTIGEN CONCENTRATION ⟶

Figure 2(iv): Effect of increasing antigen concentration on the heights of the "rockets" obtained by electroimmunodiffusion.

(Laurell, Sjoholm and Johnson, 1970, Weeke, 1972b), and as a means of following the course of a disease (Driscoll, 1973). The technique is suited to large scale routine determinations, because a single plate can be set up to accommodate a large number of samples, in addition to a set of standards. Single radial immunodiffusion (Mancini et al., 1965) is also widely used in routine investigations, but claims a precision of approximately 7%. When the "rocket" techniques is used, with agarose gel as the carrier medium, the analytical error is approximately 1-3% (Krøll et al., 1970). For the techniques developed with cellulose acetate as the carrier medium, coefficients of variation of 5% have been reported (Gill et al., 1971, Pizzolato et al., 1972).

Techniques which have been described using cellulose acetate as the carrier offer certain advantages over the techniques which employ agarose. Preparation time is reduced when cellulose acetate is used, in addition to reduction in electrophoresis, washing, staining and destaining times. While the technique described by Laurell requires 2-10 hours for the electrophoretic migration and several days for the removal of non-precipitated proteins, results may be obtained within five hours when cellulose acetate is used (Watkins et al., 1970). The time required to obtain quantitative results may be reduced of the precipitate formed in the agarose gel is sufficiently distinct to enable measurement to be made by dark-field illumination. Even though precipitates formed in cellulose acetate must always be stained before measurement is possible, results are obtained more rapidly, even for proteins with relatively low mobilities.

In addition to reducing the time required for each test, electroimmunodiffusion techniques using cellulose acetate reduce the amounts of antiserum required (Krøll, 1968) and also reduce the complexity of the apparatus needed, since cooling is not usually necessary during the electrophoretic run.

Some of the applications of the techniques which employ agarose

as the carrier medium have already been mentioned. The applications in which cellulose acetate has been used include the quantitation of plasma proteins in serum (Krøll, 1968, Gill et al., 1971), the quantitation of immunoglobulins in carbamylated serum samples (Watkins et al., 1970), and the estimation of proteins in dilute solutions, such as those found in the urine of patients suffering from proteinuria (Pizzolato et al., 1972). The techniques in which agarose was used have also proved successful in cases where proteins occur in low concentration, such as the cerebrospinal fluid (Schuller et al., 1970, Hirsch-Marie, 1970). The success of these methods is relevant to the present work, since the technique employed should be sufficiently sensitive to enable the plasma proteins to be quantitated in the low concentrations which occur in blood stain extracts. Also of relevance to the present work is the successful application of an agarose method to plasma protein quantitation in samples of whole blood (Daniels et al., 1972).

2.3.2. The Electroimmunodiffusion Technique Developed for the Quantitation of Plasma Proteins in Blood Stain Extracts.

In Chapter 1, the importance of using precise methods for the quantitation of plasma proteins in blood stain extracts was emphasised. Although previous reports suggest that electroimmunodiffusion techniques using cellulose acetate are not as precise as methods using agarose gel, this medium offers certain advantages (Section 2.3.1), and was therefore used in the present work. Modification of the methods described in the literature have improved the precision of the technique.

Cellogel (Chemetron, Milan) was used in preference to other types of cellulose acetate membranes because of its handling properties. It is stored in 30% (v/v) methanol before use, and it was found advisable to soak the Cellogel strips for 10 minutes in the barbital buffer (pH 8.6, Ionic strength 0.02; sodium diethyl barbiturate (BDH Chemicals Ltd.) 4.12g, diethyl barbituric acid (BDH Chemicals Ltd.), 0.736g per litre) which was to be used in the electrophoresis tanks, to remove traces of

the storage fluid. After 10 minutes the Cellogel was transferred for one hour to a vessel containing an appropriate dilution of monospecific antiserum (Dakopatts A/S, Denmark) in barbital buffer (pH 8.6, Ionic strength, 0.02). This method was suggested by Krøll (1968) for antiserum application. Superficial application of antiserum by means of a spreader, followed by equilibration in a humid box, as suggested by Watkins et al. (1970) produced strips which were both unevenly soaked with antiserum, and too wet for reproducible sample application. Distortion of the "rockets" resulted from incomplete absorption of the samples into the Cellogel strip.

The optimum sample size was 1µl. Larger volumes were not evenly absorbed into the cellulose acetate, and smaller volumes could not be delivered with adequate precision. The disposable micropipettes (Camlab, Cambridge) employed for sample application claim to be accurate to within 1%. With practice, it is possible to apply up to eighteen samples along the long axis of the Cellogel strips (5.7 x 14cm) in a short time. Rapid sample application is a pre-requisite of low coefficients of variations for a single strip.

Variations were found to exist across the Cellogel strips. These arose not only from the different extents of diffusion between the samples which were applied to the strip at slightly different times, but also to the greater potential drop, associated with more rapid drying, across the ends of the strip compared with that across the centre. This effect was marked by greater peak heights than expected for samples placed within 2cm of the ends of the strips (Figure 2(v)). This problem was overcome by avoiding the use of the affected area, and the other source of variation was eliminated by employing a suitable order of sample application. It was found that the most accurate results were obtained if standard solutions were applied, in random order, at intervals across the strip. A number of applications of each test solution were applied in random order between the standard solutions.

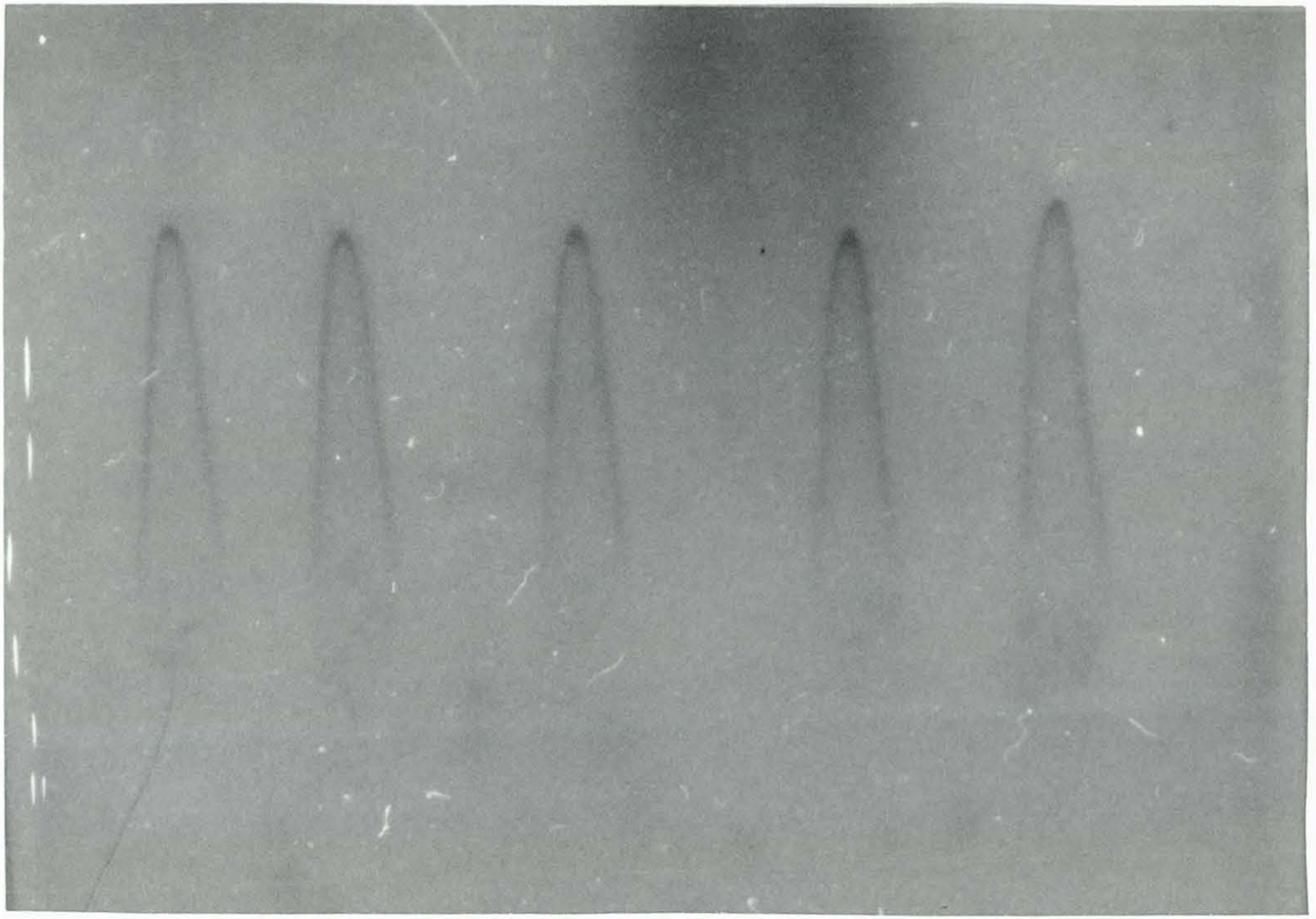


Figure 2(v): Effect of applying samples too close to the end of the Cellogel strip: A higher peak is observed for the sample at the right hand end of the strip.

An optimum ratio of number of standards to the number of sample applications (depending on the number of samples to be examined) was found. For one sample, six standards were applied, with eight sample applications. For two samples, five standards were applied with four applications of each sample, and for three samples, four standards were used and three applications of each sample were made. More than three samples could not be quantitated with adequate precision on a single Cellogel strip.

Blood stain extracts were prepared according to the method described in section 2.6. Standard solutions were prepared for α_1-antitrypsin by diluting the standards supplied in a kit for the routine determination of this protein ("Katikit", Miles Laboratories Ltd., Slough) with 0.9% w/v saline to the concentration range indicated in Table 2.I. Standard solutions were prepared for the other proteins by diluting a stabilised standard serum (Behringwerke Ltd., Hoechst Pharmaceuticals, London) in 0.9% (w/v) saline. It was necessary to prepare fresh standards every day. When standard solutions were stored in aliquots at -15°C, the amounts of specific proteins changed significantly from the amounts present when the standards were first prepared. This was possibly due to the proteins adsorbing to the glass walls of the storage tubes, or perhaps to changes in the proteins following the freezing and thawing procedure. A small quantity of bromophenol blue was added to each standard solution to aid visualisation when the samples were applied. The Cellogel strip, impregnated with antibodies, was removed from the antiserum solution, blotted lightly to remove excess moisture, and placed on a sheet of plastic while the samples were applied, in the manner described above. The loaded strip was then transferred to the electrophoresis tank, which had been made in the Chemistry Department at Loughborough University. It consisted of a perspex box, with a central divider to separate the two compartments containing platimun electrodes. The perspex bridge across which the Cellogel was placed was designed to support the long edges of the strip exactly. The apparatus is illustrated

Table 2.I.

Conditions Used for the Quantitation of Plasma Proteins in Blood Stain Extracts.

Protein	Dilution of Antiserum	Standard Range /mg $100cm^{-3}$	Voltage /v on power pack	Electrophoresis time/hrs.
Prealbumin	1:20	0.714 - 2.0	120	2
Albumin	1:20	17.5 - 35.0	120	2
Orosomucoid	1:40	2.14 - 4.5	120	2
α_1-Antitrypsin	1:20	1.80 - 10.0	120	2
Haemopexin	1:40	1.72 - 4.3	80	5
Haptoglobin	1:40	4.7 - 12.5	80	5
Transferrin	1:40	8.5 - 22.7	80	5
α_2-Macroglobulin	1:40	5.26 - 12.3	80	5
β_1C/A-Globulin	1:50	1.29 - 25.7	80	5

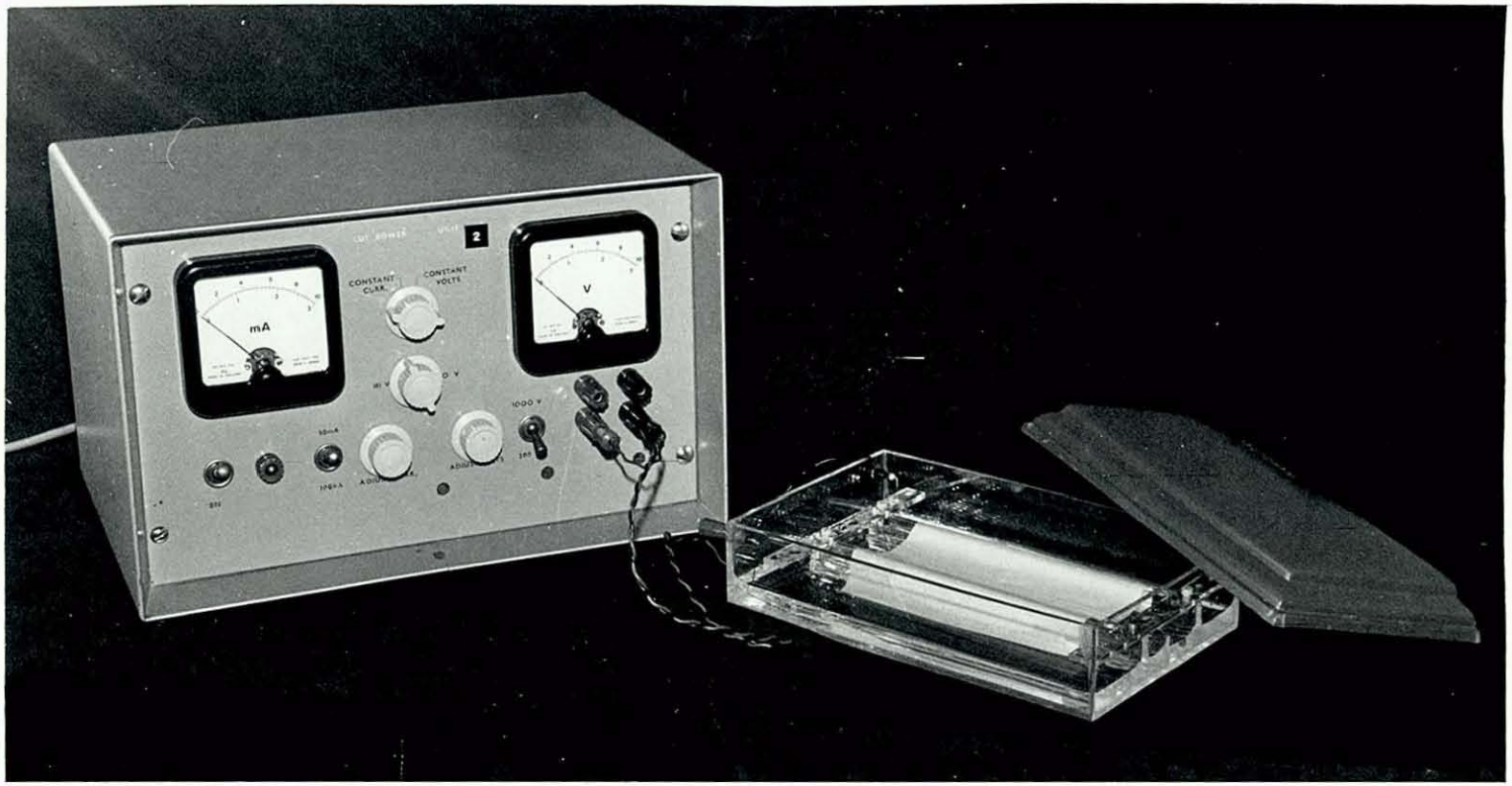


Figure 2(vi) Electrophoresis apparatus used in the electroimmunodiffusion technique.

in Figure 2(vi). Approximately 100cm^3 of barbital buffer (pH 8.6, Ionic strength 0.02) were placed in each electrode compartment, and the levels were equalised by tipping the tank. This prevented the siphoning of buffer through the Cellogel strip. The cellulose acetate was connected to the buffer by means of wicks prepared from a single thickness of chromatography paper (Whatman No.1.), 5 x 14cm, soaked in barbital buffer. The lid was placed over the tank and direct current was supplied to the electrodes, at constant voltage from power packs (Shandon Southern Ltd., Camberley, Surrey). Electrophoresis proceeded for the required time at the voltages specified in Table 2.I.

At the end of the electrophoretic migration, the current was switched off, and the Cellogel strip transferred to a tray containing 0.9% (w/v) saline. It was washed in this solution for 10 minutes to remove non-precipitated protein. The precipitates were stained in 0.25% (w/v) Coomassie Blue RL (Searle Scientific Services Ltd.) in methanol: acetic acid:water (5:1:5, v:v:v) for 5 minutes, and destained in 10% (v:v) acetic acid. The peak heights were measured from the point of sample application by means of vernier calipers.

The equation of the straight line through the standard points, with a correlation coefficient closest to one, was computed using a Sumlock Statistician calculator (Compucorp.). The concentration of the antigen in the test solutions was calculated by substituting the mean of the peak heights obtained for each solution into the equation.

The coefficients of variation obtained for dilute whole blood and dilute serum samples, applied to a single strip are given in Table 2.II.

Table 2.II.

Intra-strip Variation Using Dilute Serum and Dilute Whole Blood as Samples.

Protein	Coefficients of Variation for (i) Serum sample	(ii) Whole blood sample
Prealbumin	2.2	2.4
Albumin	4.8	1.4
Orosomucoid	2.6	3.4
α_1-Antitrypsin	2.1	3.4
Haemopexin	---*	---*
Haptoglobin	1.7	1.3
Transferrin	2.0	3.4
α_2-Macroglobulin	2.2	1.3
β_1C/A-Globulin	3.9	3.5
mean	2.7	mean 2.5

*At an early stage it was discovered that haemopexin was not a suitable protein to study, because a stable standard was not available.

Inter-strip variation was calculated for dilute serum samples only, and the results are given in table 2.III.

Table 2.III.

Inter-strip Variation in the Concentrations Calculated for a Number of Plasma Proteins in Dilute Serum Samples.

Protein	Coefficient of Variation
Prealbumin	12.4
Albumin	10.8
Orosomucoid	10.7
α_1-Antitrypsin	3.1
Haptoglobin	4.4
Transferrin	13.6
α_2-Macroglobulin	2.0
β_1C/A-Globulin	4.2
mean	7.7

2.4. Antigen-Antibody Crossed Electrophoresis.

2.4.1. A Survey of Current Methods and Applications of Antigen-Antibody Crossed Electrophoretic Techniques.

Many of the methods now available for antigen-antibody electrophoresis are modifications of the technique described by Clarke and Freeman (1968). In their method, electrophoretic separation of human serum was carried out in agarose gel. At the end of the separation, the gel containing the separated proteins was cut out and transferred to a second plate. The remainder of this plate was covered with agarose gel, containing the required concentration of antiserum. Electrophoresis proceeded with the first dimension gel on the cathode side, so that a majority of the plasma proteins migrated into the second dimension gel. At the end of the run, the plate was washed extensively in physiological saline, dried, and then stained with a suitable protein stain. Acetylated albumin acted as an internal standard, reducing sampling errors in the

technique. The corrected peak areas were then related to the areas obtained for the same protein in a reference serum. Using this technique coefficients of variation of approximately 10% were obtained.

Various media, other than agarose, have been used. Johansson and Stenflo (1971) and Giebel and Saechtling (1973) attempted to incorporate a first dimension separation in polyacrylamide into the technique, but with limited success. Pizzolato (1973) and Miller and Mutzelberg (1973) performed the technique using cellulose acetate membranes. Although results were obtained more rapidly than with agarose, large coefficients of variation (14%) suggested that the methods were unsuitable for precise quantitative work. It therefore appears that highest precision can be achieved with tehcniques employing agarose as the carrier medium, for both dimensions. Modifications have been made to the technique according to Clarke and Freeman (1968), in order to make the method more suitable for routine investigations. Firestone and Aronson (1969), for instance, described a technique which was rapid, and economical in its use of antiserum. Wright et al. (1974) have also developed a rapid technique using agarose, but in both instances, coefficients of variation were too large for successful quantitative work.

Davies et al. (1971) introduced a technique which employed smaller plates (5 x 5cm) than those (10 x 10cm) recommended by Clarke and Freeman (1968). The use of smaller plates reduced the amount of antiserum required without loss of precision in the technique. Attempts have been made to improve the precision of the technique. Weeke (1970a) claims that the coefficients of variation for the technique can be improved significantly if carbamylated transferrin is used as an internal standard. Acetylated albumin has also been used as an internal standard (Clarke and Freeman, 1968, Davies et al., 1971) but since this has an electrophoretic mobility of approximately twice that of albumin, the first dimension separation time has to be reduced in order to prevent the standard moving off the plate. Carbamylated transferrin has an electrophoretic mobility only slightly higher than albumin. Versey,

Slater and Hobbs (1973) have suggested that when serum samples are examined, the transferrin present in the sample may be used as an internal standard. However, this involves the use of an additional method, such as electroimmunodiffusion, in order to quantitate this protein.

A number of methods are employed for measuring the areas under the precipitin arcs. The plate may be enlarged, and the peak areas of the projected image measured by planimetry, either manually or with the use of an electronic integrator. A tracing may be made of the image and the relevant areas of paper cut out and weighed, or the number of millimetre squares covered by the precipitate may be counted. However precise the method of measurement may be, the accuracy with which the areas can be measured will depend upon the nature of the precipitate and of the "base-line". In the technique described by Clarke and Freeman (1968) the sample well is approximately 2-3mm from the junction of the first and second dimension gels. Thus, in the second electrophoretic stage, the proteins must migrate through a portion of the first dimension gel before reaching the antiserum-impregnated gel. This area, although originally devoid of antibodies, may contain a low concentration of antiserum through migration of antibodies towards the cathode. Thus some precipitation tends to occur in the first dimension gel, and the "base-line", i.e. the line drawn between the extremities of the precipitate, is below the junction of the two gels. The resultant "base-line" will slope up from the sample well as illustrated in figure 2(iii). A recent modification of the technique by Bradwell and Burnett (1975) in which the sample migrates along the edge of the first dimension gel removes much of the error attributed to a sloping "base-line".

A major disadvantage of the technique is encountered when the identity of the precipitates of interest is to be established. When a serum sample is examined with the use of anti-whole human serum, up to sixty different precipitates have been observed (Freeman and Smith, 1970). Identification of specific precipitates almost always involves

the use of supplementary techniques (Krøll, 1969, Weeke 1970b, Stephan and Frahm, 1970, Axelsen and Bock, 1972). Ganrot (1972) suggests the use of an antiserum raised against only a small number of proteins in the second-dimension gel, to simplify the identification procedure.

Axelsen and Bock (1972) have reviewed a number of two-dimensional methods which are currently used for the identification and quantitation of antigens and antibodies. Amongst those described is the "tandem" antigen-antibody crossed electrophoresis, which is examined in greater detail by Krøll (1973). Two samples are compared in a single run, placing them originally in separate wells in the first-dimension gel, spaced so that related proteins in the two samples give rise to precipitin peaks which fuse during the second electrophoretic migration. An electropherogram of the type illustrated in figure 2(vii) is obtained. In this way, two samples may be compared on a single plate, and height measurement may be used to determine the relative amounts of a particular antigen in the two samples, with a precision of 4-6% (Krøll, 1973).

Antigen-antibody crossed electrophoresis has been used as a means of examining genetic variants, microheterogeneity, complex formation and fragmentation. Polymorphic variants have been examined by Laurell (1965), and the changes in plasma proteins during the course of a disease followed by Rebeyrotte et al., (1970), Clarke et al., (1970) and by Versey (1973). Proteins in biological fluids other than serum have been examined (Raisys and Arvan, 1971). The technique has also been used for the examination of whole blood samples (Saint-Paul et al., 1971, Saint-Paul et al., 1972), and of blood stain extracts (Whitehead et al., 1970).

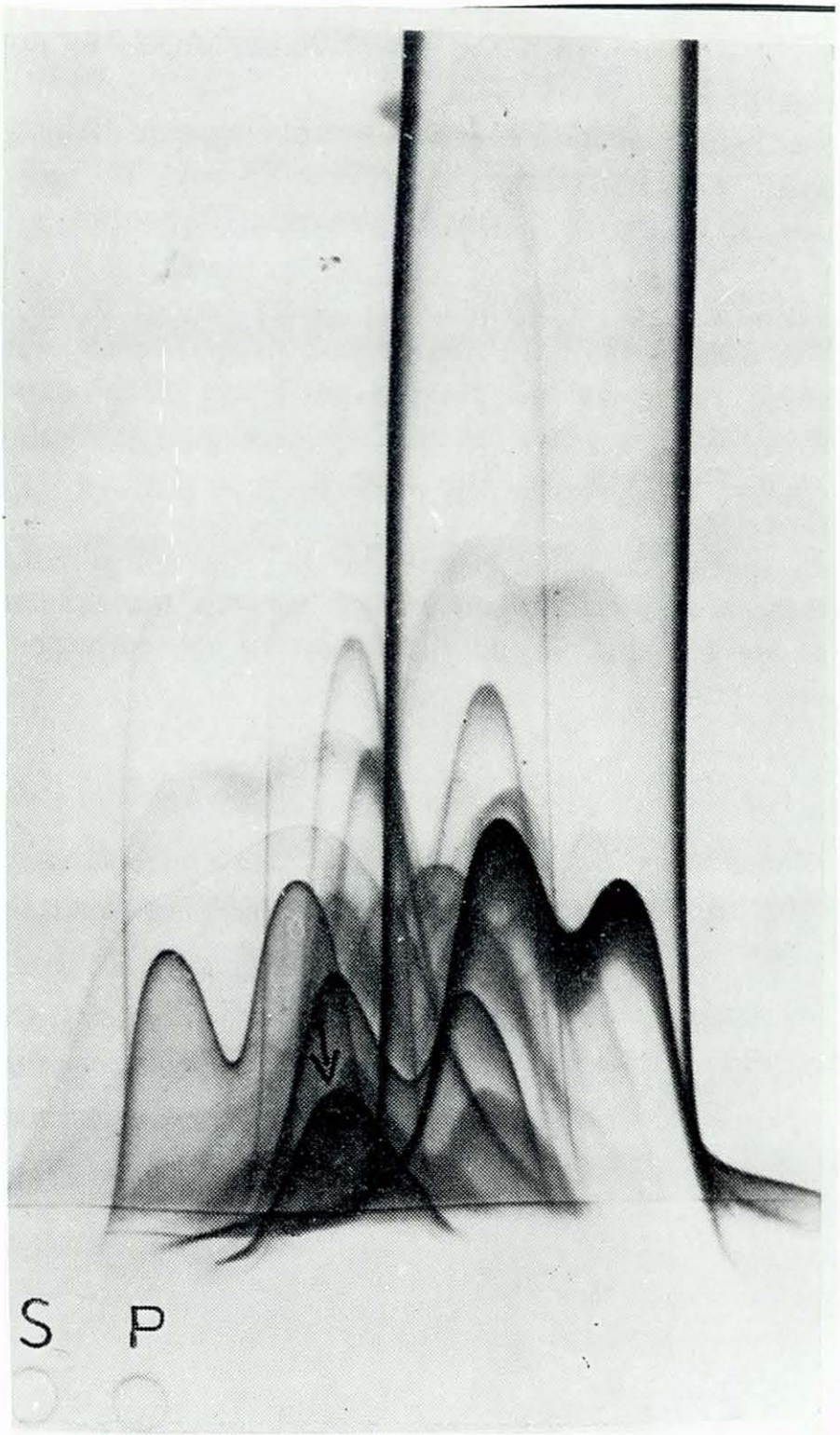


Figure 2(vii) "Tandem" antigen-antibody crossed electrophoresis: Serum (S) and plasma (P) samples examined in agarose containing anti-whole human serum in the second dimension gel.

2.4.2. The Antigen-Antibody Crossed Electrophoretic Techniques Developed for the Quantitation of Plasma Proteins in Blood Stains.

In the present work, single sample (Figure 2(viii)) and tandem antigen-antibody crossed electrophoretic techniques (Figure 2(ix)) were developed for the determination of plasma protein concentrations in blood stain extracts. In each case, an artificial antiserum was used which contained antibodies specific for five proteins only. 5 x 5cm glass plates were used as supports for the agarose gel in which the electrophoretic migrations took place.

The method used was similar to the "micro-technique" described by Weeke (1973). It differed from that developed by Davies et al. (1971) in that the first and second electrophoretic migrations took place on separate plates, several first dimension separations being performed on one large (10 x 10cm) plate (Figure 2(x)). The first dimension gel was prepared by pouring $15cm^3$ of 1% (w/v) agarose (l'Industrie Biologique Francaise, S.A., France) in barbital buffer (pH 8.6, Ionic strength 0.02; sodium diethyl barbiturate (BDH Chemicals Ltd.) 4.12g, diethyl barbituric acid (BDH Chemicals Ltd.) 0.736g per litre), containing 0.1% (w/v) sodium azide onto the clean glass plate, resting on a level surface. This gave a gel 1.5mm thick. After the gel had solidified, sample wells were cut according to the scheme illustrated in Figure 2(x), using a piece of 2mm diameter steel tube, and the plugs of agarose removed by suction. When the "tandem" technique was performed, two sample wells were cut, with their centres 5mm apart. If the wells were too close together (less than 3mm), no resolution was possible between identical proteins present in the two samples. If the wells were too far apart (more than 10mm), no fusion occurred between the precipitates of identical proteins.

The samples were prepared by adding a fixed volume of stock solution of carbamylated transferrin, prepared according to Weeke (1970a), to a known volume of blood stain extract, or standard serum. 3µl of the

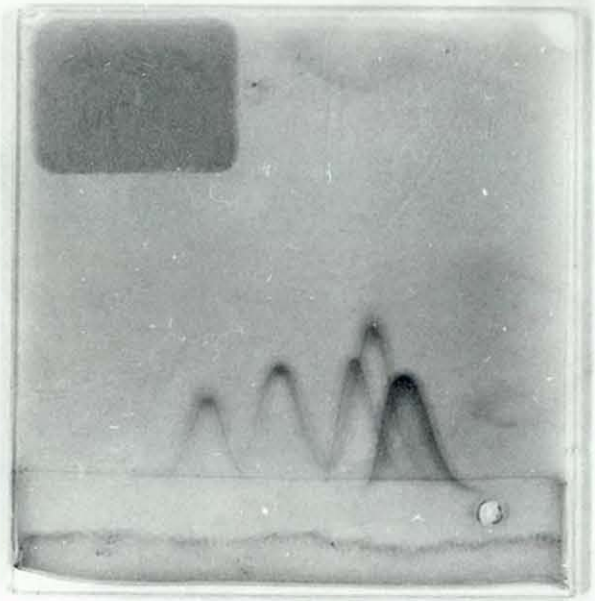


Figure 2(viii)

Antigen-antibody crossed electrophoresis:
a dilute solution of blood examined, using
a mixture of five monospecific antisera in the
second dimension gel.

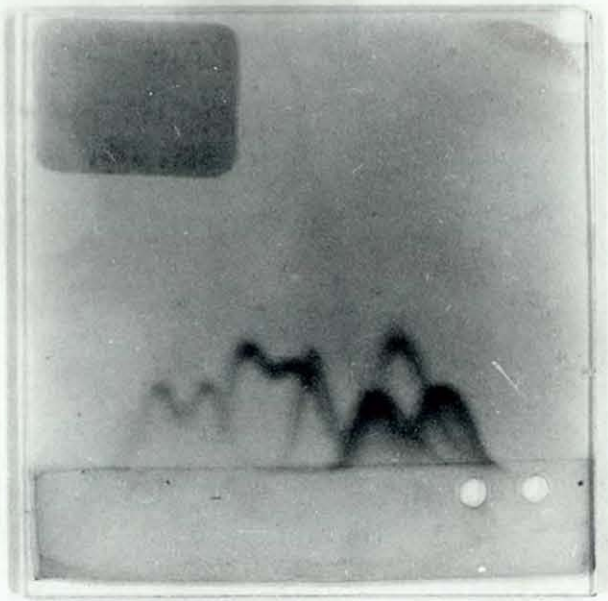


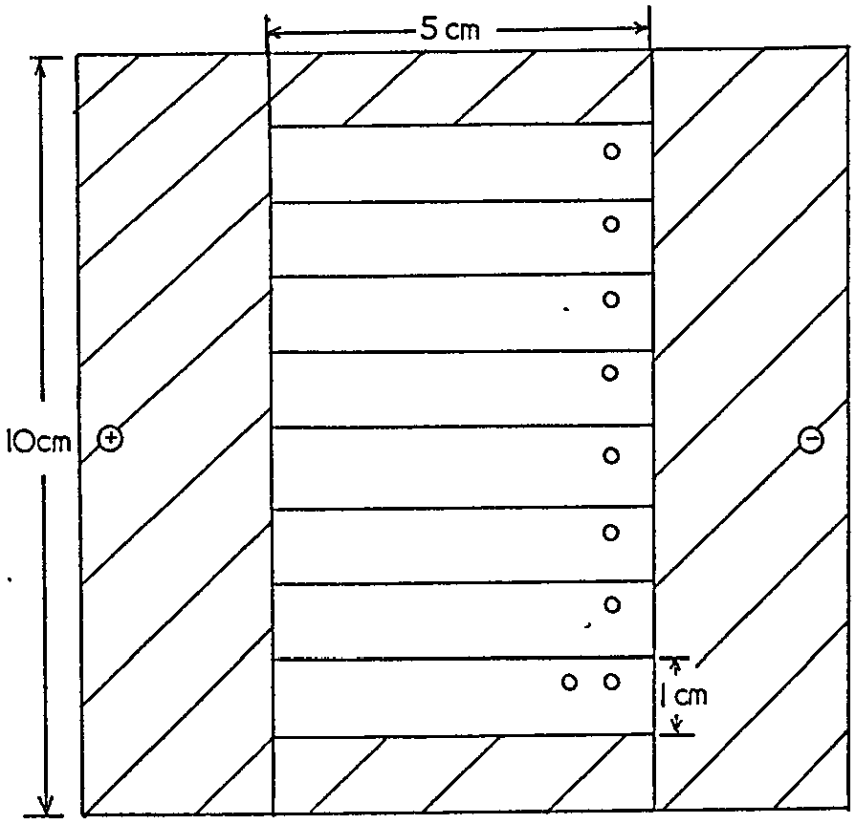
Figure 2(ix): "Tandem"

Antigen-antibody crossed electrophoresis:
Two dilute solutions of whole blood from different
donors examined, using a mixture of five
monospecific antisera in the second dimension gel.

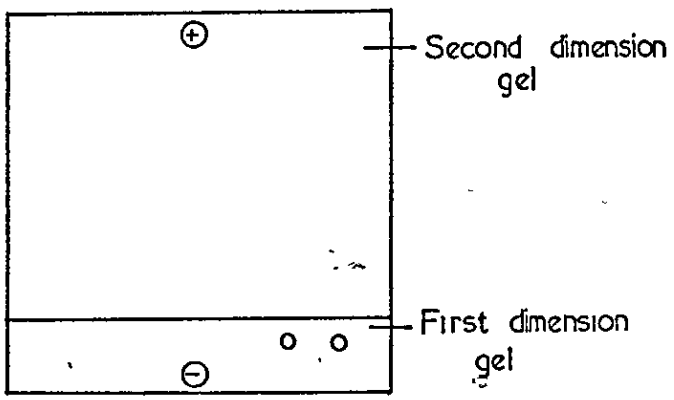
Figure 2(x).

Templates used in the antigen antibody crossed electrophoresis techniques.

A FIRST DIMENSION PLATE



B SECOND DIMENSION PLATE.



relevant solutions were then placed in the appropriate wells using a 5µl Hamilton syringe, with a straight-ended needle, and the plate transferred to the water-cooled surface of the electrophoresis tank (MBI Ltd., Ashford, Kent). Lint wicks (10 x 10cm) connected the gel to the tank buffer (barbital buffer, pH 8.6, ionic strength 0.02). Adequate contact was maintained between the gel and the buffer in this way, and although some drop in the potential occurred across the wicks, this was not as great as that encountered when paper wicks were used. When a potential drop of 200v was recorded on the power pack, a drop of 55v was measured across the second dimension gel, when lint wicks were used. When paper wicks were applied, a drop of only 28v was recorded across the gel when 200v was recorded on the power pack. Electrophoresis proceeded at 175v for 1-1½ hours. At the end of this time, the portions of the gel containing the separated proteins were carefully cut out, and transferred to the second dimension plates (Figure 2(x)). Care was taken to ensure that the gels were cut at right angles to the surface, since an oblique edge to the first dimension gel could lead to "doubling" of the precipitates formed (Clarke and Freeman, 1968).

The remainders of the second dimension plates were covered with $3cm^3$ of 1% (w/v) agarose in barbital buffer (pH 8.6, ionic strength 0.02) containing 0.1% sodium azide, to which had been added a fixed volume of an artificial antiserum. The antiserum had been prepared by mixing suitable volumes of the purified immunoglobulin fractions of five monospecific antisera (Dakopatts A/S, Denmark). Krøll (1973) emphasises the importance of using an antiserum specific for less than fifteen components in the "tandem" technique. Location of the relevant peaks becomes increasingly difficult when more precipitin arcs are present; not only is it difficult to locate the precipitin lines of the proteins of interest, but it is also difficult to locate the peaks of those lines which have arisen from identical proteins in the two samples. Antisera raised against human prealbumin, orosomucoid, haptoglobin, α_2-macroglobulin

and transferrin were used. The immunoglobulin fractions of each antiserum were prepared according to the method of Harboe and Ingild (1973). Purification was necessary to avoid any cross-reaction which might occur between the antibodies of one antiserum and any human plasma proteins which might be present as contaminants of another antiserum. Concentrations of each antiserum were chosen to give precipitin peaks of suitable size for each protein.

The second dimension plates were transferred to the electrophoresis tank, lint wicks (5 x 10cm) connected, and electrophoresis proceeded for 18 hours at 75v. At this stage it was necessary to use large volumes of tank buffer. If insufficient buffer was present, no precipitates were obtained, possibly because the pH of the gel was not maintained at 8.6. At the end of the second electrophoretic stage, the plates were washed in 0.9% (w/v) saline for three days, with frequent changes of saline to remove non-precipitated protein. The plates were then stained in 0.02% (w/v) nigrosine (Hopkin & Williams Ltd.) in 5% (v/v) acetic acid for one hour, and destained in 10% (v/v) acetic acid. The destained plates were allowed to dry at room temperature.

When only one sample was applied, quantitation was performed by enlarging the pattern obtained in a photographic enlarger, tracing around the images of the precipitin lines, and then measuring the areas by planimetry. When two samples were compared, by the "tandem" technique, the completed plates were placed on millimetre graph paper, and the peak heights measured in this way.

The coefficients of variation obtained for each of the five proteins measured on six plates are indicated in Table 2.IV. This table also shows the coefficients of variation obtained when the peak areas were related to the areas of the carbamylated transferrin precipitates.

Table 2.1V.

Coefficients of variation for the antigen-antibody crossed electrophoresis technique. Measurements were made on six plates, and the areas also related to the carbamylated transferrin peak area.

	Coefficients of Variation.	
Protein	(i) Area measurements	(ii) Areas related to the carbamylated transferrin peak
Prealbumin	4.0	3.3
Orosomucoid	6.9	4.4
Haptoglobin	3.2	5.1
α_2-Macroglobulin	8.1	7.7
Transferrin	6.0	4.2
	mean 5.6	mean 4.9

2.5. Other Immunological Techniques and their Application to the Present Study.

During the course of the experiments designed to determine the effect of the age of the blood stain on the plasma protein profile obtained, it became apparent that some of the proteins were unstable on storage. Some qualitative techniques were employed in an attempt to discover the nature of the changes that the proteins underwent. The additional methods used were classical techniques, which required only slight modifications to enable measurements to be made using blood stain extracts as the samples.

2.5.1. Immunoelectrophoresis.

Immunoelectrophoresis is a two-stage procedure, employed for

the qualitative examination of the individual proteins in a mixture. The protein mixture is first fractionated by electrophoresis in a carrier medium, and the separated proteins then allowed to react with antiserum placed in a trough, cut parallel to the direction of electrophoretic migration. Reaction occurs after the proteins and antibodies have diffused through the carrier medium.

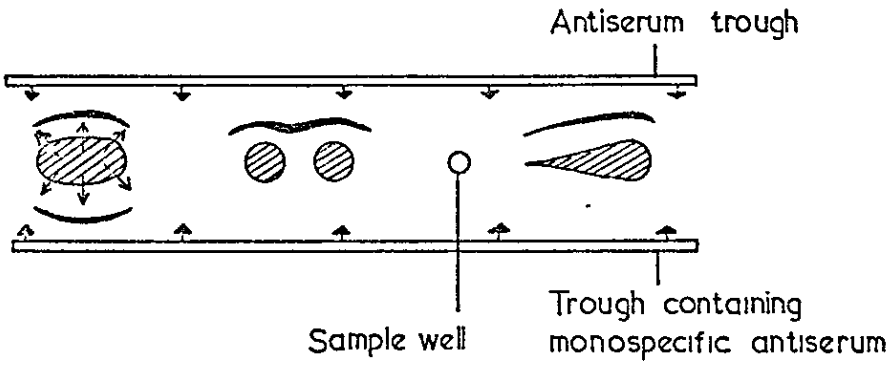
The technique was first described by Grabar and Williams (1953), but the micro-technique introduced by Scheidegger (1955), in which agar gels on microscope slides were used, has found wide application. A variety of carrier media have been employed for this technique, but for each the choice of a suitable buffer is important. A buffer chosen to give optimum electrophoretic conditions may not prove satisfactory for the immunodiffusion stage. As a general rule, the electrophoretic stage should be carried out as rapidly as possible, but avoiding the production of excessive heat. Cooling is usually necessary during the electrophoretic separation.

The shape of the precipitin arcs depends on the shapes of the individual protein bands at the end of the electrophoretic migration, and on the dimensions of the immunoelectrophoresis plate. Following the electrophoretic separation, the antigen fractions normally assume one of several shapes. They may be round or ellipsoid, they may be drawn out on either side of the centre, equally or unequally, or they may be "comet"-shaped, with a tail before or behind. During the diffusion stage, the antigen molecules will diffuse radially from their sources, while the antibodies diffuse in a single line from the antiserum trough, as indicated in Figure 2(xi). If the antigen band was originally round, a semicircular arc is formed, and if it was originally a symmetrical ellipse, an arc which is symmetrically ellipsoid is formed. A long arc of moderate curvature suggests that the antigen is electrophoretically heterogeneous.

The shape of the sample well can affect the shapes of the

Figure 2 (xi).

Diagram to illustrate the principles of immunoelectrophoresis.



Protein band

Precipitin band

Direction of antibody diffusion

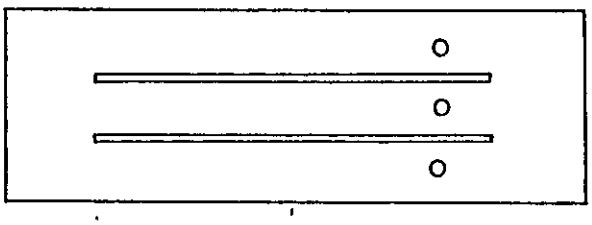
Direction of antigen diffusion

precipitin arcs formed. If the sample well is small, antigens will diffuse more rapidly from it than from a large sample well, and the resultant arc will be short, and acutely curved. If the antiserum trough is cut close to the separated proteins, precipitin arcs are formed rapidly, since the distance which antigens and antibodies have to travel before they react, is short. The arcs formed will be acutely curved.

The method adopted for the examination of individual proteins in blood stain extracts consisted of agarose gel electrophoresis of the proteins followed by immunodiffusion in the same gel. Microscope slides (2.5 x 7.5cm) were placed on a level surface and coated with $3.75cm^3$ of molten 1% (w/v) agarose (l'Industrie Biologique Francaise S.A., France) in barbitone buffer (pH 8.6, ionic strength 0.02; sodium diethyl barbiturate (BDH Chemicals Ltd.), 4.12g, diethyl barbituric acid (BDH Chemicals Ltd.)0.736g per litre) containing 0.1% (w/v) sodium azide. The gel was allowed to solidify, and sample wells then cut using a length of 3mm diameter sharpened steel tubing, using a template as illustrated in figure 2(xii), to determine the positions of the sample wells.

Figure 2(xii).

Template used for cutting sample wells and antiserum troughs on immunoelectrophoresis plates.



The plugs of agarose were removed from the wells by means of suction. It was found that the concentration of the plasma proteins in the blood stain extracts was so low that concentration of the sample was necessary before sufficient sample could be applied in the wells to produce strong precipitation bands. The samples were concentrated 3 to 4 times using Minicon concentrators (Amicon Corporation, High Wycombe), before 5-10µl of the sample was applied in the wells. Lint wicks were used to connect the agarose gels, placed on a water-cooled surface in an electrophoresis tank, to the barbitone buffer (pH 8.6, ionic strength 0.02) in the electrode vessels. Electrophoresis proceeded at 175v for 1-1½ hours. At the end of this time, troughs were cut, according to the template pattern, using a razor blade, and filled with 75µl of the relevant monospecific antiserum (Dakopatts, A/S, Denmark). The plates were then left in a humid box for 48-72 hours, when the precipitin bands developed. The gels were washed in 0.9% (w/v) saline for 3-4 days, with frequent changes of saline to remove non-precipitated protein, stained in 0.5% (w/v) Procion blue in 10% (v/v) acetic acid. Destaining was achieved using 10% (v/v) acetic acid. Finally, the plates were dried at room temperature.

2.5.2. Double Diffusion in Two Dimensions, (Ouchterlony Method).

Ouchterlony (1948) and Elek (1948) independently devised a method which facilitated the qualitative comparison of the antigens present in two or more solutions. Diffusion of both the antigen and antibody was necessary before precipitation was possible, and the nature of the precipitin lines formed gave some indication of the serological relationship between the anitgens present in adjacent positions.

In the Ouchterlony method, a central well, containing antibody, is symmetrically surrounded by a number of wells in which the antigens are placed. The antiserum and antigens diffuse radially from the application wells and when the equivalence ratio is reached between them,

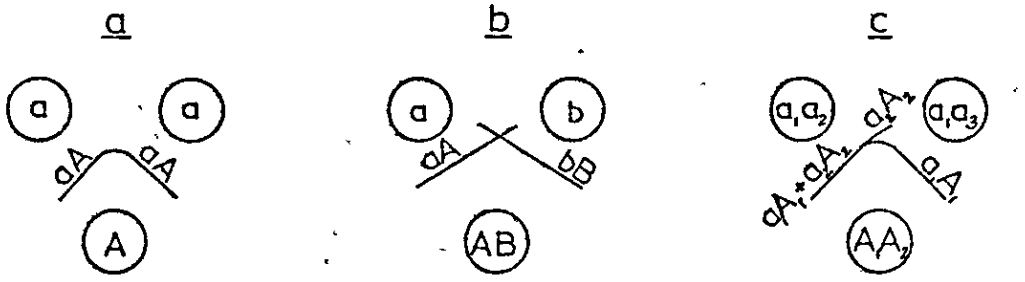
precipitation occurs. Usually, proportions of antigen and antibody are chosen which are approximately equivalent, but small differences in the antigen-antibody ratio are compensated for once the two reactants meet. As the antigen diffuses, for instance, it will encounter antibodies, whose concentration will increase as the antigen diffuses further. Precipitation will occur when the antigen and antibody are at the equivalence ratio, and the precipitin line will therefore form closer to the origin of the weaker reactant. It will become stabilised as both antibody and antigen feed into it at equivalent rates.

When two antigens which are immunologically identical are placed in adjacent wells, with antiserum to the antigen placed in the central well, the precipitin lines forming between each sample of the antigen and the antiserum will fuse. The pattern obtained is indicated in figure 2(xiii)a, which suggests that the two samples were identical. It the two samples contained antigens which were different, and antibodies to both were present in the antiserum, then precipitin lines would form, but would not fuse, indicating non-identity between the antigens (Figure 2(xiii)b). When the two samples contained antigens which were partially identical, i.e. when they have only a proportion of their antigenic groups in common, and the antiserum is capable of reacting with the groups which are common to both samples, as well as those which only one sample possesses, then a pattern of partial identity is obtained (Figure 2(xiii)c). The formation of the "spur" at the point of confluence is caused by the reaction between the antigen groups, present in one sample, but absent in the other, and specific antibodies to these. In a well-controlled system, the length and intensity of a spur is inversely proportional to the degree of antigenic similarity between the two antigens being studied.

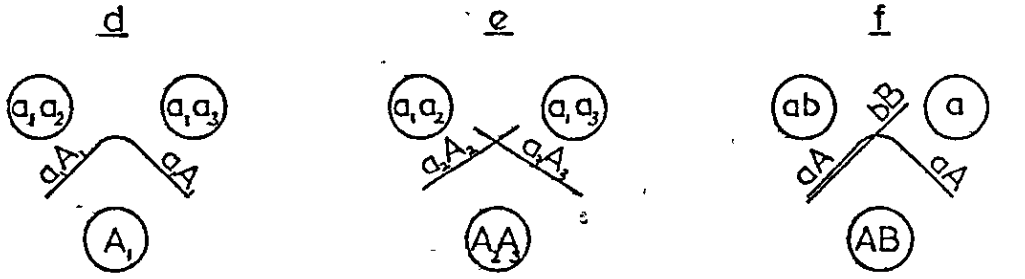
In all cases, but especially when spurs are formed, careful interpretation of the patterns obtained by the Ouchterlony method must be made. Incorrect analyses may be made because of the inability to

Figure 2.(xiii)

Precipitin reactions commonly observed in double diffusion plates



Identity | Non-identity | Partial identity



False reaction of identity | False reaction of non-identity | False reaction of partial identity

evaluate the pattern obtained correctly, or because the conditions used cannot satisfactorily illustrate the nature of the identity of the proteins concerned (Crowle, 1961). For instance, false reactions of identity may occur if the antiserum only contains antibodies directed against the common antigenic groups of two related, but non-identical proteins (Figure 2(xiii)d). A false pattern of non-identity will result if two samples are partially identical, and the antiserum contains only antibodies to the non-common sites (2(xiii)e). False spurring can arise if precipitation occurs between two antigen-antibody systems in one plane. Apparently, a single line, extending into a spur is formed, but actually, two lines are present (2(xiii)f).

It was necessary to concentrate the blood stain extracts used in the present study, in order to apply enough antigen in the sample wells to give distinct precipitin lines. Plates were prepared by pouring $5cm^3$ 1% (w/v) agarose (l'Industrie Biologique Francaise, S.A.) in barbital buffer, (pH 8.6, ionic strength 0.02) containing 0.1% (w/v) sodium azide onto glass plates (5 x 5cm), and allowing it to solidify before the sample wells were cut. This was performed using a cutter prepared in the Chemistry Department at Loughborough University, which enabled four patterns to be accommodated on one plate. The sample wells were 3.5mm in diameter, and the six sample wells were equidistant from the central antiserum well, with the centres of the wells 6mm apart. 2-10μl of blood stain extract (prepared as indicated in section 2.5, and then concentrated 3-4 times using a Minicon concentrator (Amicon Corporation)) was placed in each sample well, and 10μl of monospecific antiserum (Dakopatts A/S, Denmark) placed in the central well. The plates were then left in a humid box for 24-48 hours until the precipitate formed.

Evaluation of the patterns obtained was possible after non-precipitated protein was washed from the agarose with several changes of 0.9% (w/v) saline, the precipitates stained with 0.5% (w/v) Procion

blue in 10% (v/v) acetic acid, and background staining removed by washing in 10% (v/v) acetic acid. The plates were dried at room temperature.

2.5.3. Quantitative Immunoprecipitation.

The precipitin reaction described by Heidelberger and Kendall (1929), and referred to in section 2.1, has been used for the quantitative estimation of antigens and antibodies. Antigens can be quantitated in the region of antibody excess, employing this part of the precipitin curve as a standard range. In addition, the precipitin curve may be used to give further information concerning the nature of the antigen-antibody reaction, if the entire curve, from antibody excess to antigen excess is constructed.

The antibody population of any serum is heterogeneous, and the precipitation reaction observed between a particular antigen and the antibodies raised against it consists of a large number of specific reactions. The precipitin curve describes the average behaviour of the heterogeneous antibody population towards the antigen. All of the factors which contribute to the shape of the precipitin curve are not known, but the solubility of the antigen-antibody complex is important.

The reaction between antigen and antibody is influenced by a number of factors (Maurer, 1971), and hence it is important that the quantitative precipitin reaction is performed under carefully controlled conditions. The pH and ionic strength of the buffer in which the reaction takes place should be carefully chosen. The temperature should be controlled during the incubation period. Other factors which influence the amount of precipitate formed are the rate at which antigen and antibody are mixed, the volume of the reaction mixture, and the state of aggregation of the antigen (Maurer, 1971).

The amount of precipitate formed can be estimated by a number of methods, both physical and chemical (Kwapinski, 1965). Quantitative evaluation may be achieved by gravimetric techniques, or by turbidimetric

techniques. The precipitate formed may be washed and dissolved and the absorbance of the resultant solution measured. The chemical methods employed estimate the amount of protein precipitated.

The classic method of Heidelberger and Kendall (1929) employed the Kjeldahl technique for the estimation of total nitrogen precipitated. More recently, other techniques of protein quantitation have been used, including the ninhydrin, Folin-Ciocalteau or Biuret methods.

The procedure adopted in the present work is similar to that described by Li and Williams (1971), measuring the extent of precipitation by the optical density of the suspension formed. The relationship between turbidity and antigen concentration is very complex, but an approximate equation has been put forward by Wells (1927):

$$T = k \frac{cld^3}{d^4+\alpha\lambda^4}$$

where T = turbidity

c = concentration

l = thickness of medium layer

d = average thickness of suspended particles

λ = wavelength of the illuminating light

k and α are constants

From this equation it can be seen that the turbidity increases with decreasing wavelengths. Therefore, when economical use of antiserum is important, or, as in the present case, when there is a limited supply of antigen, it is important to measure the turbidity of the solutions at low wavelength. In determining the optimum wavelength for a particular antigen-antibody system, regions in which substances, present in either the antigen solution or antiserum, strongly absorb should be avoided. In the present study, the choice of a suitable wavelength was mainly affected by the absorption bands of haemoglobin. This absorbs strongly between 400 and 420nm and less strongly at 540nm. Although measurements might be made at 500nm, a shorter wavelength was preferred

since higher values were obtained for the turbidities of the solutions. 320nm was chosen as the optimum wavelength, but a complete series of "antigen blanks" (containing no antiserum) had to be prepared to provide suitable controls.

Precipitin curves were obtained for albumin, extracted from blood stains of different ages. It was impractical to attempt to obtain precipitin curves from the other plasma proteins studied in ageing blood stains, because they are present in such low concentrations that large amounts of sample would be required to provide sufficient material. Dilutions of blood stain extract were prepared in phosphate buffered saline (pH 7.5; 85.2cm^3 of a solution of $Na_2HPO_4.2H_2O$ (BDH Chemicals Ltd.), 11.9g per litre of 0.9% (w/v) saline, mixed with 14.8cm^3 of a solution of KH_2PO_4 (BDH Chemicals Ltd.), 9.1g per litre of 0.9% (w/v) saline). The range of albumin concentrations covered (determined by the "rocket" technique) was approximately 0-100μg per cm^3. 0.5cm^3 of each dilution was transferred to test tubes, in duplicate, and allowed to equilibrate at 37°C. A 1:10 dilution of anti-albumin serum (Dakopatts A/S, Denmark) in phosphate buffered saline was prepared, and also allowed to equilibrate at 37°C for 15 minutes. After 15 minutes, 0.5cm^3 of the antiserum dilution was added, at timed intervals, to each tube in the first series of diluted extracts. The solutions were carefully mixed, and left at 37°C for 1 hour. The second series of dilutions were to provide the "antigen blanks", and 0.5cm^3 of phosphate buffered saline was added to each. The solutions were mixed and left at 37°C for 1 hour.

At the end of the incubation period, the optical densities of the solutions were read using an SP 500 spectrophotometer (Pye-Unicam) with silica cells (2mm x 10mm path length) at 320nm. Each of the test solutions was read against an antiserum blank (containing 0.5cm^3 antiserum dilution mixed with 0.5cm^3 buffered saline) and each of the "antigen blanks" against phosphate buffered saline.. The difference in optical

density between the test series and the "antigen blanks" corresponded to the turbidity due to the antigen-antibody precipitate. The turbidities recorded were plotted against the number of μg of albumin present, as estimated by the "rocket" technique.

Experimental error was minimised by careful timing and controlled incubation temperature. All solutions used were filtered, using membrane filters (Sartorius, Membranfilter GMBH). The test was performed using extracts of blood stains 5 and 27 days old, and using fresh haemolysed blood to provide a reference albumin curve.

2.6. Preparation, Storage and Extraction of Blood Stains.

The amount of material left at the scene of a crime may vary considerably from small splashes of blood, containing perhaps 1μl of blood, to much larger volumes. In the present work, stains were prepared from 5μl of blood, although in practice a profile of the quantities of six plasma proteins, measured by the electroimmunodiffusion technique developed could be constructed using only 2μl, and by the "tandem" technique using approximately 0.5μl.

Blood stains were prepared from a small cut made in the subject's thumb using a blood lancet ("Sera-Sharp", Propper Mfg. Co.). 5μl aliquots of blood were measured using disposable micro-pipettes (Camlab, Cambridge), and placed at intervals on a piece of dry cotton material, previously rinsed in several changes of distilled water to remove traces of the detergent in which it had been washed. The samples were allowed to dry at room temperature, with the cotton suspended horizontally. 5μl aliquots of blood gave stains approximately 5mm in diameter.

For those experiments where the effect of the age of the stain on the plasma protein profile was to be examined, a large number of stains was prepared on one occasion, and the stains extracted at intervals over

the period of study. The concentrations of the plasma proteins in the extracts were measured by the electroimmunodiffusion technique (section 2.3.2.) and by the "tandem" antigen-antibody crossed electrophoresis (section 2.4.2). For the qualitative tests, carried out on extracts of stains of different ages, 5µl blood stains were prepared from the same subject, at intervals, and extracted at the same time. In this way, a direct comparison of the extracts could be made on the same immuno-electrophoresis and Ouchterlony plates.

When the blood stains had dried for one hour at room temperature, the material was labelled with the donor's name and the date, and then transferred to a sealed box in a dark cupboard, at room temperature. Humidity and light are both believed to affect the stability of proteins in blood stains (Culliford, 1973).

Extraction of the stains involved cutting the stain from the material, and placing it in 75µl 0.9% (w/v) saline for 18 hours (see section 3.1.3). The extract was separated from the piece of cloth by centrifugation. The cloth and extract were placed in a plastic centrifuge tube (Walter Sarstedt (UK) Ltd), which had a small hole in the bottom of it. The centrifuge tube was placed over a second one and secured by means of adhesive tape (Figure 2(xiv)). On centrifigation, the extract collected in the lower tube, while the piece of cloth remained in the upper one.

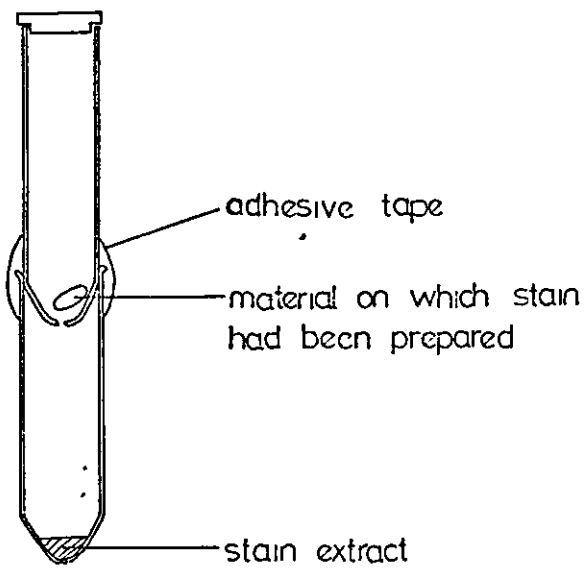
Stain extracts prepared by the method described contained the plasma proteins at concentrations which could be quantitated satisfactorily by the electroimmunodiffusion method described in section 2.3.2. Sufficient extract was available from one stain for the quantitation of nine proteins by electroimmunodiffusion, and analysis of the protein solution by "tandem" antigen-antibody crossed electrophoresis.

The efficiency of removing 5µl aliquots of blood from the subject and the efficiency of the extraction procedure were measured by estimating the amount of protein present in the samples by the Folin-

Lowry method (Lowry et al., 1951). The efficiency of the extraction procedure was also estimated for the individual proteins by the electro-immunodiffusion technique. This technique was also used to determine the effect of time of soaking of the stains in saline on the amount of the individual proteins extracted (section 3.3).

Figure 2(xiv)

Centrifuge Tubes Prepared for the Separation of Blood Stain Extract.



3. RESULTS.

3.1. Determination of the Precision with which Blood Stains were Prepared.

In the experiments in which the stabilities of plasma proteins in blood stains were examined, stains prepared from 5µl of blood were used. The precision with which 5µl aliquots of blood could be removed from the subject was determined, so that the extent to which the error of this procedure was influencing later results could be assessed.

Ten 5µl aliquots of blood were placed directly into 75µl of 0.9% (w/v) saline. The amount of protein present in the samples was then determined by the Folin-Lowry method (Lowry et al., 1951). A coefficient of variation of 4.6% was calculated from the results. This figure includes the error due to the Folin-Lowry method for protein quantitation.

3.2. Determination of the Precision with which Plasma Proteins could be Extracted from Blood Stains.

Simultaneously with the above experiment, ten blood stains, each prepared from 5µl blood on cotton material were allowed to dry for one hour, and then extracted into 75µl saline for 90 minutes. After this time, the amounts of protein present were assessed, using the Folin-Lowry method, and also the amounts of prealbumin, albumin and orosomucoid in each were measured using the "Rocket" technique.

The amounts of protein measured corresponded to a 94% extraction of protein from the stain.

The coefficients of variation for the total protein, prealbumin, albumin and orosomucoid measurements are given in Table 3.I.

Table 3.I.

Coefficients of variation for the extraction of total protein, prelbumin, albumin and orosomucoid from ten blood stains.

Protein	Coefficient of Variation
Total Protein	5.0
Prealbumin	6.0
Albumin	3.7
Orosomucoid	4.0
Mean =	4.7

The results of this experiment indicate that the extraction procedure is very reproducible, bearing in mind the coefficient of variation of 4.6% calculated for the stain preparation procedure, (section 3.1.).

Figure 3(1)

Extraction of proteins from fresh blood stains and from stains 19 days old using the peak heights of the precipitates as a measure of the amount of protein extracted.

a) Fresh stains

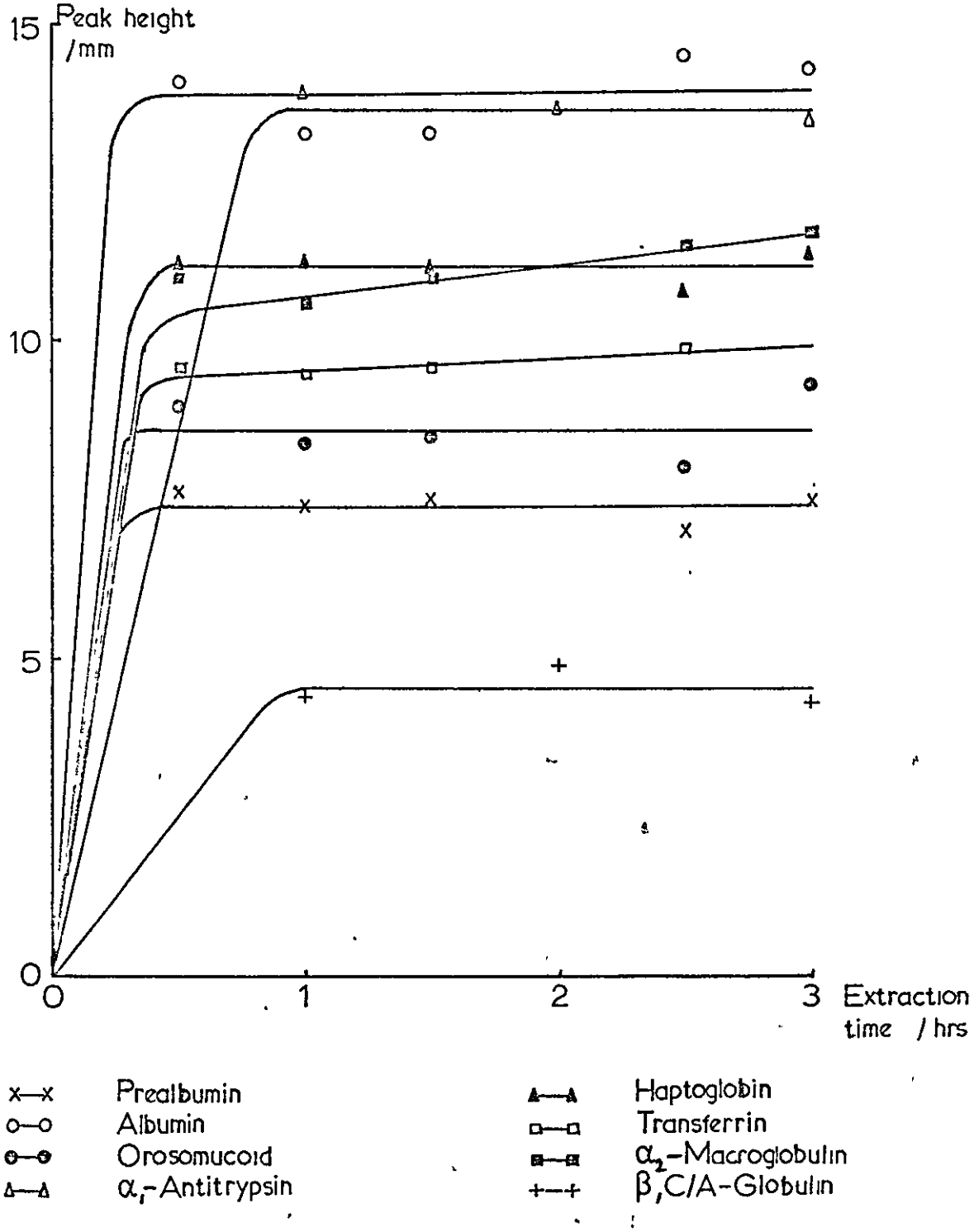
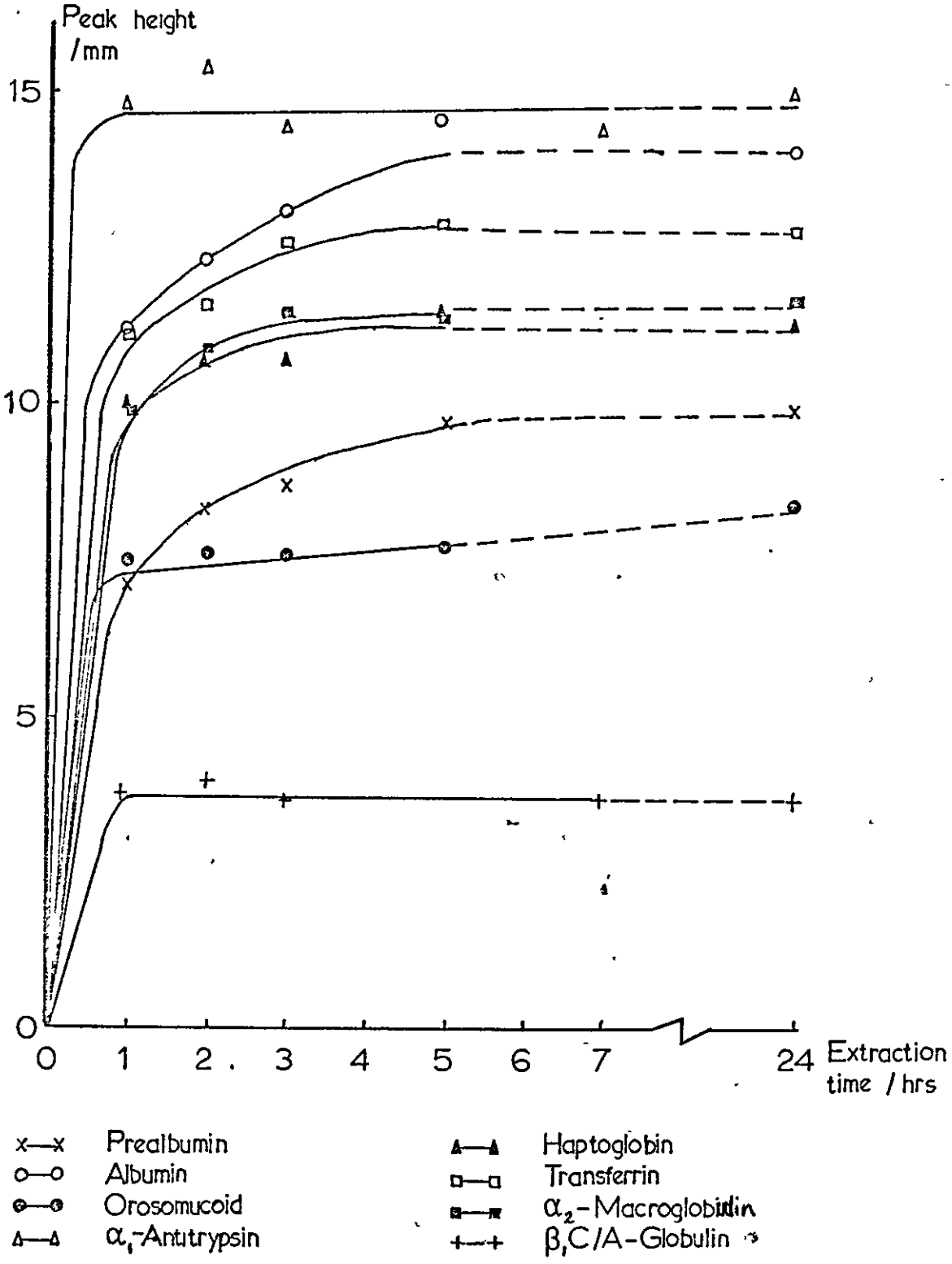


Figure 3(i)

b) 19 day old stains



3.3. Determination of the Optimum Time for Blood Stain Extraction.

The time required for maximum extraction of protein from blood stains was examined.

The amounts of total protein and of individual proteins in extracts, prepared by soaking 5µl blood stains in saline for different lengths of time were determined. Fresh stains and stains 19 days old were examined, total protein being measured by the Folin-Lowry method, and individual proteins by electroimmunodiffusion. The results are illustrated in Figure 3(i).

It appears that all the proteins studied, except transferrin and α_2-macroglobulin, are maximally extracted from fresh blood stains after one hour. For older stains, longer periods are required for maximum extraction of some proteins. α_1-Antitrypsin and β_1C/A-globulin are maximally extracted from stains 19 days old after one hour, transferrin, α_2-macroglobulin and haptoglobin after five hours, while albumin, orosomucoid and prealbumin require longer periods of soaking.

It was concluded that blood stains, whatever their age, should be extracted overnight (18 hours) in order to extract maximally all the proteins to be quantitated.

(Unfortunately, this was not realised until after the first experiment to determine the stability of plasma proteins in blood stains had been performed).

3.4. Examination of the Stability of Plasma Proteins in Blood Stains by Electroimmunodiffusion.

Three experiments were carried out to determine whether any of the plasma proteins chosen for study remain stable in blood stains up to 50 days old. In each experiment, a number of 5µl blood stains were prepared from two male subjects, the same donors being used in

Experiments 2 and 3 (i.e. subjects 2 and 3).

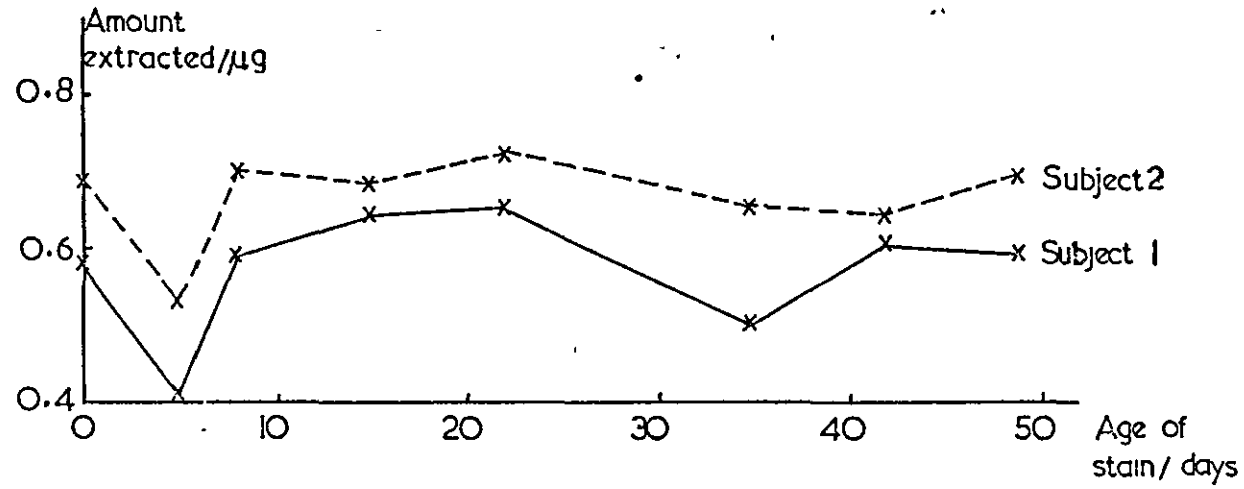
In experiments 1 and 2, prealbumin, albumin, orosomucoid, haptoglobin, α_2-macroglobulin and transferrin were quantitated in stain extracts prepared at intervals over the 50-day period. In Experiment 3, two additional proteins, α_1-antitrypsin and β_1C/A-globulin were quantitated.

The results obtained in Experiment 1 are influenced by the fact that the stains were not extracted adequately. They were extracted for 90 minutes only, while the stains used in Experiments 2 and 3 were extracted for 18 hours. According to the information given in section 3.3, this will affect the results obtained in Experiment 1 for all stains but fresh ones. Both Experiments 1 and 2 were carried out before the optimum sample order across the Cellogel strip was determined. Although the results from Experiments 1 and 2 cannot be directly compared with those of Experiment 3, they can be examined independently since, each time stain extracts were examined, the standard and test solutions were applied to the cellulose acetate strips in the same order. The results of Experiment 3 are believed to represent more precisely the behaviour of the plasma proteins in ageing blood stains.

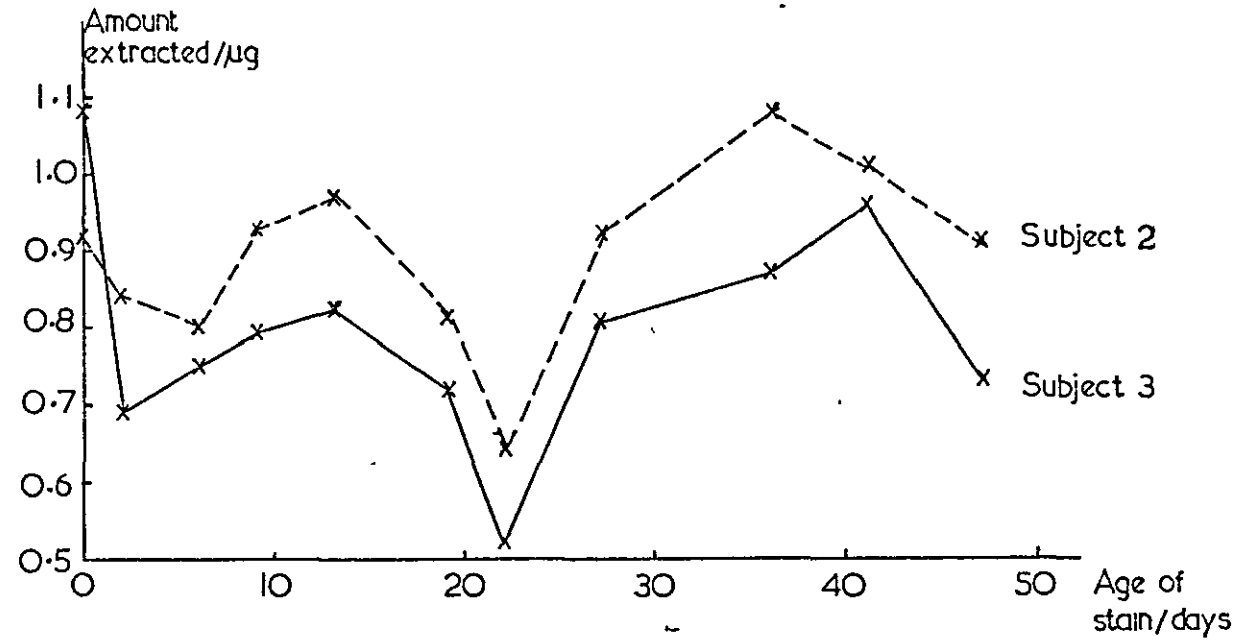
In the three experiments described, it was possible to calculate the total amount of each protein in the stain extracts prepared, since the stains had been made from known volumes of blood. The amounts of each protein recovered over the periods of study are tabulated in Appendix I, and these results presented as graphs in Figure 3(ii)a-h. When stains are prepared from unknown volumes of blood, the total amount of each protein present in the stain extracts cannot be calculated, and because of this, it is impossible to compare two stains. However, if a reference protein was present in the stain extracts so that the concentration of a particular protein could be related to the concentration of the reference protein in the stain extract, then comparison of two stains would be possible. The reference protein should be stable in blood stains. Inspection of the graphs showing the stabilities of the plasma proteins in stains (Figure 3(ii)) suggests that orosomucoid may

Figure 3.(ii)a Extraction of prealbumin from blood stains.

Experiment 1



Experiment 2



Experiment 3

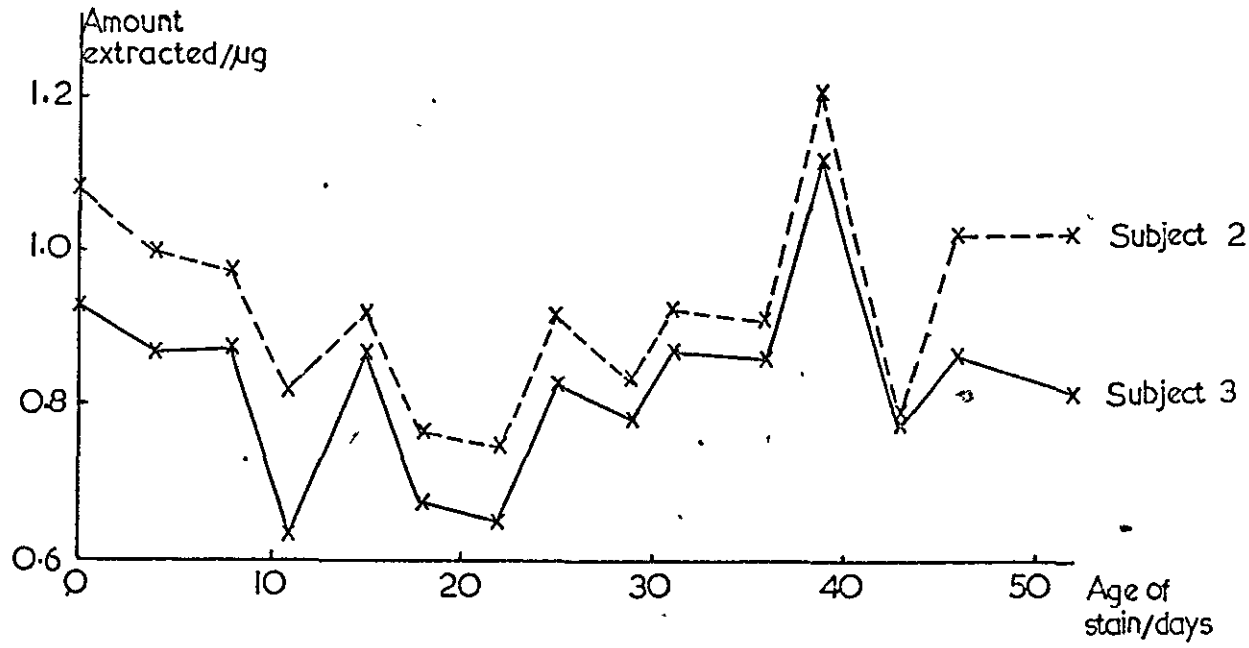
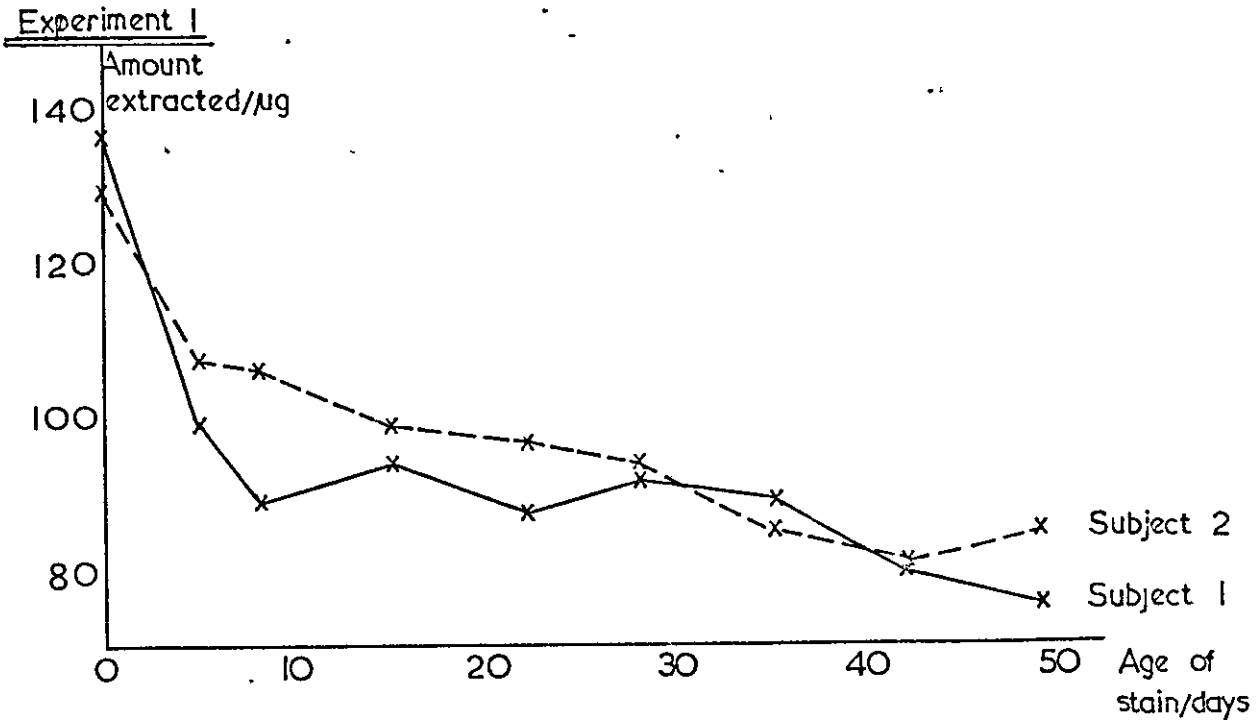
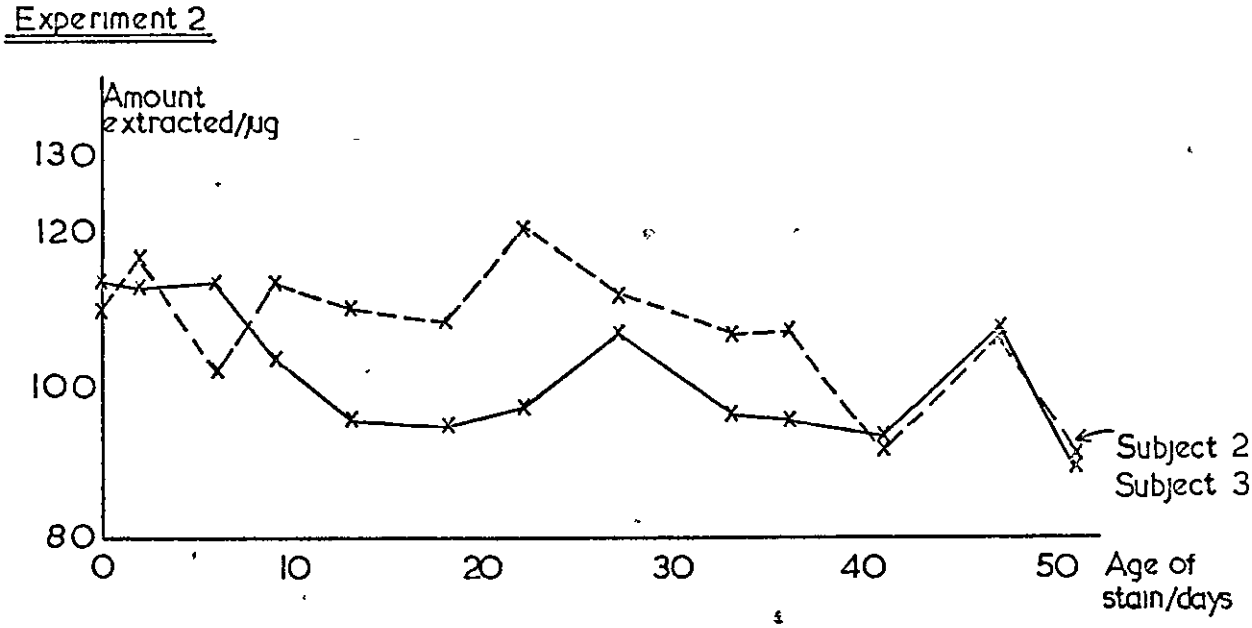


Figure 3(ii) b Extraction of albumin from blood stains.





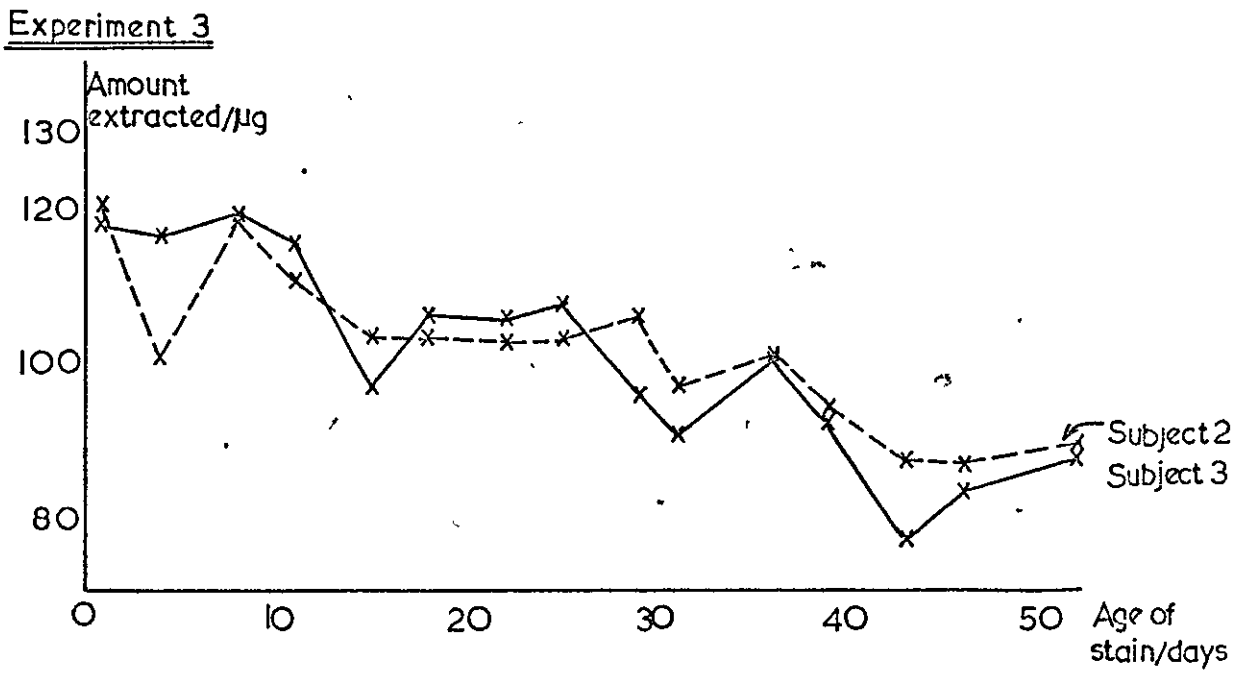


Figure 3.(ii) c. Extraction of orosomucoid from blood stains.

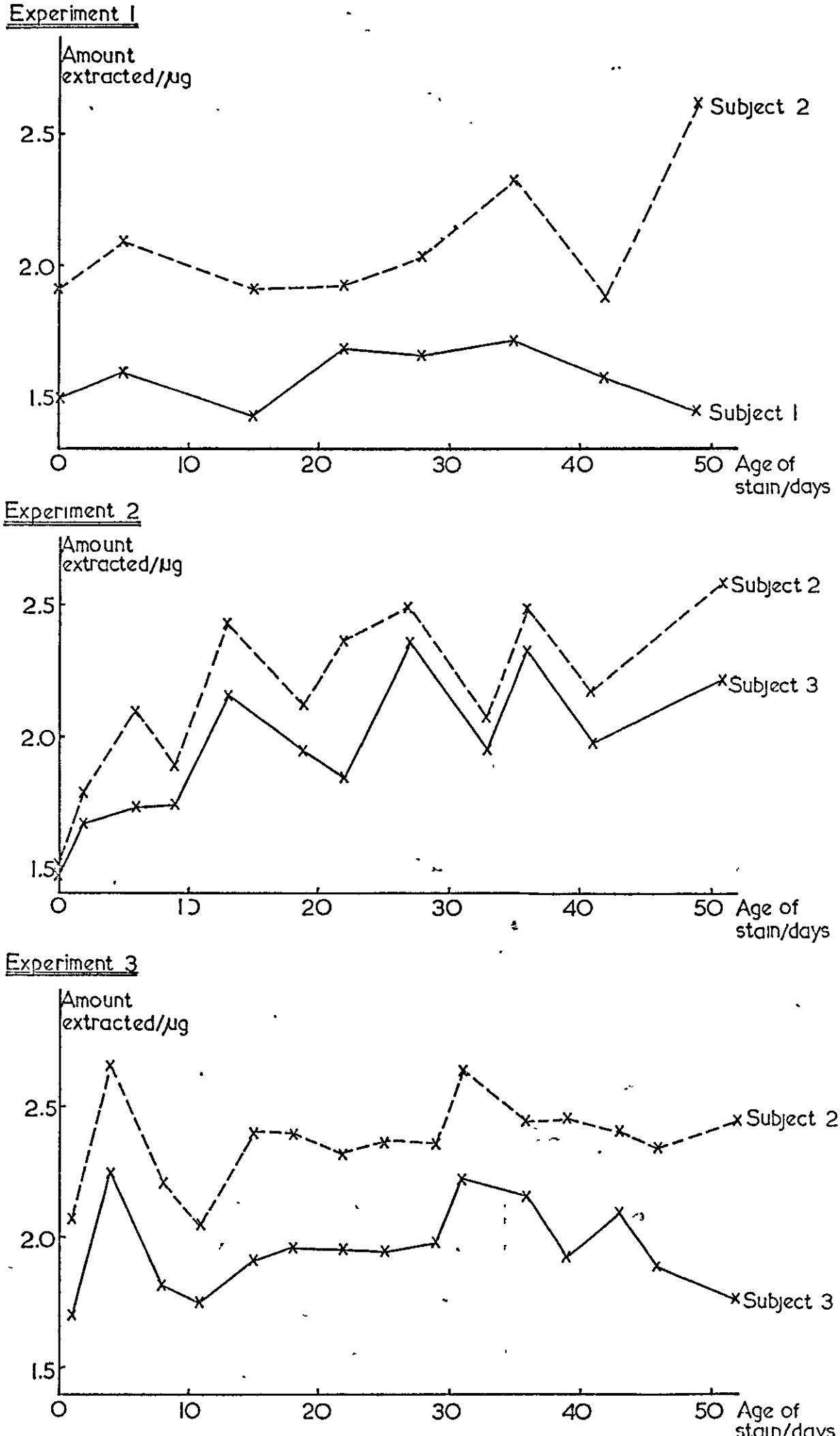


Figure 3.(11).d. Extraction of haptoglobin from blood stains.

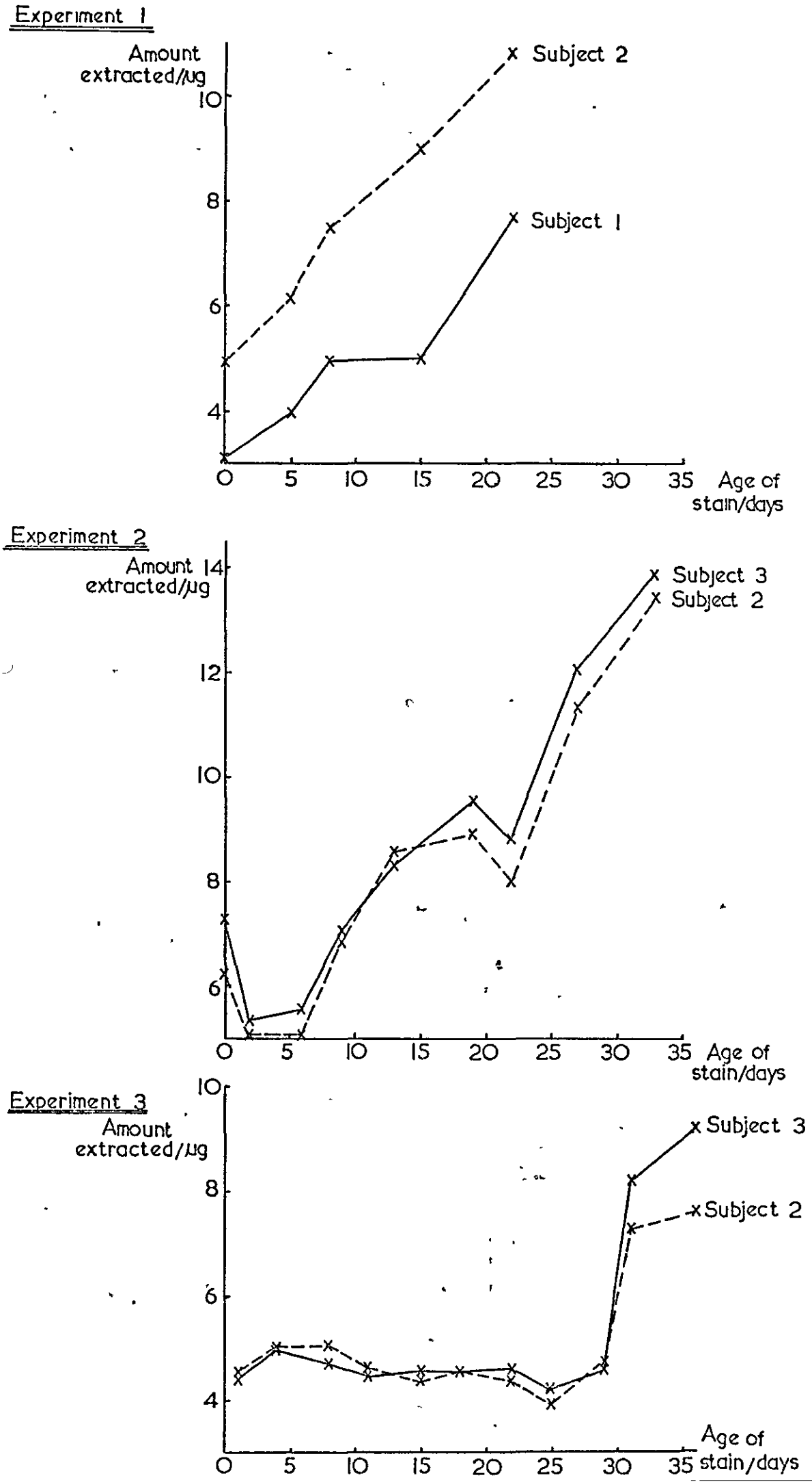


Figure 3(ii).e. Extraction of transferrin from blood stains

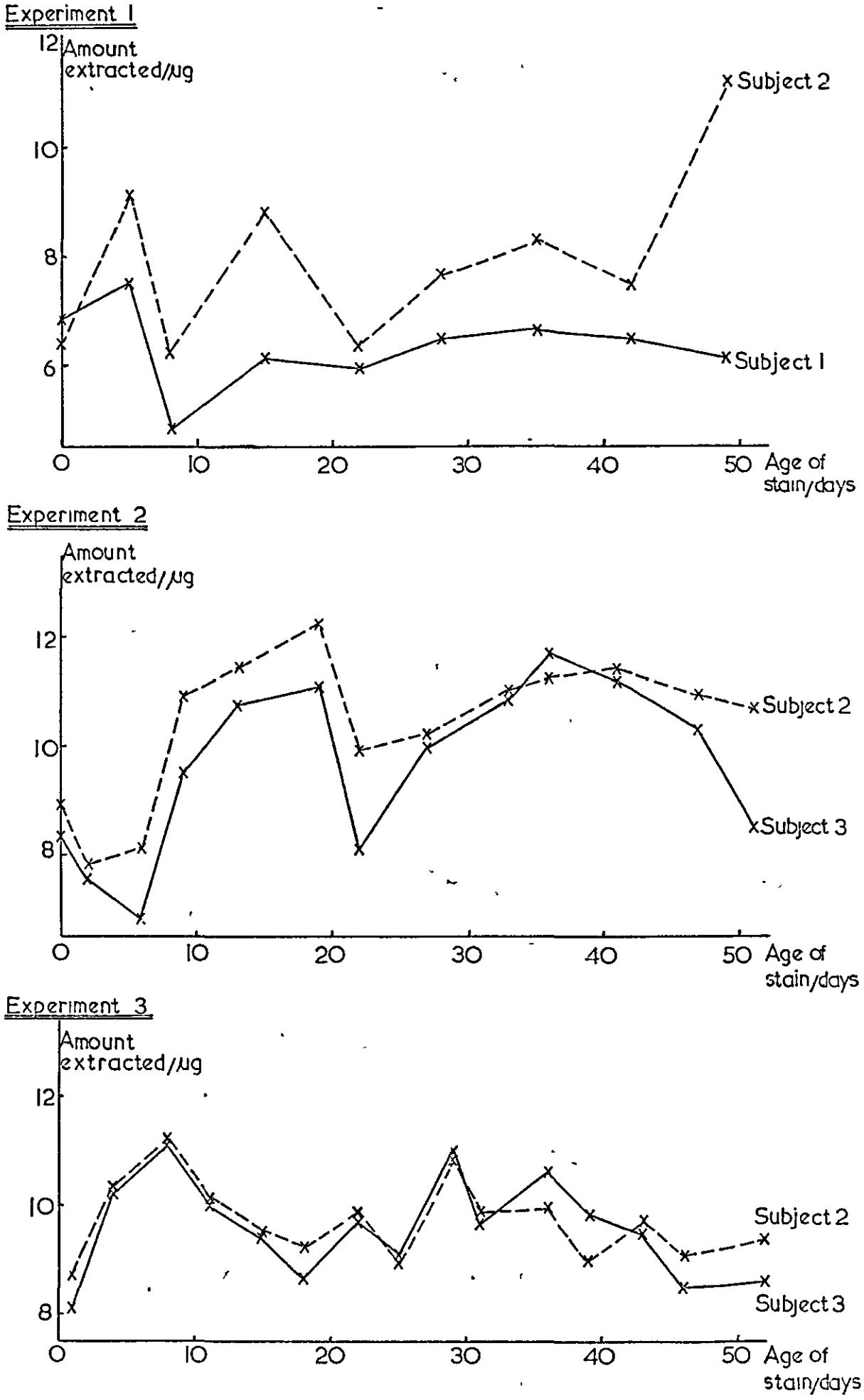


Figure 3.(ii).f Extraction of α_2- macroglobulin from blood stains

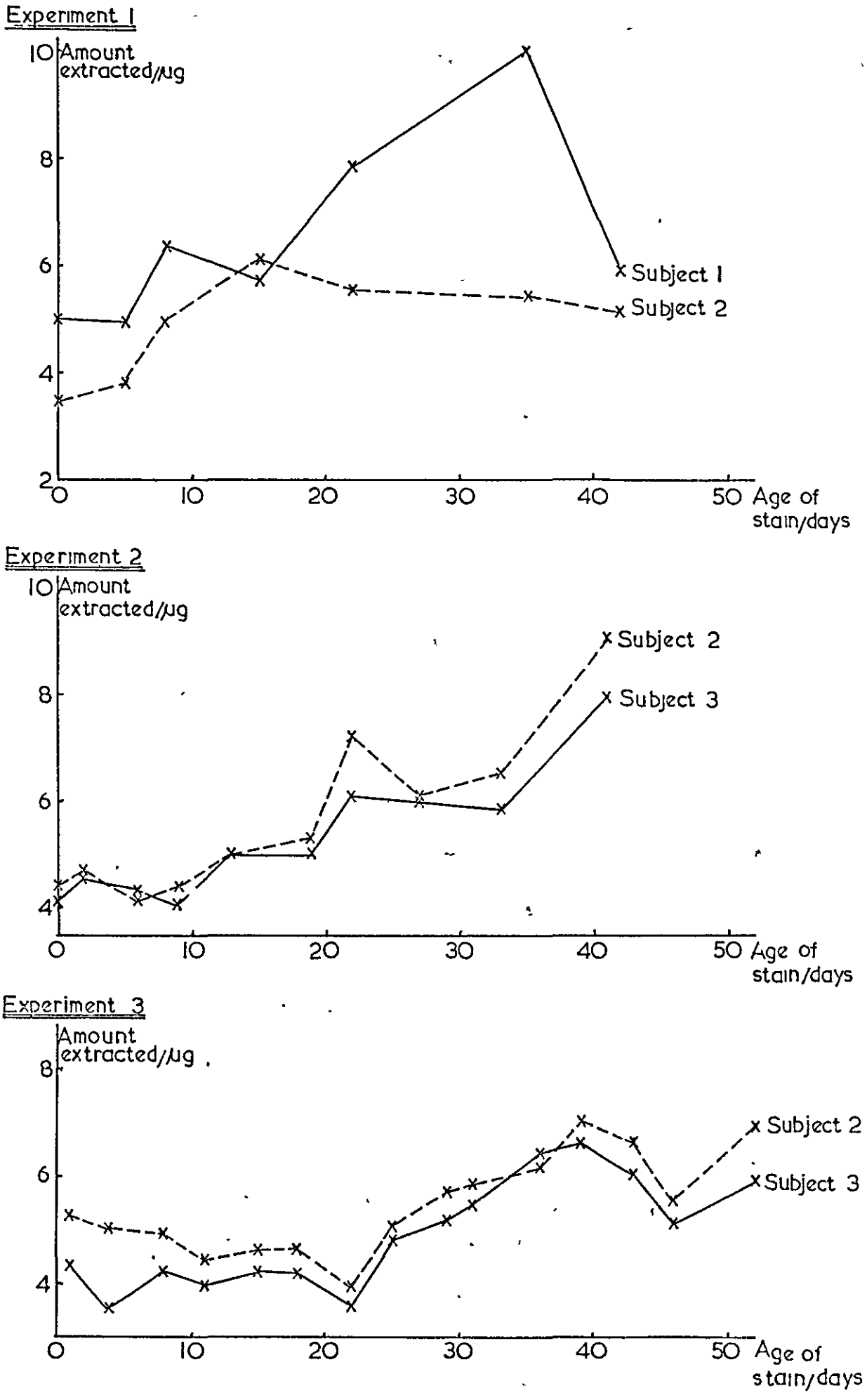


Figure 3(ii).g Extraction of α_1-antitrypsin from blood stains

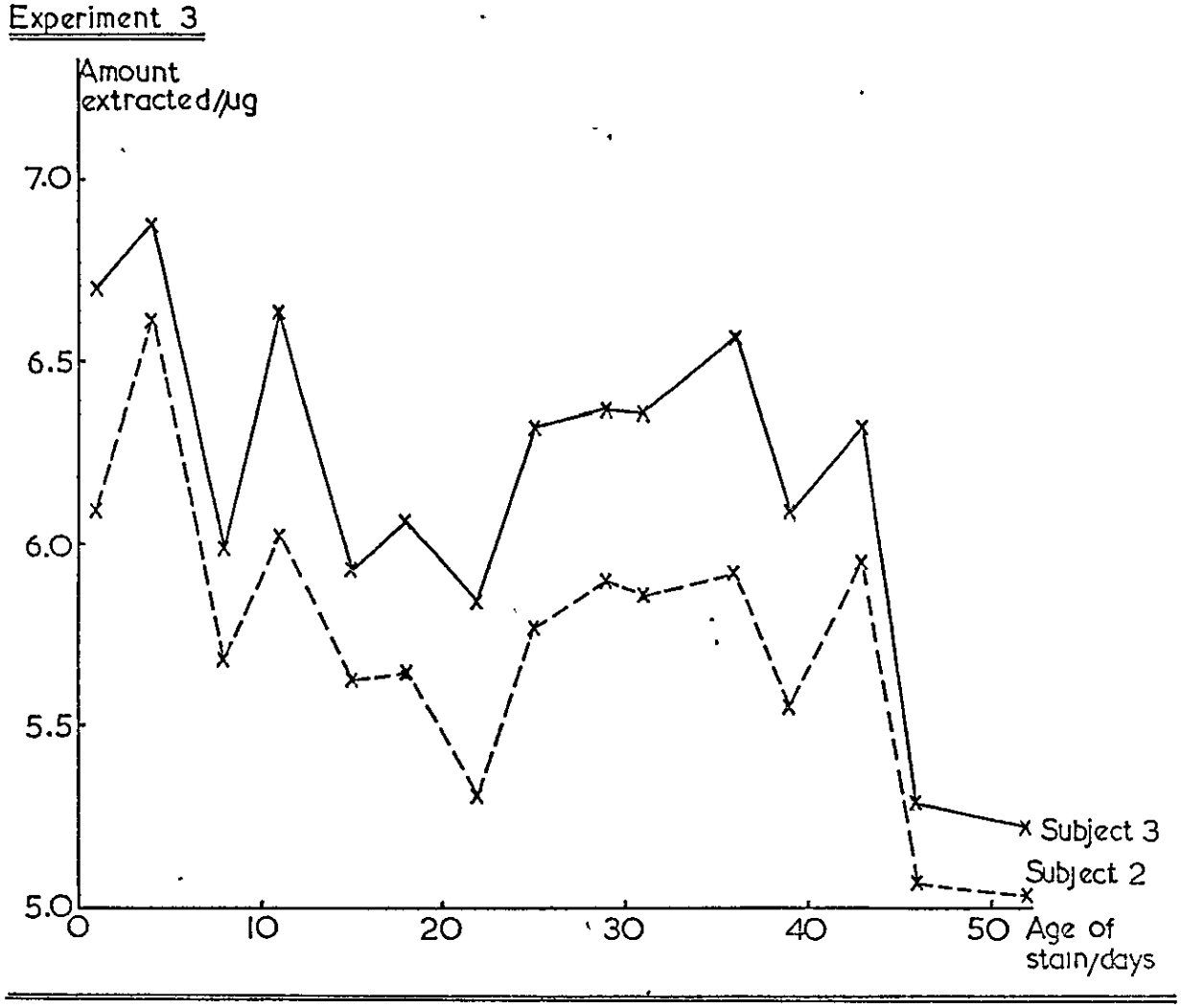
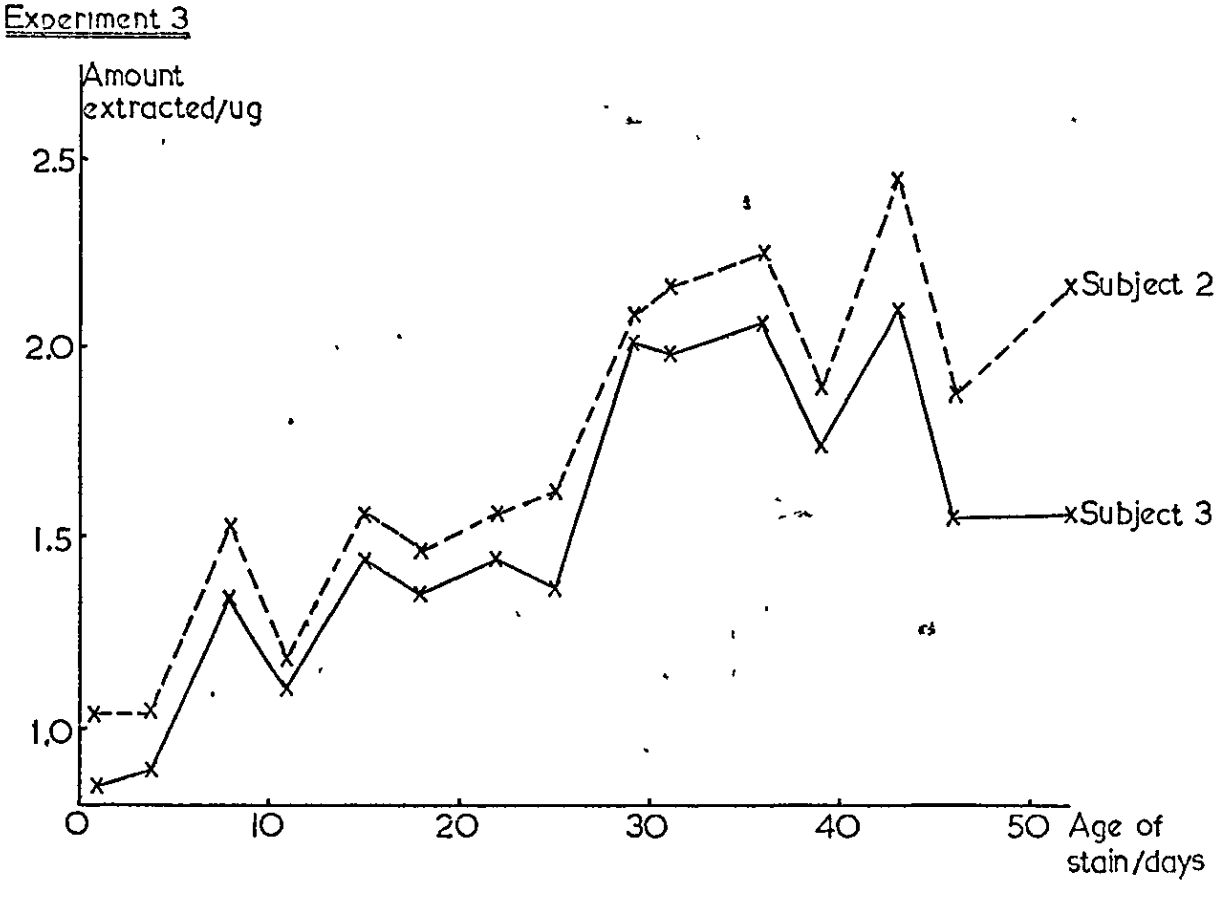


Figure 3.(ii).h Extraction of β_1C/A-globulin from blood stains



be a suitable reference protein. Although the line of regression suggests that the amounts of orosomucoid recovered decline over the 50 day period, the slope is not significant, and the protein may be assumed to be stable.

In addition to acting as a suitable reference, orosomucoid acts, in some cases, as an internal standard, removing some of the error introduced at the blood-sampling stage. Inspection of the results obtained for blood stains from subject 3, in Experiment 3, shows that relating the amounts of each protein to the amounts of orosomucoid removes some of the fluctuations associated with the error in blood-sampling. The variance of the orosomucoid curve for subject 3 is 0.029, calculated from

$$\text{variance, } \sigma^2 = \frac{(1 - r^2) \cdot (Y - \bar{Y})^2}{n - 2}$$

$$\text{where} \qquad r^2 = \frac{(X - \bar{X})(Y - \bar{Y})}{(X - \bar{X})^2 \cdot (Y - \bar{Y})^2}$$

$\bar{Y}$ = mean amount of protein extracted/μg

$\bar{X}$ = mean age of stain/days

n = number of extracts examined.

The variance of the transferrin curve for the same subject is 0.911. When the amounts of orosomucoid are subtracted from the amounts of transferrin extracted on each sampling day, the variance of the "difference curve" so produced is 0.806. If the amounts of orosomucoid and transferrin recovered from the stains were completely independent, then the expected variance of the "difference" curve would be equal to the sum of the variances for the orosomucoid and transferrin curves alone, i.e.

$$\sigma^2_{\text{diff.}} = \sigma^2_{\text{Oroso.}} + \sigma^2_{\text{Transf.}}$$

Since the variance for the difference curve is less than this expected value, then it is assumed that orosomucoid is acting as an internal

standard. The effect of relating the amounts of the other proteins extracted from stains prepared from subject 3 to the amounts of orosomucoid was examined, and improvement in the results obtained for α_1-antitrypsin, haptoglobin (for results from stains up to 29 days old), prealbumin, and β_1C/A-globulin. The results were not improved for albumin or for α_2-macroglobulin (for results from stains up to 22 days old).

Since orosomucoid is believed to act as an internal standard, the amounts of each protein, in each experiment were related to the amounts of orosomucoid. The corrected results are tabulated in Appendix II, and represented as graphs in Figures 3(iii)a-g. Inspection of these results gives an indication of the stability of the plasma proteins studied.

Prealbumin appears to be fairly stable over the entire period, but after approximately 50 days, it ceases to give measurable "rockets". This is due mainly to the presence of denatured non-specific protein in the samples, which masks the presence of any precipitate. Since the precipitates formed for prealbumin are weak, owing to the very low concentrations of this protein in blood stain extract, the effect of the non-specific protein is most marked.

Albumin appears to be unstable, and the amounts recovered appear to decline over the period of study. The slopes of the regression lines through the two sets of results obtained in Experiment 3 correspond to a 30-35% loss in the amounts recovered over the 52 day period.

The results of Experiment 3 suggest that haptoglobin is fairly stable in stains up to 29 days old. The results of the two earlier experiments suggest that this protein is not stable at all, but this could be due to sub-optimal experimental conditions. Haptoglobin ceases to give measurable "rockets" after 36 days. Transferrin appears to be stable for the entire period, but from the results of Experiment 3, α_2-macroglobulin appears to be stable in blood stains up to approximately three weeks old. α_1-Antitrypsin does not appear to be completely stable

Figure 3(iii).a Amount of prealbumin extracted relative to orosomucoid

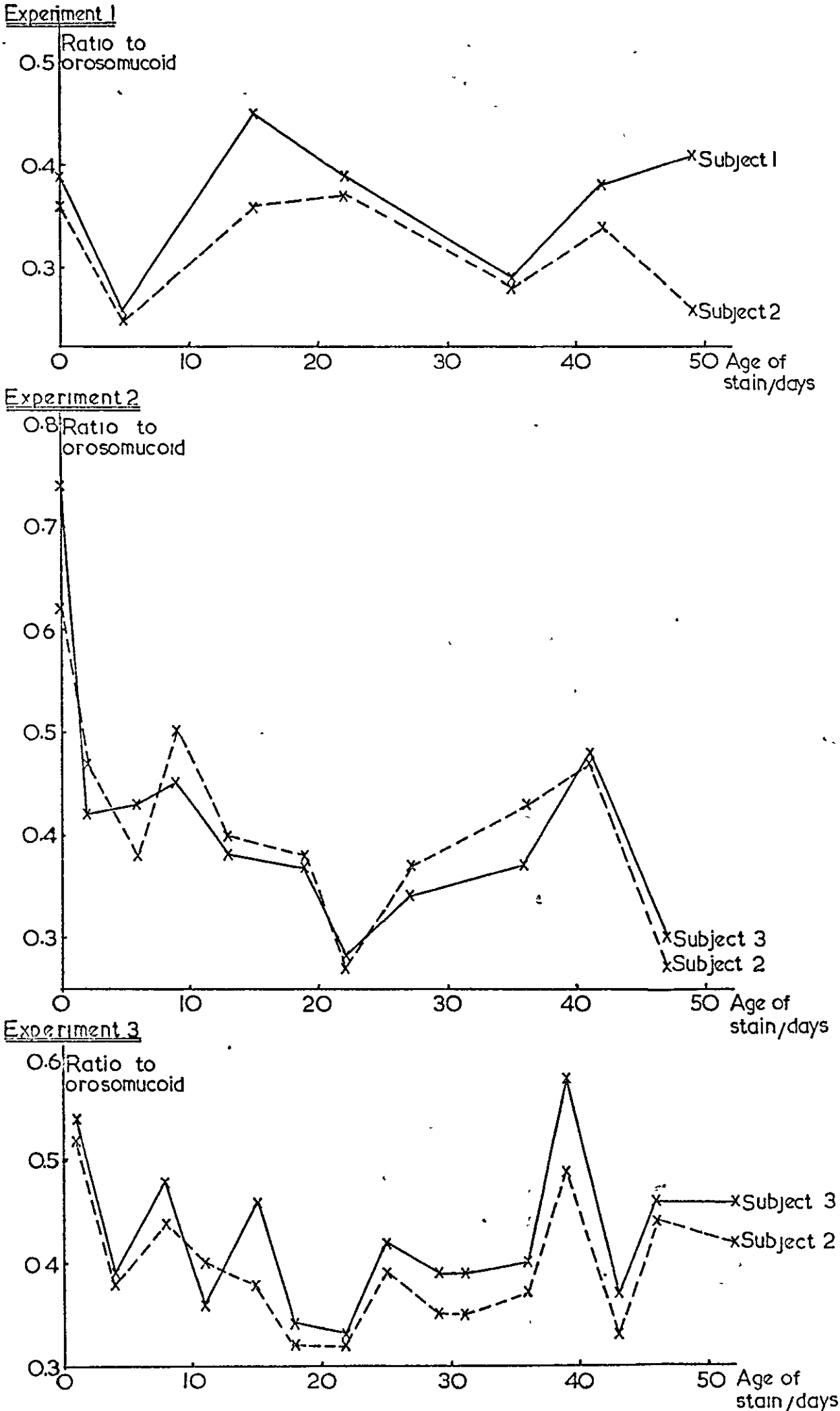


Figure 3.(iii).b Amount of albumin extracted relative to orosomucoid

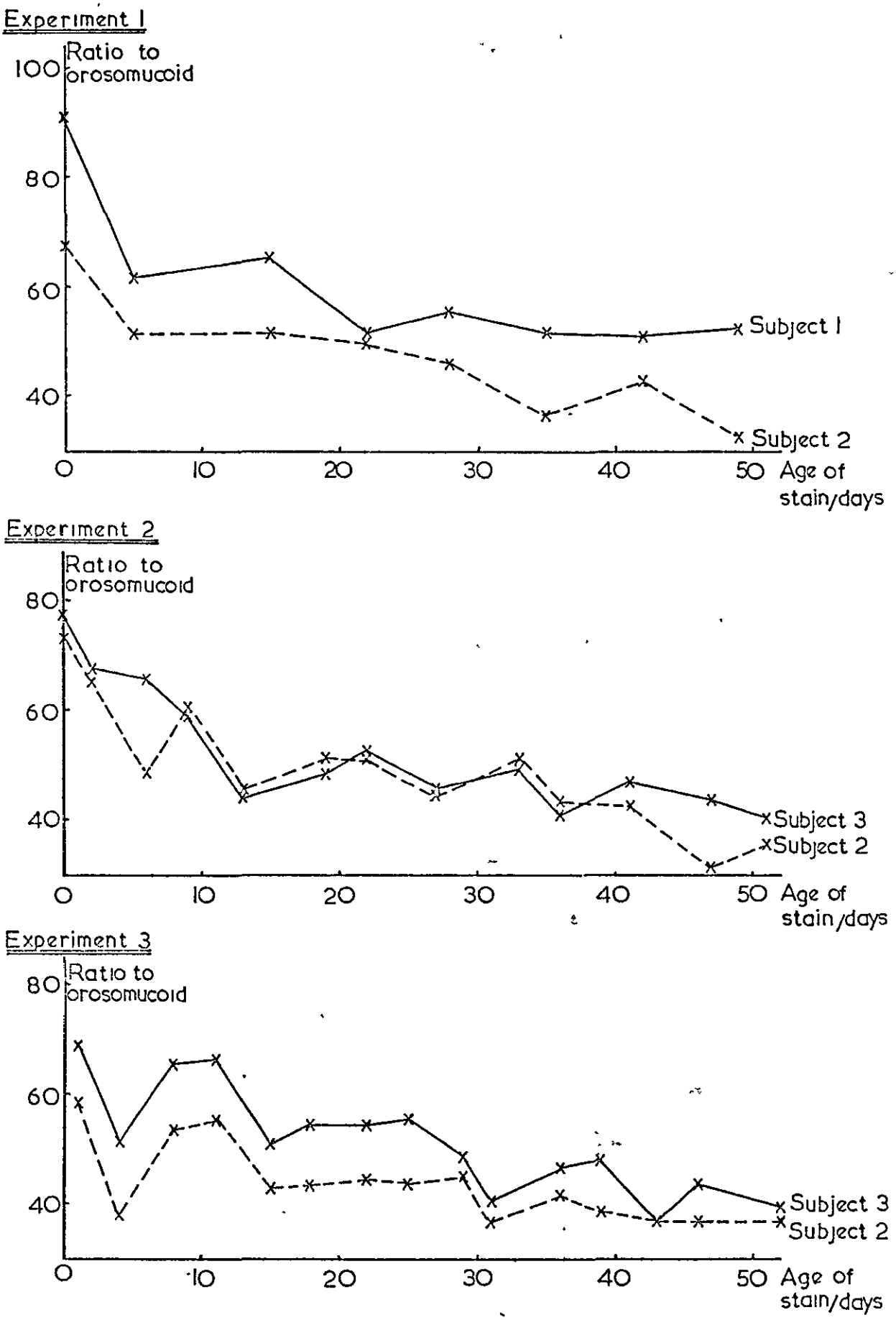


Figure 3(iii).c Amounts of haptoglobin extracted relative to orosomucoid

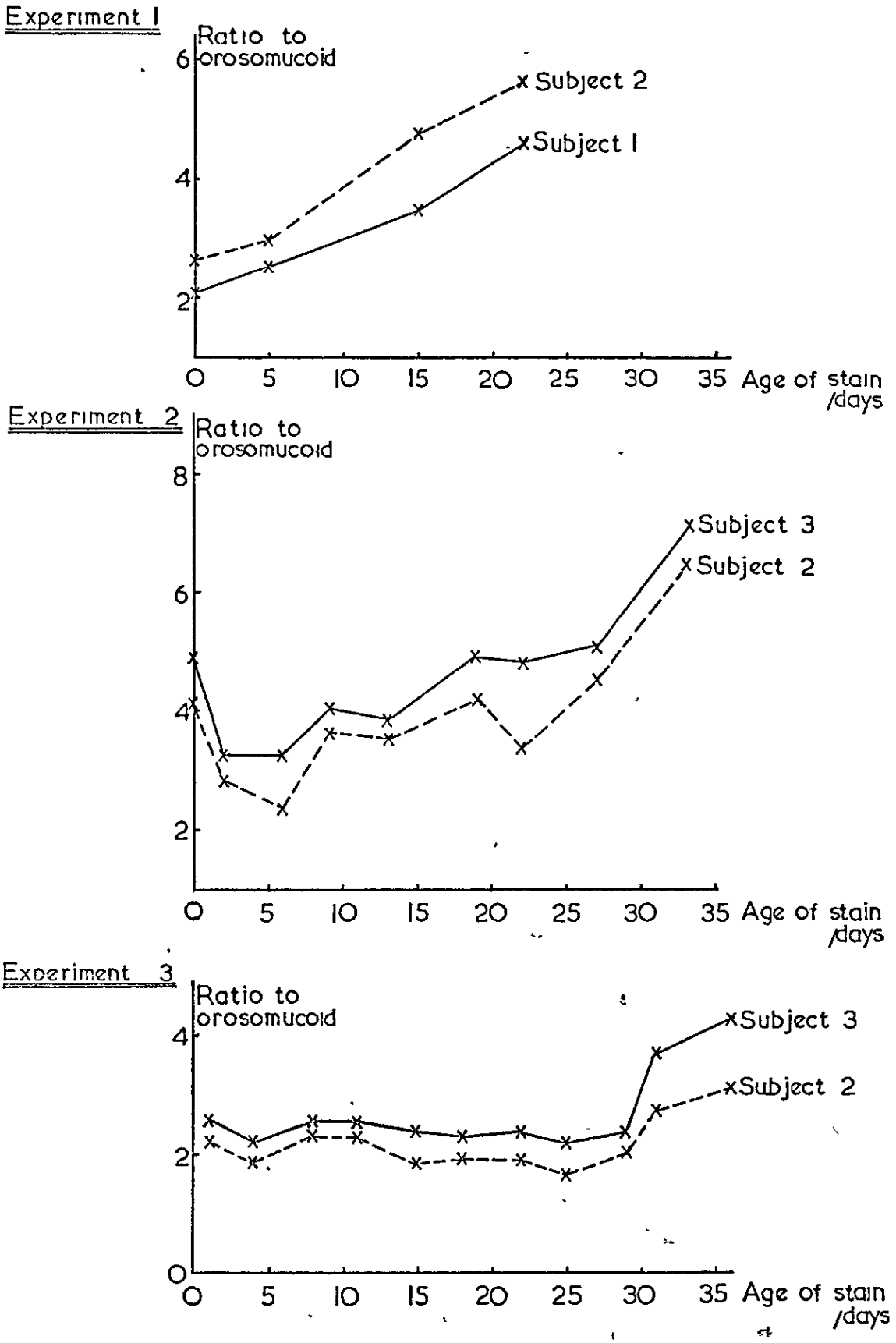
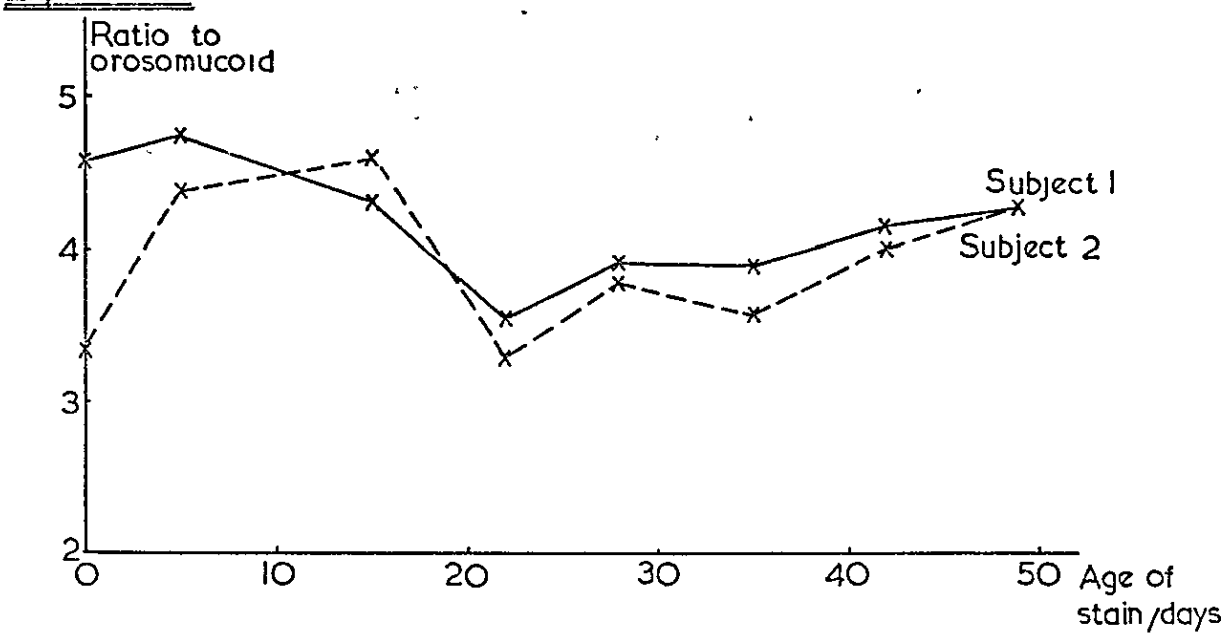
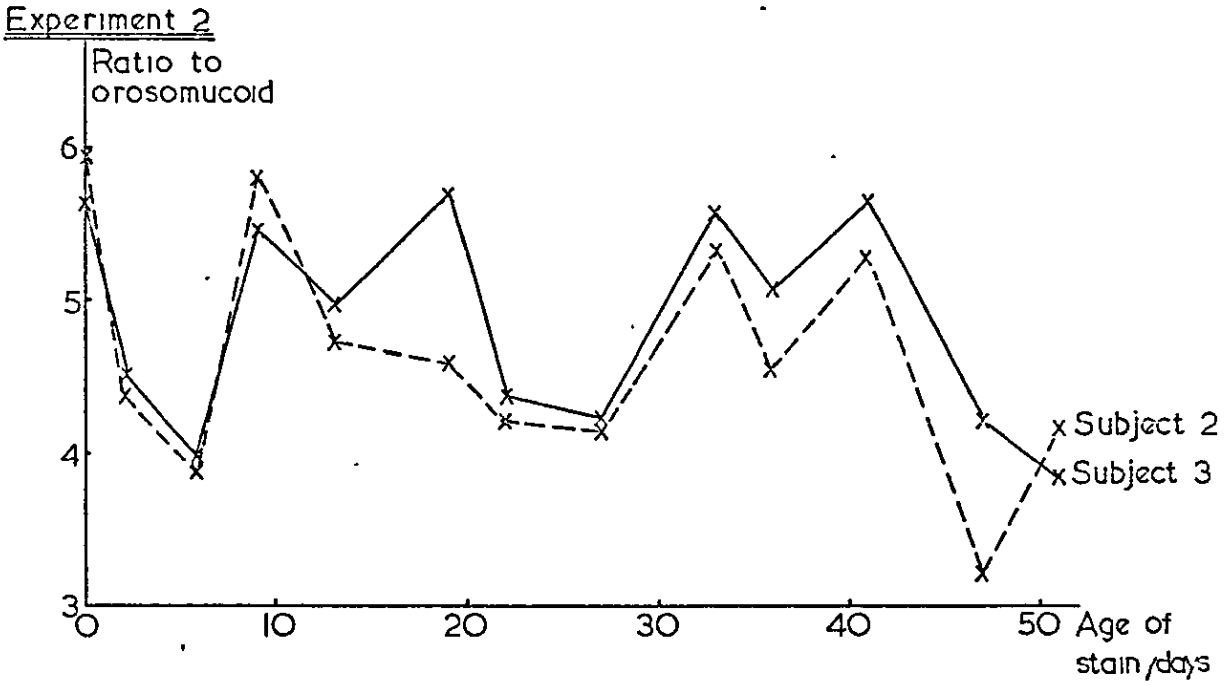


Figure 3.(iii).d Amounts of transferrin extracted, relative to orosomucoid





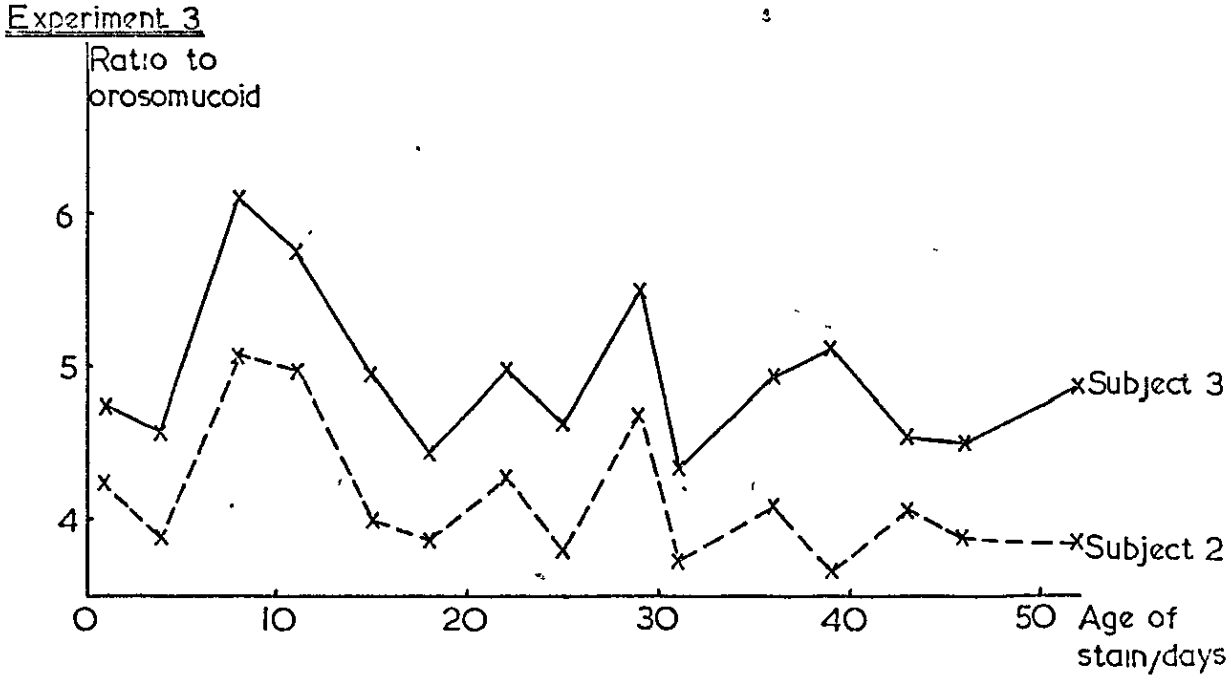
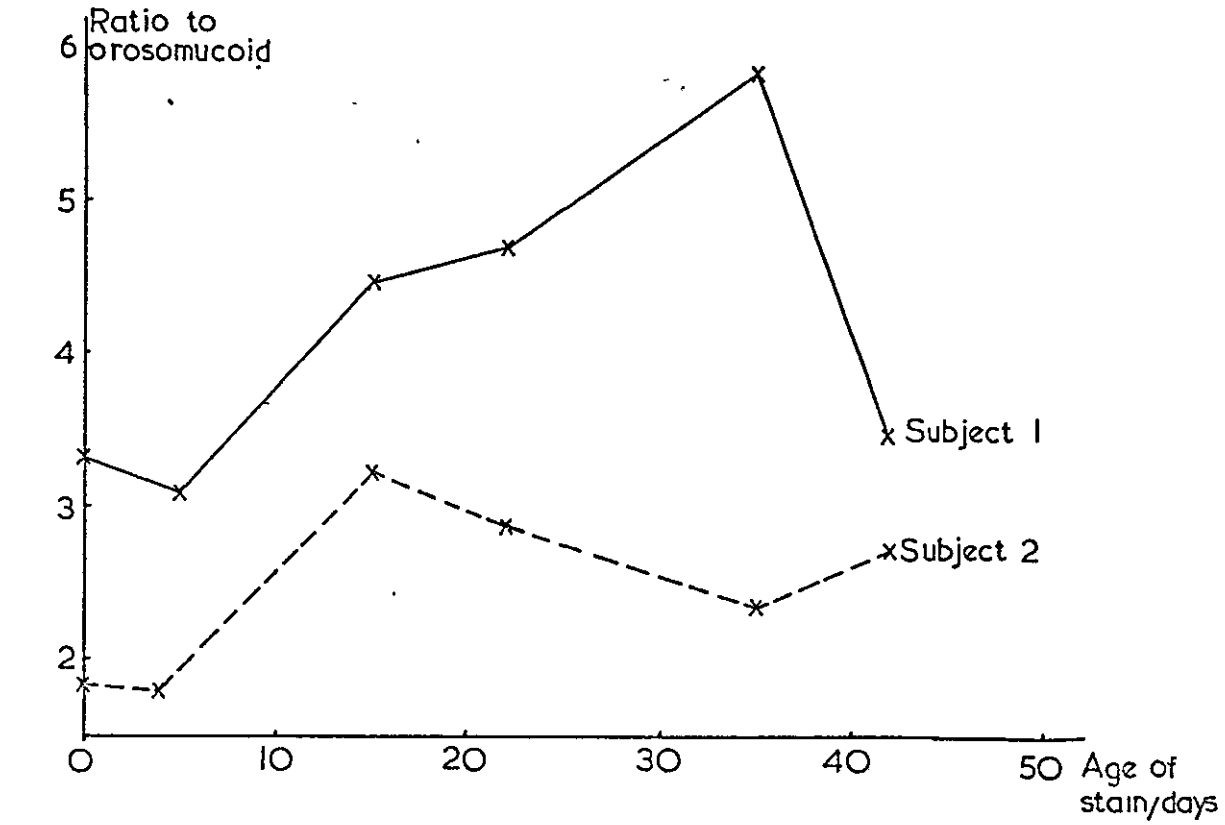
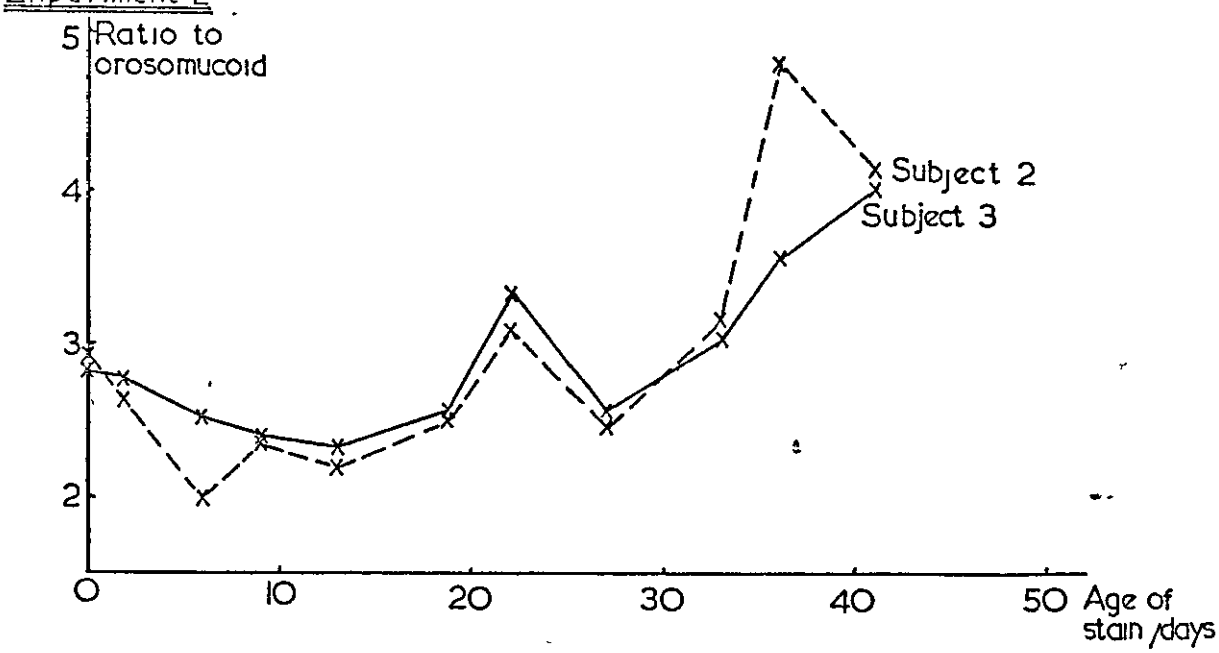


Figure 3(iii)e Amounts of α_2-macroglobulin extracted, relative to orosomucoid.

Experiment 1



Experiment 2



Experiment 3

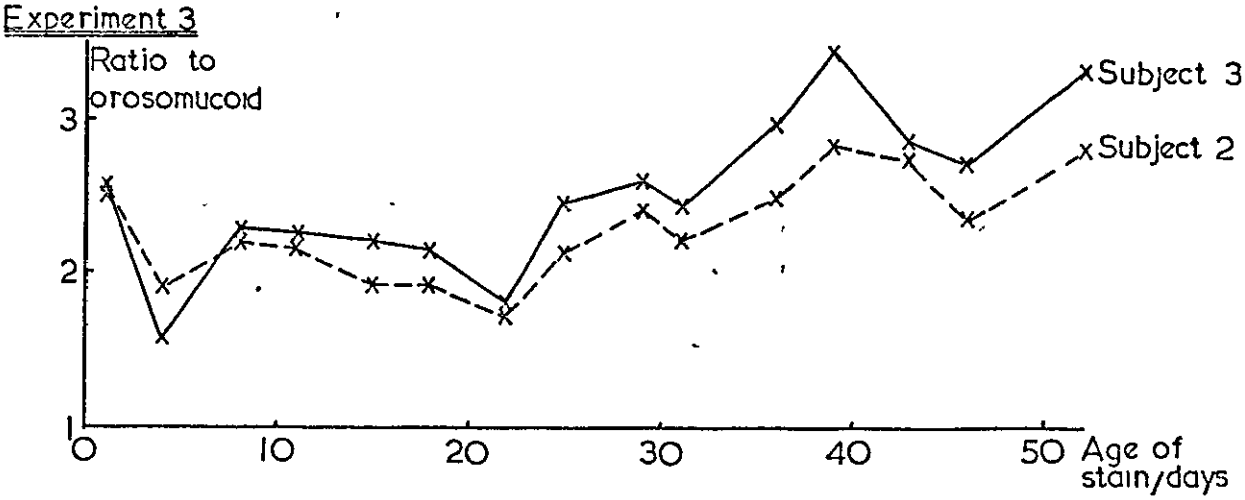


Figure 3.(iii).f Amounts of α_1-antitrypsin extracted, relative to orosomucoid

Experiment 3

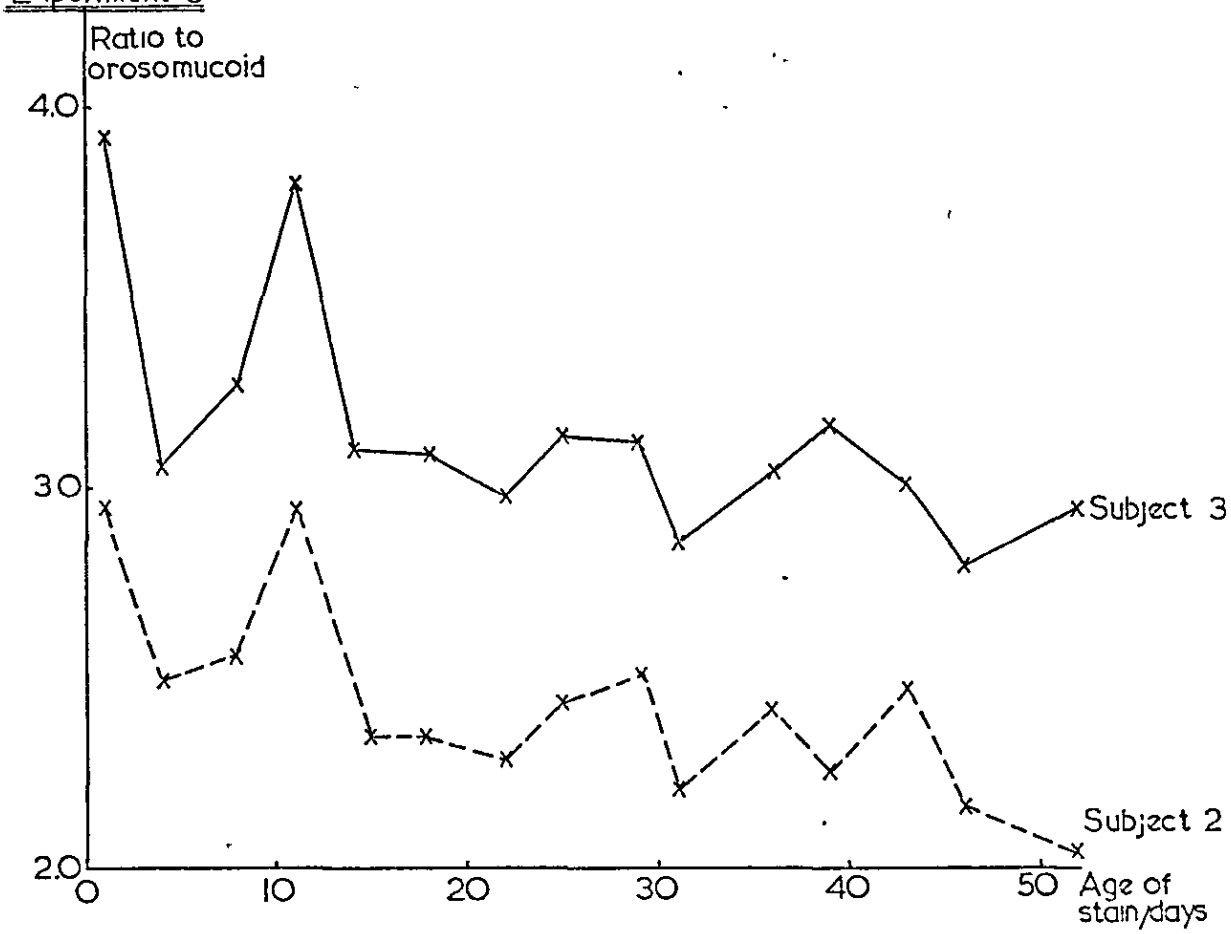
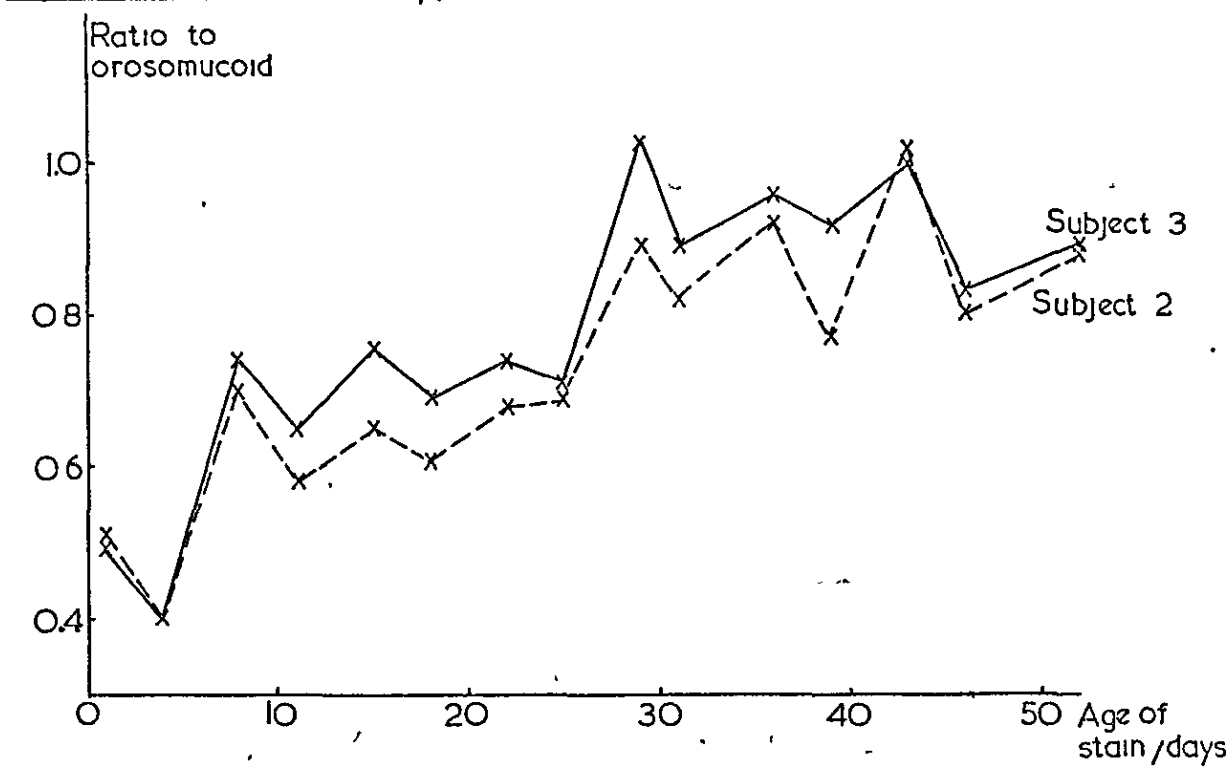


Figure 3(iii).g Amounts of β_1C/A-globulin extracted, relative to orosomucoid



over the 50 day period and β_1C/A-globulin is also unstable.

The slopes of the stability curves obtained for subjects 2 and 3 in Experiment 3, and the errors in the slopes are given in Table 3.II. For haptoglobin and α_2-macroglobulin, only the results for the period over which these proteins remained stable are considered. The figures given in Table 3.II indicate that prealbumin, haptoglobin (in stains up to 29 days old), transferrin and α_2-macroglobulin (in stains up to 22 days old) may be considered to be stable, since the slopes calculated are not significant. The amounts of albumin, α_1-antitrypsin and β_1C/A-globulin extracted do appear to change significantly over the period of study.

Figures 3(ii) and 3(iii) indicate that there are large fluctuations in the amounts of each protein and in the ratios of each protein to orosomucoid from day to day. These fluctuations, in all experiments, and for each pair of subjects, are approximately parallel. The extent of these parallel fluctuations can be accounted for, in part, by the error in preparing the standard solutions. However, the major part of the fluctuations is probably caused by variation between different Cellogel strips, over which there is little control. The effect can be minimised by expressing the amount of a particular protein in one sample relative to the amount of the same protein in the second sample, both measured on the same strip of Cellogel. In the experiments described above, where a known volume of blood was used to prepare the stains, it is possible to compare the amounts of a particular protein in two samples directly. When unknown volumes of blood are used, a direct comparison cannot be made. However, if the concentration of the protein is first related to the concentration of orosomucoid in the stain extract, then comparison between the two samples is possible, i.e. the value of $\frac{Pr_1}{Or_1} \cdot \frac{Or_2}{Pr_2}$ is calculated, where Pr_1 and Pr_2 are the concentrations of the protein in samples 1 and 2, and Or_1 and Or_2 the concentrations of

Table 3.II.

Subject 2.

Protein	Slope	Error in Slope
Prealbumin	0.000	± 0.001
Albumin	-0.333	± 0.084
α_1-Antitrypsin	-0.0113	± 0.003
Haptoglobin	-0.0122	± 0.0073
Transferrin	-0.0124	± 0.0068
α_2-Macroglobulin	-0.0273	± 0.0100
β_1C/A-Globulin	+0.0085	± 0.0014

Subject 3.

Protein	Slope	Error in Slope
Prealbumin	+0.002	± 0.001
Albumin	-0.438	± 0.107
α_1-Antitrypsin	-0.0129	± 0.004
Haptoglobin	-0.0078	± 0.0052
Transferrin	-0.0097	± 0.0085
α_2-Macroglobulin	-0.011	± 0.018
β_1C/A-Globulin	+0.0092	± 0.0017

orosomucoid in samples 1 and 2 respectively. The values for each protein for each pair of subjects, calculated in this way, are expressed in Appendix III, and a summary of the results is given in Table 3.III.

Table 3.III.

Summary of the results obtained by comparing the ratios of each protein to orosomucoid, for each pair of subjects.

Expt. No.	Protein	$\frac{Pr_1}{Or_1} \cdot \frac{Or_2}{Pr_2}$ in whole blood	Mean value of $\frac{Pr_1}{Or_1} \cdot \frac{Or_2}{Pr_2}$ in stain extracts	C.V.
1	Pre.	1.17	1.16	16.8
	Alb.	1.29	1.28	13.3
	Hpt.	0.94	0.80	6.0
	Trf.	1.19	1.07	11.9
	α_2M.	1.97	1.72	25.3
2	Pre.	1.66	1.03	12.3
	Alb.	1.00	0.94	12.3
	Hpt.	1.01	0.84	9.0
	Trf.	1.07	0.95	9.7
	α_2M.	1.21	1.00	13.7
3	Pre.	----	0.93	6.8
	Alb.	----	0.84	8.3
	α_1AT.	----	0.77	4.4
	Hpt.	----	0.82	6.7
	Trf.	----	0.84	5.4
	α_2M.	----	0.92	9.6
	β_1C/A.	----	0.94	6.3

The low coefficients of variation calculated for these results indicate that a good comparison can be made between two stains of the same age when the plasma proteins are measured on the same Cellogel strips.

3.5. Examination of the Stability of Plasma Proteins in Blood Stains by the "Tandem" Technique.

The "tandem" technique was used to quantitate prealbumin, orosomucoid, haptoglobin, and transferrin in a number of the extracts of ageing blood stains examined by electroimmunodiffusion in Experiment 3. (Section 3.4.). Extracts of stains made from the blood of subjects 2 and 3, were compared in adjacent wells in "tandem" plates. Two plates were used for each determination, the first with stain extract from subject 2 in the well nearer the first dimension anode, and the second with the samples in the reverse order. The peak heights of the precipitates were corrected for sampling errors, by relating them to the peak height of carbamylated transferrin. The ratios of the amounts of each protein for subject 2 to the amounts of the same protein for subject 3 were calculated, and compared with the results obtained by the "rocket" technique. The results are indicated in Table 3.IV.

Figure 3(iv) illustrates the correlation between these two sets of results. The correlation coefficient calculated from the data presented in Table 3.IV is 0.58. If the figures calculated when the stains were 11 days old are omitted from the calculation, the correlation coefficient is improved (0.78). Whereas in the "rocket" technique improvement of the results is observed when the amounts of each protein are related to the amounts of orosomucoid, this is not the case for the results of the "tandem" technique. A smaller correlation coefficient is obtained if this comparison is made.

Figure 3.(iv).

Graph to represent the correlation between the results of the 'tandem' and 'rocket' techniques for the amounts of some plasma proteins extracted from blood stains of different ages.

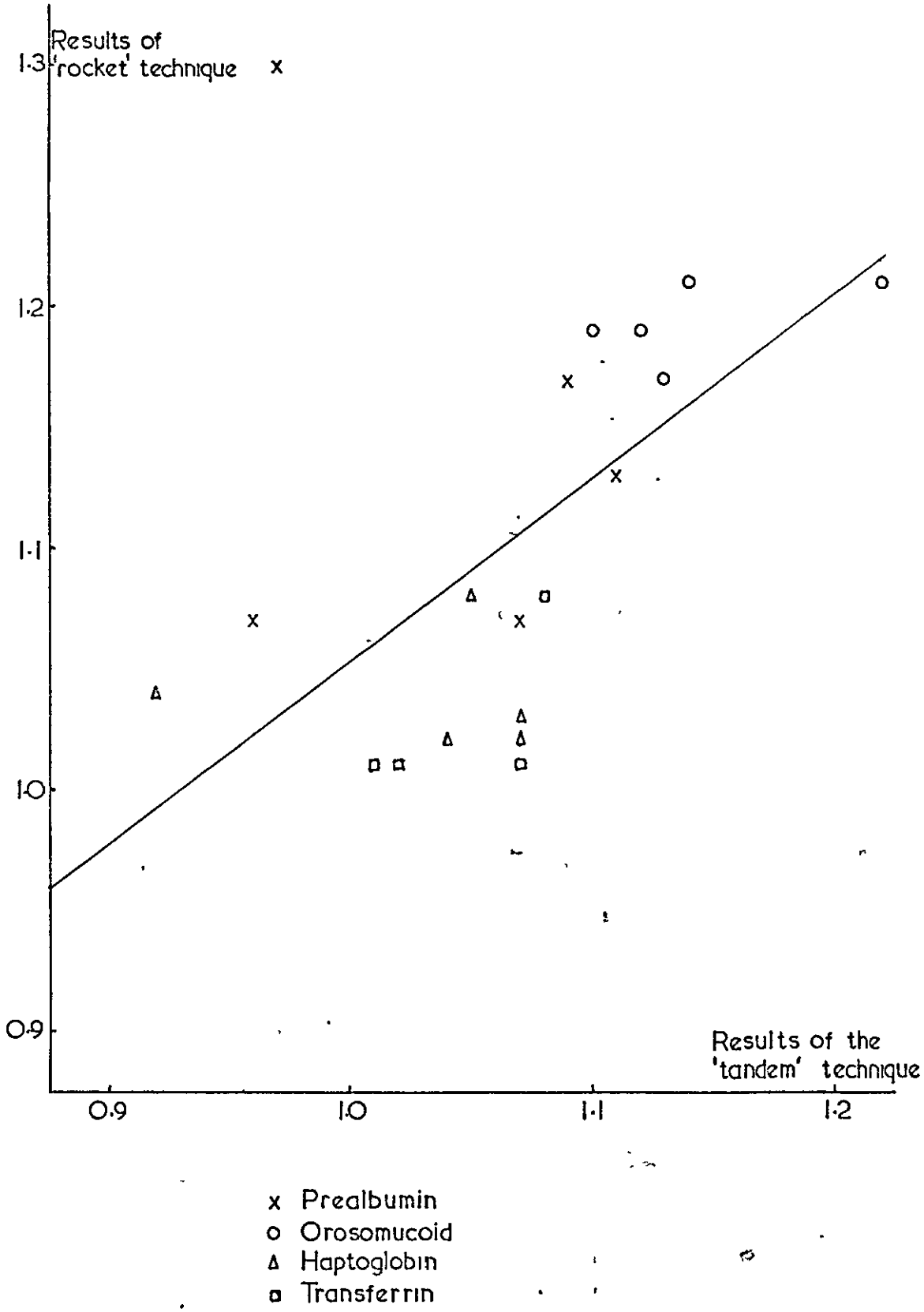


Table 3.IV

Comparison of the results obtained by the "tandem" ("T") and "rocket" ("R") techniques for the amounts of some plasma proteins in blood stains of different ages.

Age of stain/days	Pre_2/Pre_3		Oro_2/Oro_3		Hpt_2/Hpt_3		Trf_2/Trf_3	
	"T"	"R"	"T"	"R"	"T"	"R"	"T"	"R"
1	1.09	1.17	1.14	1.21	1.07	1.03	1.08	1.08
8	1.11	1.13	1.22	1.21	1.05	1.08	1.01	1.01
11	0.97	1.30	1.13	1.17	0.92	1.04	1.07	1.01
22	1.07	1.07	1.12	1.19	1.07	1.02	1.02	1.01
29	0.96	1.07	1.10	1.19	1.04	1.02	1.01	1.01

3.6. Examination of the Stability of Plasma Proteins in Blood Stains by Other Methods.

3.6.1. Immunoelectrophoresis.

The results of the immunoelectrophoresis experiments, designed to illustrate any changes in electrophoretic mobility or homogeneity are demonstrated in Figure 3(v). Three samples were applied to each plate. In the well on the left hand side was placed extract prepared from stains 1 day old, in the central well, a sample of extract from stains 17 days old, and in the right-hand well, extract prepared from stains 100 days old. The relevant monospecific antiserum was placed in each of the two troughs at the end of the elctrophoretic stage.

Prealbumin shows no change in electrophoretic mobility, even after 100 days, though only a faint precipitate was obtained for the oldest sample.

Albumin in the two older stain extracts gives a longer precipitation arc, extending towards the application well, than is observed

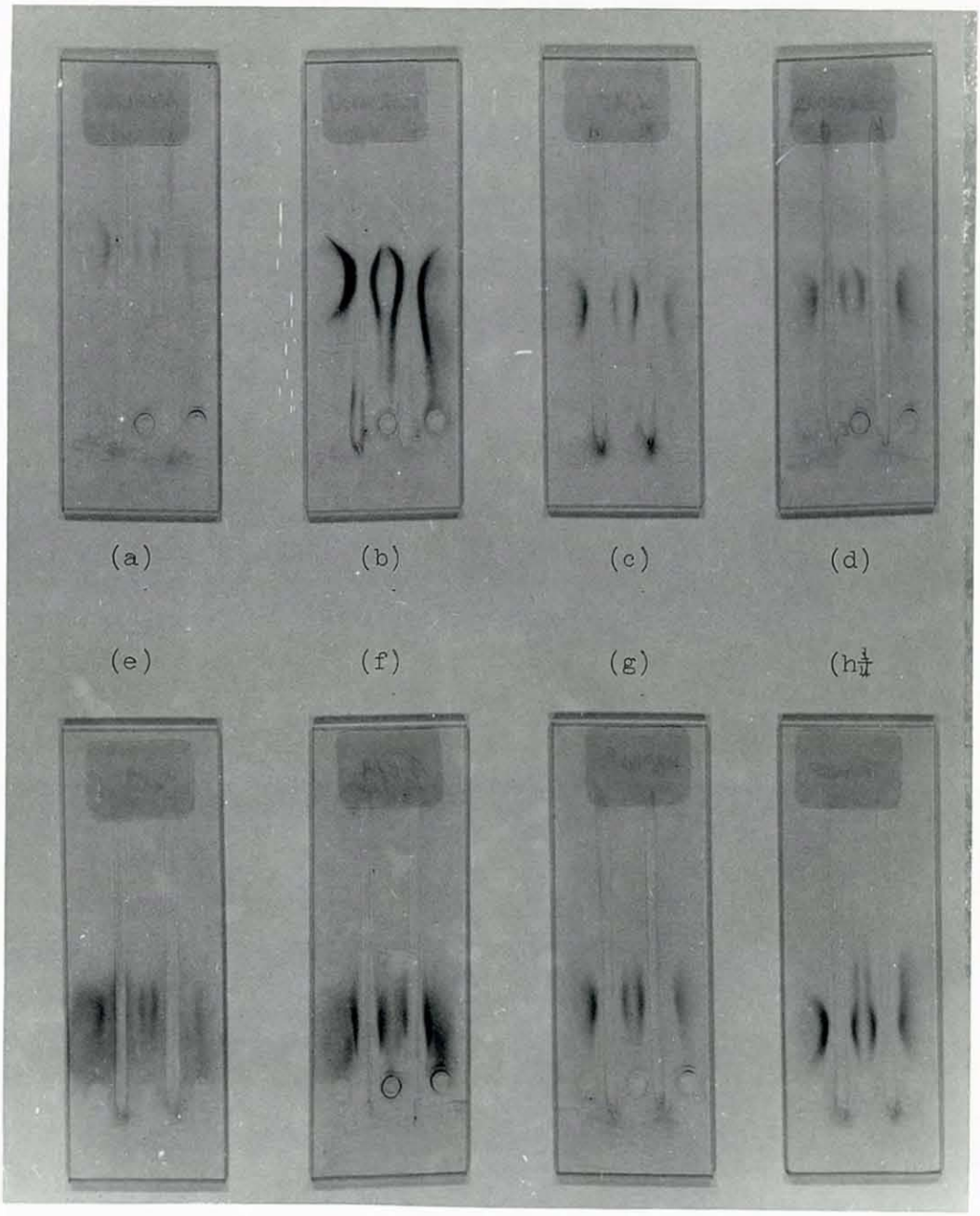


Figure 3(v): Results of the immunoelectrophoresis experiments for

(a) Prealbumin
(b) Albumin
(c) α_1-Antitrypsin
(d) Orosomucoid
(e) α_2-Macroglobulin
(f) β_1C/A-Globulin
(g) Haptoglobin
(h) Transferrin

for the albumin present in the 1 day old sample. Also, as the stain ages, the curvature of the precipitin arc observed for albumin decreases.

α_1-Antitrypsin shows no change in electrophoretic mobility, but, like prealbumin, a more feeble precipitate was obtained for the oldest sample.

Orosomucoid shows no change in mobility .

α_2-Macroglobulin in the 17 day old stain extract shows no change in mobility, but the oldest sample contains α_2-macroglobulin which gives rise to a longer arc which is of lower curvature.

β_1C/A-Globulin gives two precipitates for each sample; a heavier precipitate is formed nearer the antiserum trough, and another, more diffuse precipitate forms further from the antiserum trough.

Haptoglobin produces arcs which become longer and less curved as the stain ages.

Transferrin in the extracts of stains 17 and 100 days old gives an additional precipitate, arising from protein that has a higher mobility than the protein present in the most fresh sample.

3.6.2. The Results of the Ouchterlony Tests.

Extracts were prepared from blood stains 2, 3, 17 and 80 days old. Ouchterlony plates were set up as described in section 2.5.2, placing monospecific antiserum in the central wells and aliquots of the concentrated stain extracts in the surrounding wells, according to the scheme indicated in Figure 3(vi)a. Samples of fresh, haemolysed blood were placed in wells 1 and 4, for comparison.

Difficulty was encountered in obtaining satisfactory results for β_1C/A-globulin. The application of insufficient stain extract led to precipitation occurring in the antigen wells.

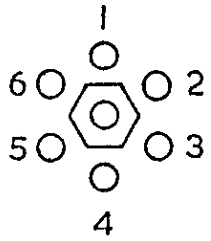
For prealbumin, albumin, orosomucoid, α_1-antitrypsin and transferrin, no change in antigenic properties was observed between the stain extracts and the fresh blood sample.

The results for haptoglobin suggest that one of the two

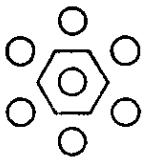
Figure 3.(vi)

The results of the Ouchterlony tests.

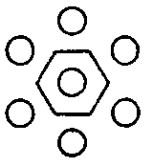
a) Prealbumin



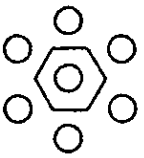
b) Albumin



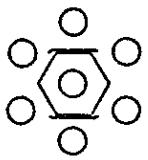
c) Orosomucoid



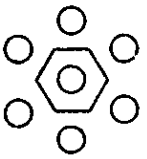
d) α_1-Antitrypsin



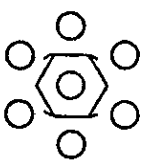
e) Haptoglobin



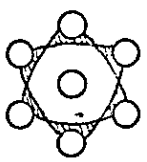
f) Transferrin



g) α_2-Macroglobulin

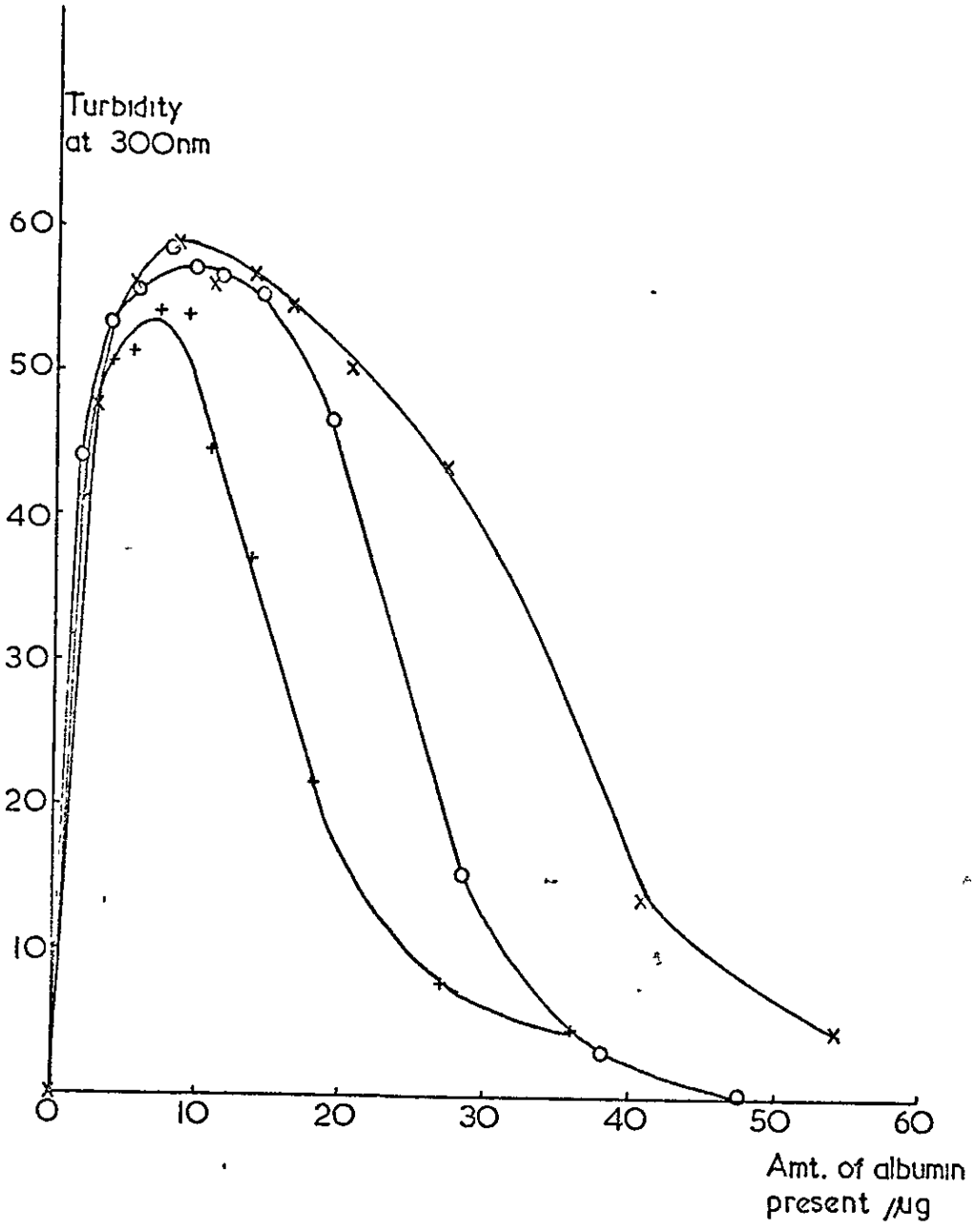


h) β_1C A-Globulin



Well number : 1 : Fresh haemolysed blood.
2 : Extract of stains 2 days old
3 : " " " 3 " "
4 : Fresh haemolysed blood.
5 : Extract of stains 17 days old
6 : " " " 80 " "
Central well : Monospecific antiserum.

Figure 3.(vii). Immunoprecipitin curves for albumin in a fresh blood sample and in extracts of blood stains.



x—x : Fresh haemolysed blood sample.
o—o : Extract of stains 5 days old.
+—+ : Extract of stains 27 days old.

precipitation lines present in the fresh blood sample is absent from the extracts of stains of all ages.

Spurs are formed between the α_2-macroglobulin in fresh blood and that in the extracts of blood stains. Thus some change in identity is believed to occur in this protein, either in the stain preparation or the extraction stage.

3.6.3 Quantitative Immunoprecipitation.

Extracts of stains 5 and 27 days old were used to construct complete immunoprecipitation curves for albumin. A fresh haemolysed blood sample was used to prepare a reference curve. Increasing amounts of stain extracts were added to constant amounts of antiserum. Controls were used as indicated in section 2.5.3, to give the resultant immunoprecipitation curves illustrated in Figure 3(vii).

The curves for each sample do not vary significantly in the antibody excess region, or in the position of equivalence, (within the limits of experimental error). The amount of precipitate formed at the equivalence point is reduced slightly with increasing age of the stain. The most marked difference between the curves is observed in the antigen excess region. As the stain ages, less precipitate is formed in the antigen excess region, suggesting that the antigen-antibody complex formed with older samples is more susceptible to the presence of excess antigen, and is therefore probably less stable.

3.7. Results of "Blind Trials."

In order to test the feasibility of a plasma protein profile as a means of distinguishing between the donors of whole blood samples and of blood stains, a number of blind trials were performed. In each case, 5μl blood samples were taken, and seven proteins (Prealbumin, albumin, orosomucoid, α_1-antitrypsin, haptoglobin, transferrin and

α_2-macroglobulin) were measured by electroimmunodiffusion. The heights of the precipitin arcs were used as a measure of the amount of protein present (no standard solutions were used), and related to the peak height of the orosomucoid in the sample.

3.7.1. Matching Whole Blood Samples.

Three samples of whole blood in saline were provided, labelled A, B and C. The results for the amounts of each protein present, relative to the amounts of orosomucoid are given in Table 3. V, and the plasma protein profiles for the three samples illustrated in Figure 3(viii)a.

Table 3. V.

Results of First Blind Trial.

	Ratios of Proteins to Orosomucoid.					
Sample	Pre.	Alb.	α_1AT.	Hpt.	Trf.	α_2M.
A	2.09	2.74	3.41	1.93	2.26	1.82
B	1.73	2.92	2.13	1.92	1.78	2.06
C	2.09	2.74	3.43	1.98	2.21	1.83

Samples A and C were correctly identified as originating from the same donor.

3.7.2. Matching Whole Blood Samples with Blood Stains up to One Week Old.

Two whole blood samples in saline (C and D) were provided, each of which matched with one of the two blood stains (A and B). Again, seven proteins were measured in each sample, and the peak heights related to those of orosomucoid. The results are given in Table 3.VI, and the plasma protein profiles illustrated in Figure 3(viii)b.

Figure 3.(viii).a Profiles for the samples examined in the first blind trial.

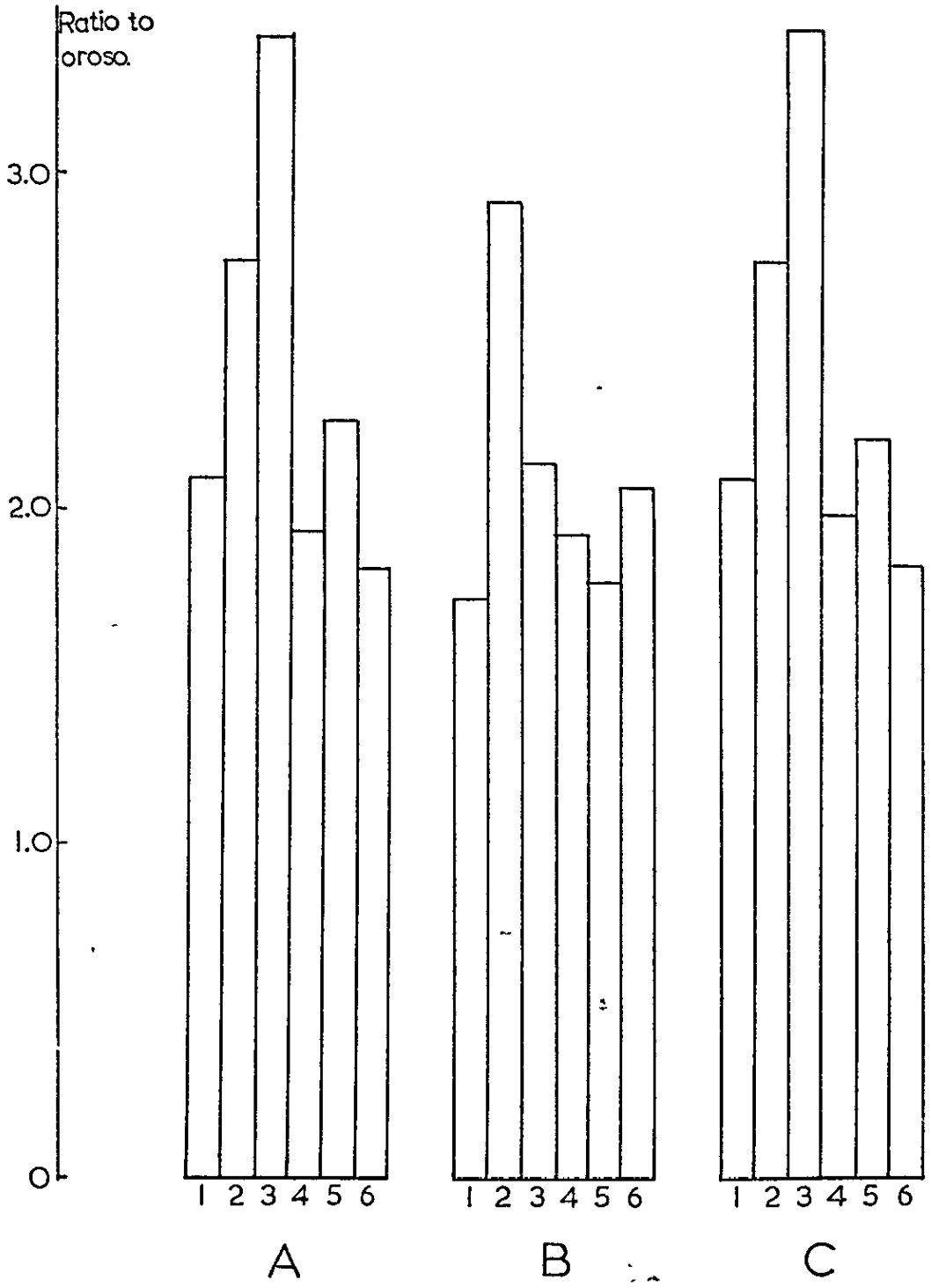
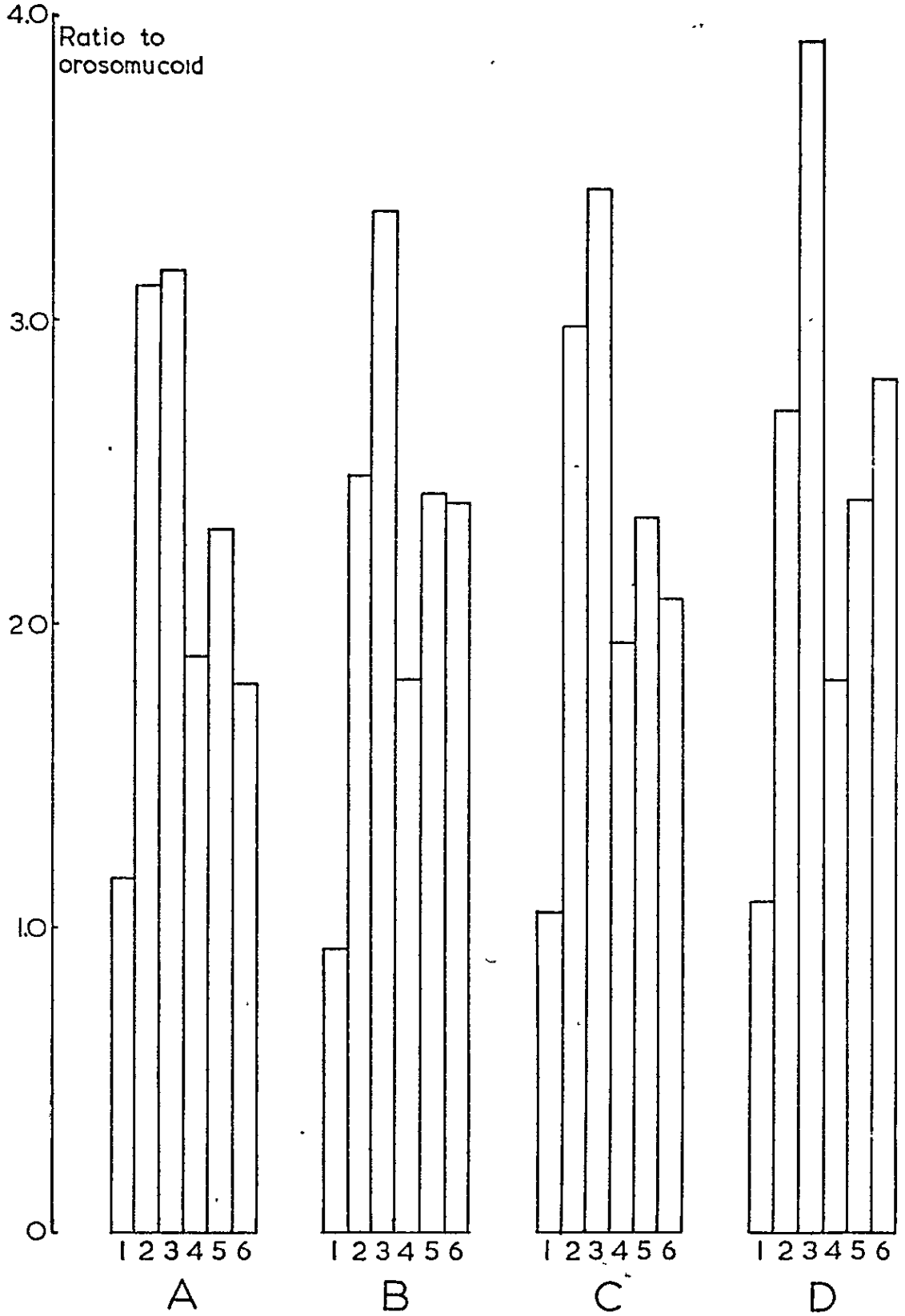


Figure 3.(viii).b Profiles for the samples examined in the second blind trial.



1 : Prealbumin
2 : Albumin
3 : α_1-Antitrypsin
4 : Haptoglobin
5 : Transferrin
6 : α_2-Macroglobulin

Table 3.VI.

Results of Second Blind Trial.

Sample		Ratios of Proteins to Orosomucoid				
		Pre.	Alb.	α_1AT.	Hpt.	Trf.	α_2M.
A	Stain	1.16	3.11	3.16	1.89	2.31	1.80
B	Extracts	0.93	2.49	3.36	1.82	2.43	2.40
C	Whole	1.05	2.98	3.43	1.94	2.35	2.09
D	Blood	1.09	2.71	3.92	1.82	2.41	2.81

Samples A and C, and B and D were correctly paired. (Actually, stain A was 3 days old, and stain B 7 days old).

3.7.3. Matching Stains up to One Week Old.

Six 5µl blood stains were provided, labelled A - F. All were five days old, and might have included stains prepared from blood from the same person. The results are tabulated in Table 3.VII, and the profiles obtained illustrated in Figure 3(viii)c.

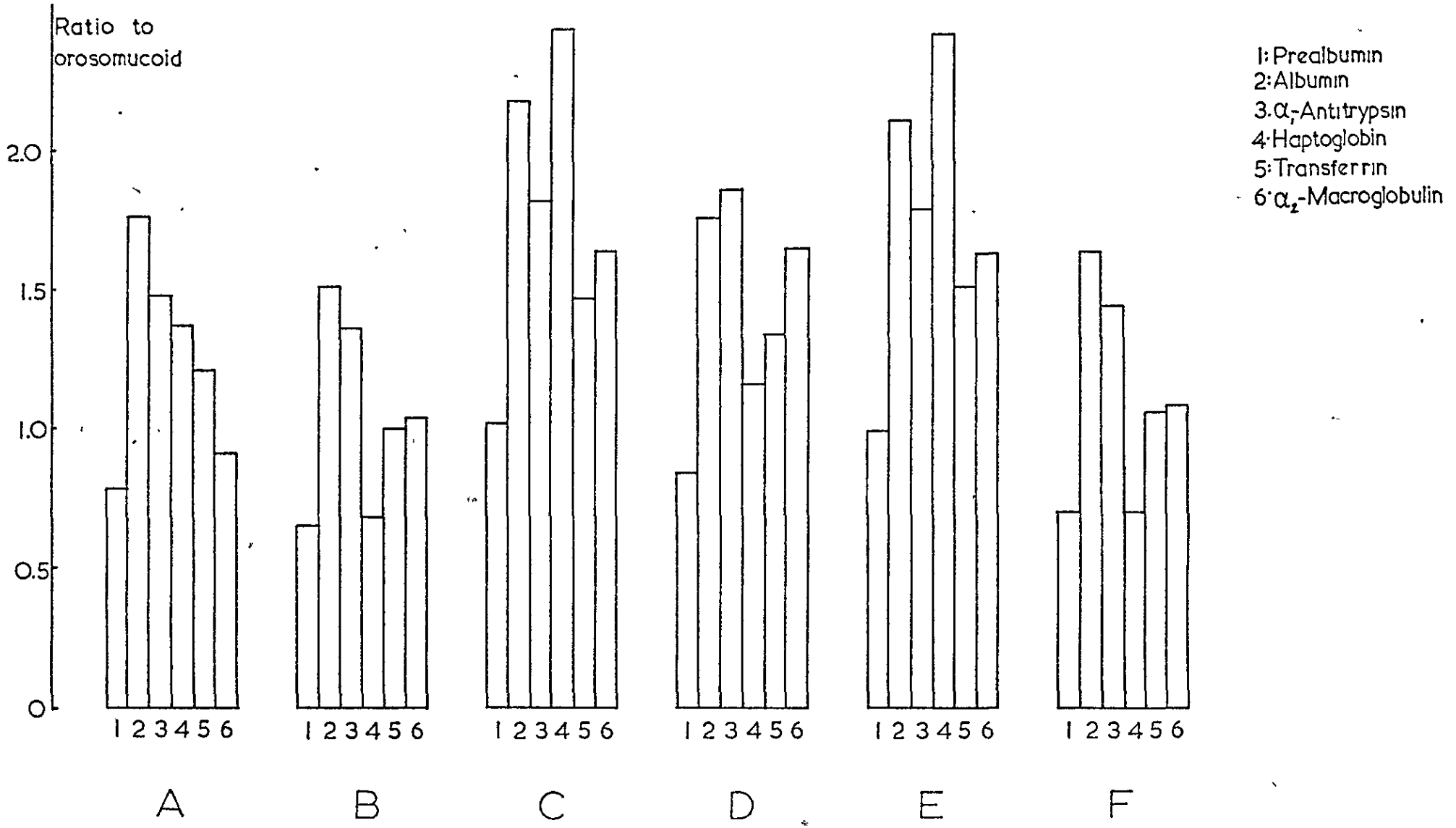
Table 3.VII.

Results of Third Blind Trial.

Sample	Ratios of Proteins to Orosomucoid.					
	Pre.	Alb.	α_1AT.	Hpt.	Trf.	α_2M.
A	0.78	1.76	1.48	1.37	1.21	0.91
B	0.65	1.51	1.36	0.68	1.00	1.04
C	1.02	2.18	1.82	2.45	1.47	1.64
D	0.84	1.76	1.86	1.16	1.34	1.65
E	0.99	2.11	1.79	2.42	1.51	1.63
F	0.70	1.64	1.44	0.70	1.06	1.08

B and F, C and E were correctly paired, since the plasma protein profiles drawn (Figure 3(viii)c) matched closely. Stains A and

Figure 3.(VIII).c Profiles for the samples examined in the third blind trial.



D were prepared from the blood of different donors.

3.7.4. Matching Stains up to 30 Days Old.

Six stains were provided, labelled A - F, which were up to 30 days old. They were known to include stains made from the same person at different times. The results are given in Table 3.VIII, and illustrated in Figure 3(viii)d.

Table 3.VIII.

Results of the Fourth Blind Trial.

Sample	Ratios of Proteins to Orosomucoid					
	Pre.	Alb.	α_1AT.	Hpt.	Trf.	α_2M.
A	1.53	1.84	4.02	1.66	1.11	3.09
B	1.61	2.36	3.45	1.52	1.11	2.08
C	1.32	1.84	2.46	1.41	0.83	1.30
D	1.61	2.01	3.37	1.52	1.15	2.20
E	1.58	1.83	4.12	1.42	1.10	2.94
F	1.47	2.17	3.73	1.41	1.04	2.05

After inspection of the plasma protein profiles, A and E were correctly paired, and B, D and F correctly identified as being donated by the same subject. (Actually, stain A was 28 days old, and stain E 21 days old. Both were from the same donor. Stains B and F were 1 day old, stain B being donated in the afternoon, and stain F in the morning. Stain D was prepared from the donor of stains B and F, but was 27 days old. Stain C, from a different donor, was 5 days old).

3.7.5 Matching Stains up to 50 Days Old.

Six stains, up to 50 days old, were provided, labelled A - F, which included samples, prepared from the same donor at different times. The results are given in Table 3.IX, and illustrated in Figure 3(viii)e.

Figure 3.(viii)d Profiles for the samples examined in the fourth blind trial

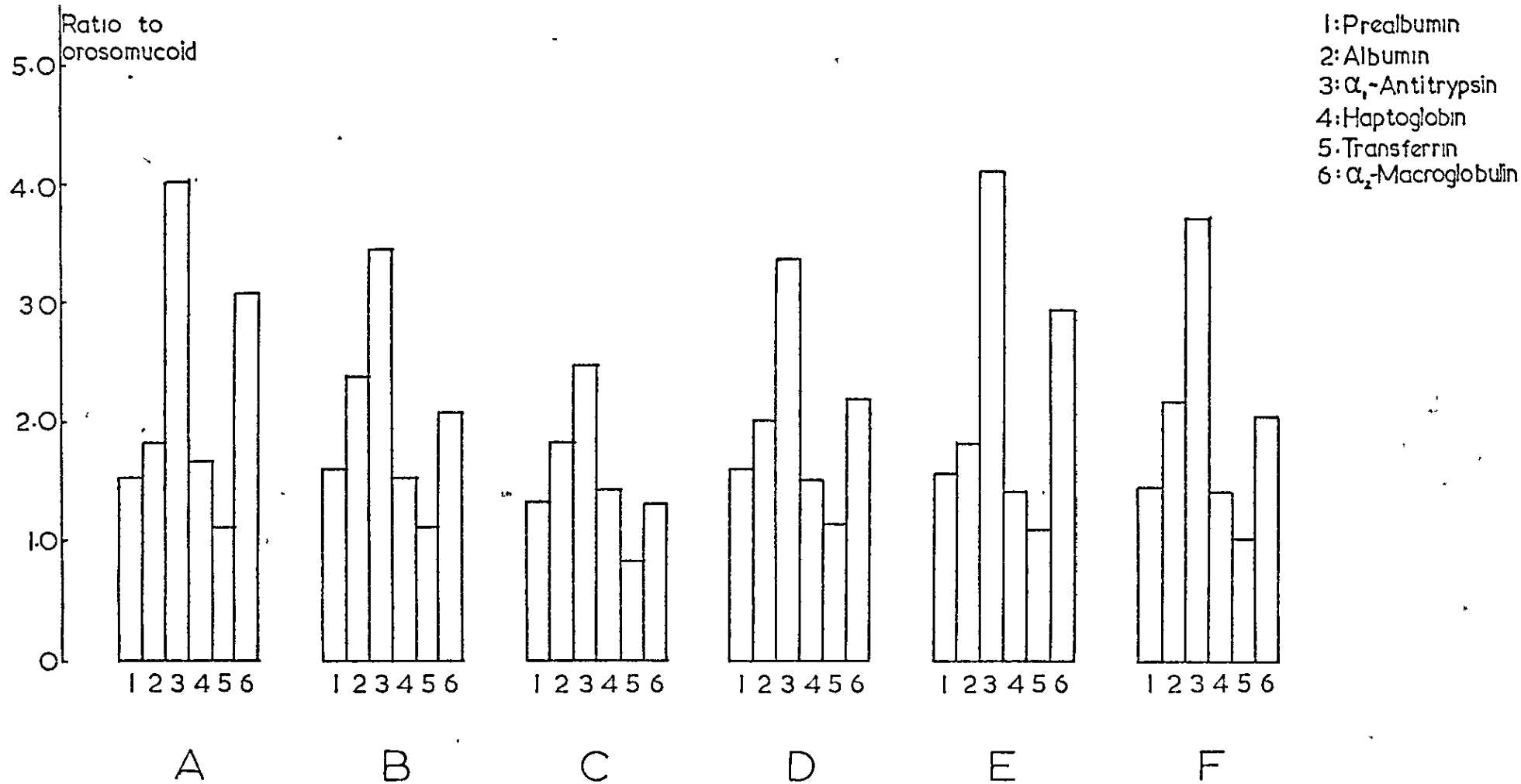


Figure 3(viii) e Profiles for the samples examined in the fifth blind trial

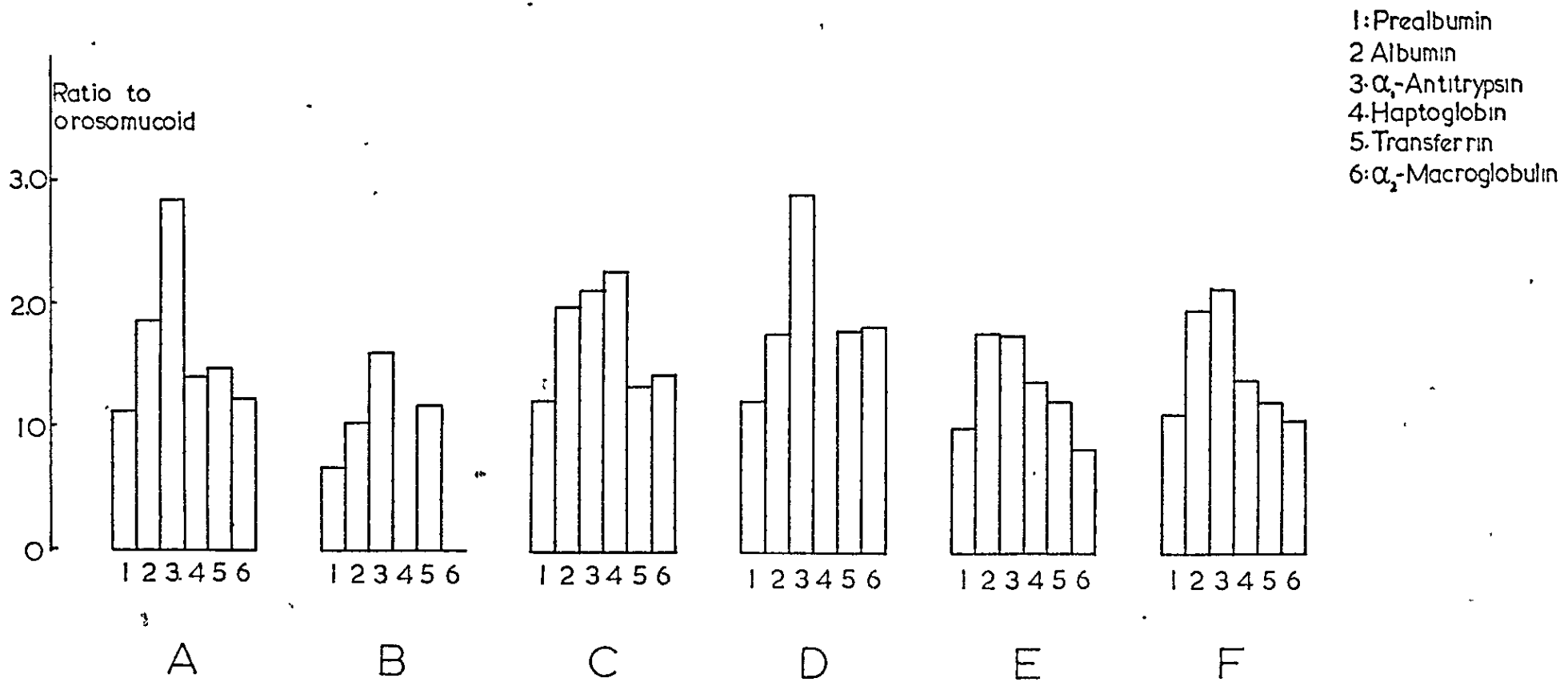


Table 3.IX.

Results of Fifth Blind Trial.

Sample	Ratios of Proteins to Orosomucoid. Pre.	Alb.	α_1AT.	Hpt.	Trf.	α_2M.
A	1.11	1.86	2.84	1.39	1.46	1.22
B	0.67	1.04	1.60	----	1.18	----
C	1.22	1.98	2.11	2.27	1.34	1.42
D	1.21	1.76	2.90	----	1.79	1.82
E	1.01	1.78	1.76	1.39	1.21	0.83
F	1.13	1.96	2.14	1.40	1.22	1.07

"----" = not detectable.

Since haptoglobin could not be detected in stains B and D, it was concluded that they were more than 36 days old. α_2-Macroglobulin could not be detected in stain B, suggesting that this stain was older than stain D. Because these stains were older than those encountered in the previous blind trials, the profiles obtained for them were examined carefully, since the levels of some of the other proteins (albumin, α_1-antitrypsin) may have altered significantly since the time of stain preparation. Of the other stains, samples A and F were tentatively matched. No other conclusions were drawn.

(Actually, A, D and F originated from the same donor, and were 12, 41 and 1 day old respectively. Stains B and E were donated by the same suject, and were 46 and 10 days old respectively. Stain C was not paired with another, but was 10 days old).

3.7.6. The Use of the "Tandem" Technique in the Blind Trials.

The samples provided in the third and fourth blind trials were also examined by the "tandem" technique. Prealbumin, orosomucoid, α_2-macroglobulin and transferrin were quantitated. Each sample was

compared with a standard serum sample (Behringwerke, Hoechst Pharmaceuticals), on a single "tandem" plate. A known volume of carbamylated transferrin was added to fixed volumes of each stain extract and the standard serum solution. The carbamylated transferrin peaks on each plate were used to correct for sampling errors in the technique. The corrected peak heights for the precipitin bands of the test solution were then compared with the peak heights corresponding to the same protein in the standard solution, i.e. the values of

$$\frac{\text{Pk.ht. for protein in test}}{\text{Pk.ht. for protein in standard}} \times \frac{\text{Pk.ht. for C.T. in standard}}{\text{Pk.ht. for C.T. in sample}}$$

were calculated.

Although comparison between the amounts of a particular protein in each of the sample solutions was thus made possible, the results of these blind trials, given in Tables 3. X and 3.XI, show these corrected amounts relative to the amounts of orosomucoid. Relating the amounts of each protein to the amounts of orosomucoid may eliminate some of the error incurred during the stain preparation procedure.

Table 3. X.

Results of the Third Blind Trial, Obtained by the "Tandem" Technique.

Sample	Ratios of Proteins to Orosomucoid		
	Prealbumin	α_2-Macroglobulin	Transferrin
A	1.54	0.91	1.00
B	0.97	1.01	0.63
C	1.92	2.08	1.24
D	1.27	1.53	1.00
E	2.00	1.94	1.28
F	0.95	0.92	0.68

Table 3.XI.

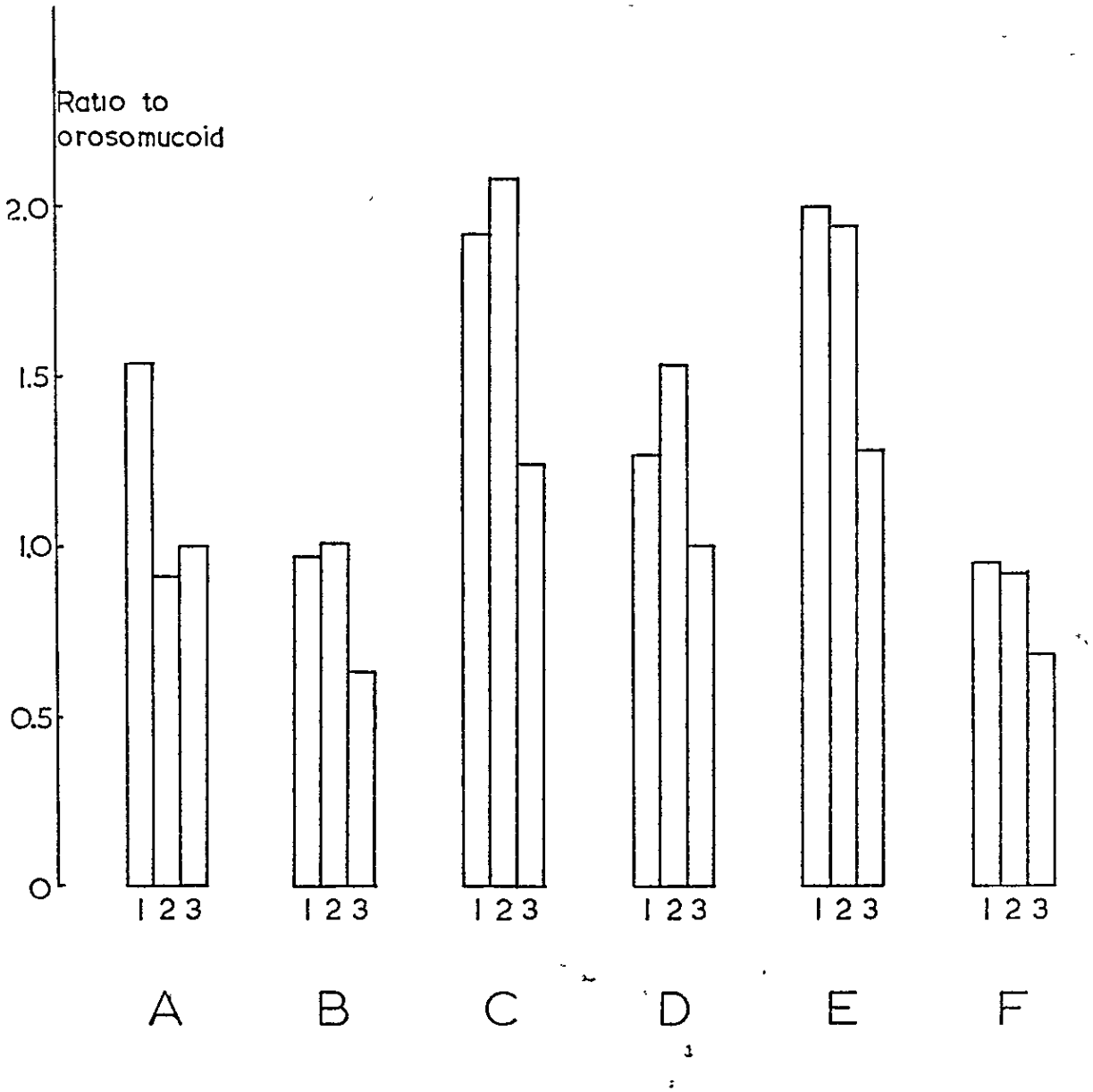
Results of the Fourth Blind Trial, Obtained by the "Tandem" Technique.

Sample	Ratios of Proteins to Orosomucoid		
	Prealbumin	α_2-Macroglobulin	Transferrin
A	1.23	1.88	0.93
B	2.04	1.54	1.36
C	1.38	0.90	0.84
D	1.54	1.50	1.02
E	1.13	2.04	1.06
F	1.63	1.31	1.20

The plasma protein file for each sample in the third blind trial is illustrated in Figure 3(ix), and in the fourth blind trial in Figure 3(x).

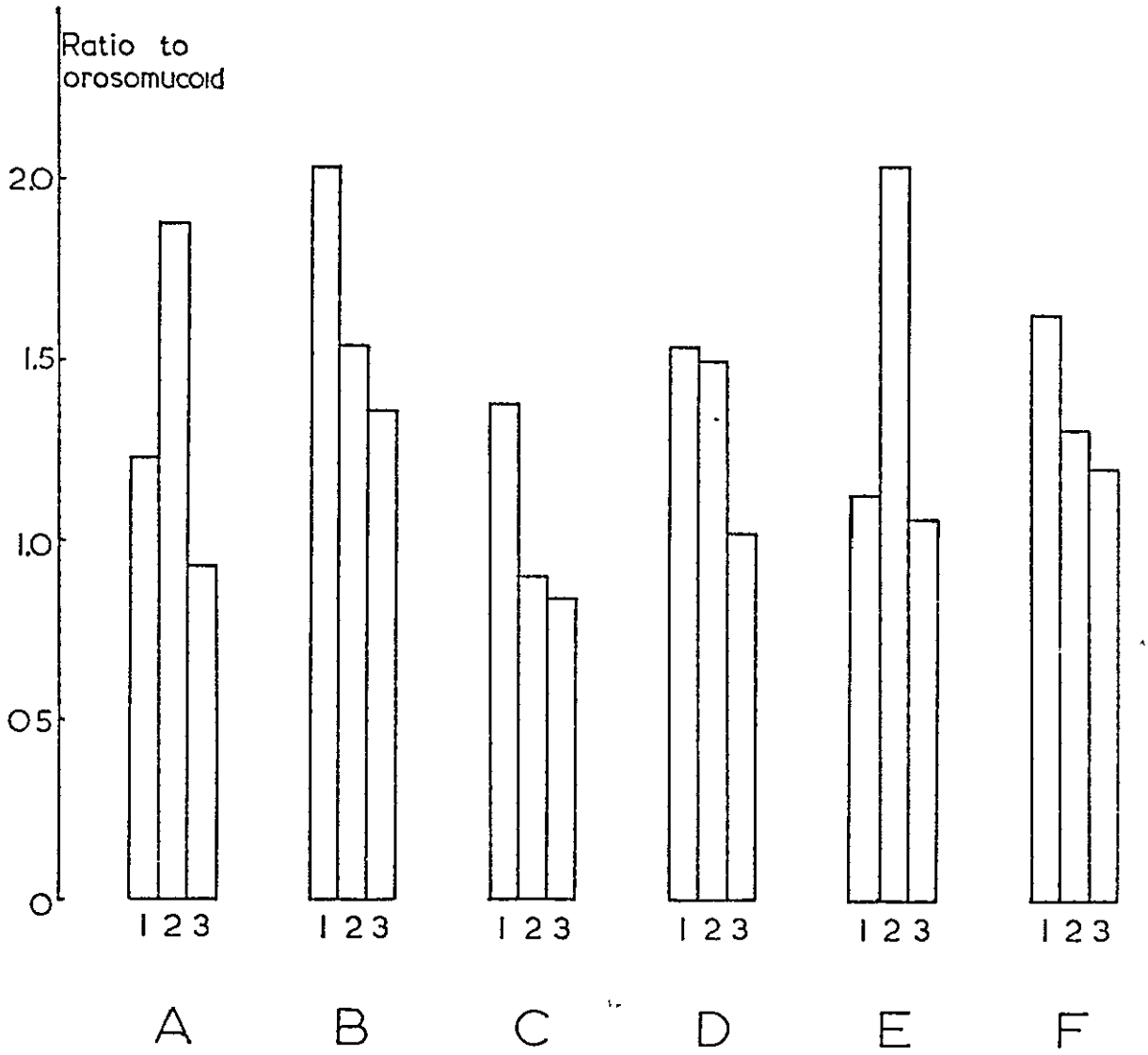
The results for the matched pairs of stains in the third trial are generally no worse for the "tandem" technique than for the "rocket" technique. Difficulty is encountered, however, in matching the profiles obtained in the "tandem" technique, mainly through lack of data, since only four proteins are included in the profile, compared with seven in the "rocket" technique. The discrepancies in the values obtained for matched samples are generally greater in the results of the fourth blind trial than for the third blind trial, but some comparisons can be drawn between the profiles drawn for each sample.

Figure 3.(ix). Profiles for the samples examined in the third blind trial by the 'tandem' technique.



1 : Prealbumin
2 : α_2-Macroglobulin
3 : Transferrin

Figure 3.(x). Profiles for the samples examined in the fourth blind trial by the 'tandem' technique.



1. Prealbumin
2: α_2-Macroglobulin
3: Transferrin

4. DISCUSSION.

The results presented in the previous chapter indicate the suitability of a plasma protein profiling system for discriminating between blood stains. Studies of the stabilities of a number of plasma proteins in ageing blood stains have indicated the suitability of each protein for inclusion in such a profile. As suggested in the Introduction, a satisfactory appraisal of the role of individual proteins in a profiling system cannot be achieved without adequate information concerning personal and population variations, and also the stabilities of the proteins in blood stains. Although each protein studied cannot be surveyed thoroughly, owing to lack of data concerning personal and population variations, the success of the blind trials indicates the potential of a profiling system. Some of those proteins which were included in the profiles for the samples examined in the blind trials may not be suitable, because the variations within an individual may be almost as great as the population variations (e.g. albumin, transferrin). Some of the proteins studied were not completely stable (e.g. albumin, α_1-antitrypsin), and a more efficient profiling system could

be developed if only stable proteins with large population variations were included.

A number of the proteins studied appear to be stable in blood stains for a certain period. The amounts of prealbumin, orosomucoid and transferrin extracted from blood stains were stable over the entire period of study. The results of the immunoelectrophoresis and Ouchterlony tests indicated that no change in electrophoretic mobility or immunological identity occurred in either prealbumin or orosomucoid on storage. Transferrin extracted from blood stains appeared to be immunologically identical with that in a fresh blood sample. On immunoelectrophoresis, however, two precipitin bands were observed for the transferrin extracted from blood stains, compared with a single arc for the transferrin in the fresh blood sample. The presence of the additional protein of higher electrophoretic mobility does not affect the apparent amount of this protein in stain extracts, so it is still suitable for inclusion in a plasma protein profile, in this respect.

Both haptoglobin and α_2-macroglobulin appear to be stable for a period, and then undergo more marked changes, which lead to apparent increases in the amounts of these proteins. The results of the Ouchterlony tests suggest that changes occur in these proteins, possibly during the drying stage. (Spurs are formed between the α_2-macroglobulin in fresh blood samples and fresh stain extracts. One of the two precipitin lines observed for haptoglobin in fresh blood is absent from fresh stain extracts.) The lengthening of the precipitin arcs observed on the immunoelectrophoresis plates suggests that the proteins become less homogeneous on storage in stains. The apparent increase in the amounts of α_2-macroglobulin and haptoglobin, after 22 and 29 days, respectively, could be due to gradual denaturation of the proteins. It has been shown (Bock and Axelsen, 1972) that the presence of partially identical proteins leads to impairment of quantitation by electroimmunodiffusion. It is possible that, on storage, the proteins undergo conformational changes such that some of the antigenic sites present

in the 'native' protein are lost. At the same time, unfolding of the polypeptide chains may lead to the exposure of different antigenic sites, which can then react with antibodies specific to them, present in the antiserum. The presence of such antibodies may be accounted for if the protein originally used to elicit the production of antibodies was partially denatured before it was injected into the animal, or if denaturation of the protein occurred within the animal before antibody production commenced. (This is believed to occur with anti-human orosomucoid production in the rabbit: Barker and Whitehead, 1963).

β_1C/A-Globulin also shows an apparent increase in concentration as the blood stains age. This may be due to the gradual conversion of β_1C- to β_1A-globulin. β_1C-globulin is the native form of complement factor C3. It is found only in fresh samples, and on storage is converted to β_1A-globulin (Alper, 1974). β_1C-globulin has a lower electrophoretic mobility than β_1A-globulin, and when the two forms are compared by antigen-antibody crossed electrophoresis (Alper, 1974), β_1C-globulin gives a broader-based, flatter precipitin arc than β_1A-globulin which gives rise to a more pointed "rocket". The two bands observed in the immunoelectrophoresis patterns may correspond to the two forms of this protein, the more diffuse precipitation arising from the presence of β_1C-globulin.

α_1-Antitrypsin and albumin both appear to decline in concentration as the age of the blood stains increases. No change in identity or electrophoretic mobility of α_1-antitrypsin was observed, so the gradual decline in the amounts extracted may be due to the increased difficulty in extracting it from the stained material.

Although no change in identity was indicated from the results of the Ouchterlony test, albumin appears to become less homogeneous as the stains age. This is indicated by the lengthening of the precipitin arc towards the sample well of the immunoelectrophoresis plate as the blood stains age. Also, a gradual change in the shape of the quantitative immunoprecipitin curve with increasing age of the stain was observed.

A similar observation was made by Levine and Brown (1957), when they constructed immunoprecipitation curves for albumin in its dimeric and monomeric forms. The curves were similar in the antibody excess and equivalence regions, but in the antigen excess region the dimer was less effective in inhibiting the formation of the precipitate. Although, by analogy, the curves obtained in the present work could imply that dimeric albumin (which occurs to an appreciable extent in serum) is being converted to the monomer, the results of the immunoelectrophoresis tests suggest that this is not the case. Monomeric albumin has a higher electrophoretic mobility than the dimer (Schultze and Heremans, 1966), and since there is an increase in the amount of protein with a lower electrophoretic mobility than the "native" albumin, it is suggested that other changes occur. It may be possible that the protein undergoes proteolytic denaturation.

It was suggested in the Introduction, that six proteins, each with a discriminating power of 0.42 would be required in order to give satisfactory discrimination between blood stains at the 99% confidence level. In addition to showing small variations within the individual, compared with the expected population variations, the proteins should, of course, be stable in blood stains. Of those proteins so far examined, prealbumin, orosomucoid and transferrin appear to be stable in stains up to at least 50 days old, and haptoglobin and α_2-macroglobulin in stains up to 29 and 22 days old respectively, and therefore satisfy the requirement concerning the stability of the protein in ageing stains. The expected personal and population variations for all of these proteins is not known, so a detailed discussion of the use of these proteins in a plasma protein profiling system cannot be given. α_1-Antitrypsin declined in concentration as the stains aged. Although the concentration fell significantly, this protein would still be of use in a profiling system, if the stain was only a few days old, so that the fall in concentration was not significant compared with the expected

inter-person variation. Albumin appears to be unstable in blood stains, and since this protein has also been shown to offer only low discriminating power (King, 1974), it would seem an unsuitable protein for a plasma profiling system. β_1C/A-Globulin, although it offers a high discriminating power would also appear to be unsuitable for inclusion in a plasma protein profile because it is unstable in blood stains.

Thus, it would be of value to determine whether other proteins remain stable in stains, to augment the list of those already found suitable for a profiling system. In determining which other proteins to investigate, some useful information may be obtained from the results for the proteins studied in the present work. Orosomucoid and transferrin, which appear to be stable in blood stains, are single chain glycoproteins of relatively low molecular weight. Prealbumin, is also stable, and although it is a multi-subunit protein, is of low molecular weight, and contains some carbohydrate (Table 1.V). Haptoglobin and α_2-macroglobulin are both multi-subunit proteins of fairly high molecular weight. They both appear to be stable for a period, and then undergo marked changes. Albumin, which is unstable, is a single chain protein, containing no carbohydrate. From these results it might be suggested that small, single chain glycoproteins are the most stable in blood stains. However, the results for α_1-antitrypsin, which is a protein of this type, would suggest that it is untrue. α_1-Antitrypsin may be exceptional, however, since it has been shown to be a fragile protein, undergoing denaturation on storage, (Koj, 1974).

It has already been emphasised that the suitability of a plasma protein for inclusion in a profiling system cannot be judged by its stability in blood stains alone. The blind trials performed indicate the advantages of including proteins which have a large variation within the population, compared with the intra-person variation, even though they may not be completely stable in blood stains. α_1-Antitrypsin is a protein of this type. The serum level of α_1-antitrypsin is genetically

determined, and marked differences occur between the serum levels of this protein in subjects of different genotype. This phenomenon was observed for the samples examined in the blind trials, where the differences between the levels for different subjects was so great that matching of paired samples was possible in stains of up to 50 days old.

The serum levels of haptoglobin are also genetically determined; haptoglobin type 2-2 is characterised by lower concentrations than type 1-1, with haptoglobin type 2-1 occurring at intermediate levels. This protein appears to be of great use in a profiling system for stains up to 29 days old, since it offers a high discriminating power. Similarly, α_2-macroglobulin is suitable for inclusion in a profiling system for stains less than 22 days old, since the levels of this protein vary significantly with the age and sex of the subject. In stains more than four weeks old, the levels of haptoglobin and α_2-macroglobulin recorded are probably not true estimates of the amount of each protein present.

The blind trials were designed to simulate situations in which plasma protein profiles might be of use in discriminating between blood samples. The first trial was exceptional, and indicated the error in the technique. In this trial, whole blood samples were compared, and the errors involved in stain preparation and extraction, and variations between different Cellogel strips were removed. The errors introduced by each of these factors were present in the stability experiments, but would not be evident in a real trial, where stains of unknown volume would be provided, and every effort would be made to compare all the samples on the same Cellogel strip. In this trial, excellent agreement (mean error - 1.2%) was obtained between the amounts of each protein in the two matched samples.

In the second blind trial, blood stains up to one week old were compared with fresh blood samples donated by the same subjects. This is a situation which would arise fairly often, if a single stain was left at the scene of a crime. The suspects would be required to

provide fresh blood samples, and a match made between the profile of one of the fresh samples and that obtained for the blood stain. For a number of the proteins included in the profiles, close agreement between the concentrations in fresh blood and in stain extracts was not obtained. It would have been of value to attempt more trials of this nature, to establish whether the discrepancies which existed between the amounts of some of the proteins in the matched pairs were the result of large variations within the individuals, or due to changes in the proteins in stains. It is possible that some proteins are extracted less efficiently from blood stains than others, or that some proteins undergo changes on drying, which are not reversed during the extraction procedure. A more thorough inspection of the plasma proteins examined in this work, and possibly an examination of more proteins would be necessary before confidence could be placed in a profiling system for comparing whole blood samples with stain extracts.

In the third blind trial it was shown that two blood stains shed by one person at a particular time could be matched without confusion with blood shed by different subjects at the same time. Although this trial was performed using stains which where 5 days old, it should be possible to construct profiles with similar accuracy for stains of greater age, if they were prepared at the same time and stored under similar conditions. In the fourth and fifth trials, stains made from the blood of the same donor, but shed at different times were compared, in the presence of other blood stains of different ages. The success of these trials depended on the proteins included in the profiles remaining stable within the individual, and also remaining stable on storage in dry stains. Correct matching of stains up to 30 days old was possible constructing profiles which included seven proteins. The close agreement observed between the profiles of matched samples may be influenced by the fact that much of the intraperson variation in plasma protein levels is caused by changes in haemato-concentration. Although

the absolute amounts of the proteins recorded will vary, relating the protein concentrations to the concentration of a reference protein may reduce much of this variation.

In the fifth blind trial, it was found that stains between 40 and 50 days old could not be successfully matched with fresh stains from the same donor. The lack of success was partly due to the failure to quantitate α_2-macroglobulin and haptoglobin in the oldest stains. While it may be possible that other plasma proteins remain stable in stains for longer periods than α_2-macroglobulin and haptoglobin, it may not be of value to develop a system for characterising stains more than 30 days old, since a majority of the specimens examined in the forensic laboratories will be less than one month old.

The results of the blind trials using the "rocket" technique indicate the potential of a plasma protein profiling system for discriminating between blood stains. Studies of additional plasma proteins may lead to the identification of further proteins which are suitable for inclusion in a plasma profiling system. Even though the proteins included in the blind trials were not ideal, the success of these trials suggests that a system of the type developed may be of use in forensic laboratories. The electroimmunodiffusion technique developed is rapid, requires simple apparatus, small quantities of sample, and has a standard method for each of the proteins to be measured. Some of the advantages of using a "tandem" two-dimensional technique for the construction of plasma protein profiles have already been stated; the method would enable quantitation of several proteins in two samples on (in theory) a single plate. (In practice, more plates would be required to incorporate adequate standardisation). Small quantities of sample and antiserum would be required, and preparation time would be minimal. However, the results presented in the present work suggest that the technique developed is not sufficiently precise. The precision achieved by the "rocket" technique for the samples examined in the third and

fourth blind trials was not matched by that of the "tandem" technique. It is likely that the technique will require modification before its use for the forensic examination of blood stains could be contemplated. The technique suggested by Bradwell and Burnett (1975) which unfortunately was not published until the present work was complete, may be preferable to the one developed. They claim that an overall precision of 3.6% may be obtained if the sample well is correctly placed. If this precision can be achieved in the "tandem" technique, then it may be more suitable for the construction of plasma protein profiles than the "rocket" technique. Although results are obtained more rapidly by the "rocket" method on Cellogel than by the "tandem" technique using agarose, preparation time is shorter, using the latter technique, and a permanent record of the results is more readily obtained. Some time and apparatus might be saved in the "rocket" technique if two proteins were quantitated on one strip of Cellogel. Laurell (1972) suggests that two proteins of similar electrophoretic mobility may be quantitated in this way, using agarose as the carrier medium.

Some of the advantages of a plasma protein profiling system over existing techniques of blood stain classification have already been discussed. The main advantage is that it should be possible to discriminate between individuals who have identical genetic classifications. The parameters included in the profiles should be carefully chosen, so that maximum discriminating power is obtained for the minimum number of attributes. The parameters measured cease to be of value when the variation within the population is less than the intra-person variation. In general, intra-person variation is less than the inter-person variation (Mondorf and Kollmar, 1969), variations in the personal plasma protein concentrations occurring as a result of changes in haematoconcentration (following periods of exercise or postural changes) or as a result of diurnal or seasonal fluctuations (Lyngbye and Krøll, 1971). It has already been indicated that the effect of changes in

haemato-concentration may be eliminated if the protein concentrations are related to a reference protein. Diurnal or seasonal fluctuations in the concentrations of individual plasma proteins, or marked changes in the plasma concentrations of the "acute phase" reactants will, however, affect the profiles obtained for samples taken from a subject at different times. In this respect, the polymorph-typing system may offer advantages, since the genotype of the subject will not change. However, some of the proteins which are included in the polymorph-typing system may give ambiguous results following storage in blood stains. Therefore it may be preferable to develop a system for blood stain characterisation where a number of continuously variable attributes with known personal and population variations are measured.

The present work has indicated the practical advantages of a plasma protein profiling system over existing techniques of blood stain analysis. Since relatively large volumes of sample are required for the typing of some polymorph systems, care must be taken to allocate any specimens left at the scenes of crimes to those tests which offer maximum discriminating power. Thus, preliminary tests are usually carried out on fresh blood samples taken from the suspects, to determine the optimum systems for discrimination. These tests are then performed on the specimen, in order to effect a match with one of the fresh blood samples. In order for this system to operate successfully, each forensic laboratory should be able to perform each of the tests developed for stain characterisation. Different tests involve the use of different electrophoretic systems, all of which may not be available. In a profiling system, some preliminary tests may be necessary, to establish the variations in the plasma protein levels within the suspects, before confidence could be placed in the match between the profiles for the subject's blood and that left at the scene of the crime (King, 1974). Since the plasma proteins can all be quantitated using similar electrophoresis apparatus and identical techniques, this should not be a major disadvantage.

One main advantage of the plasma profiling technique is that very small volumes of material are required. The "tandem" technique enables information to be obtained from smaller stains than could be analysed by the "rocket" technique.

One disadvantage of a plasma profiling system would be the storage of data concerning particular criminals. Because the protein concentrations are constantly fluctuating within the individual, it would not be feasible to classify a subject's blood at one time and use this classification on a subsequent occasion. In this respect, the polymorph-typing system may be of great value. The genetic classification of a subject will not alter, so such information can be kept on record. Reference to such a record may be of value in developing a more rapid system of stain classification, indicating those tests which should be performed to give maximum discriminating power.

It is possible therefore, that a plasma profiling system could be used in conjunction with existing methods of stain analysis. Profiles could be drawn, using only small volumes of material, which would give precise measurements of the amounts of the plasma proteins present. If proteins were included whose levels in the plasma were genetically determined, then additional information concerning the genetic classification of the suspect could be obtained. Further tests could then be performed to confirm the genetic groupings, and possibly related to classifications kept on record.

A number of criteria have not been investigated in the present work, but would require examination before the application of a plasma profiling technique could be contemplated. Blood stains of fixed volume only have been examined so far, and extracts have been prepared from whole stains. Although orosomucoid appears to be a suitable reference protein, which could be used to correct for changes in volume size when an entire blood stain is eluted, this may not be so when a portion of a large stain is extracted. The concentration of each protein may not be uniform over the entire area of the stain, owing to different rates

of diffusion, and fallacious results might be obtained if only a small area was tested.

Also, the effect on the plasma protein levels of storage under a range of conditions has not been studied. It is not known whether storage of identical stains under different conditions of light intensity, humidity and temperature would cause significant differences between the plasma protein profiles constructed. Culliford (1973) suggests that the temperature of storage affects the levels of serum enzymes in blood stains, and although it is unlikely that the immunological properties of the plasma proteins are so severely affected, they may be altered to some degree.

Another factor which has not been studied in the present work is the efficiency of extraction of the plasma proteins from blood stains made on different materials. It is possible that some proteins may be more, or less difficult to extract from other natural materials, or from synthetic fabrics. Thus two stains shed at the same time by the same donor on to two different materials may give rise to dissimilar plasma protein profiles.

The present work suggests that a plasma protein profiling system may be of value in the classification of blood stains. If a number of plasma proteins, which have large variations within the population compared with intra-person variations, and which remain stable in blood stains, were incorporated in the profile, then adequate discriminating power should be achieved. A number of the proteins studied in the present work have been shown to be suitable; prealbumin, orosomucoid, transferrin, α_2-macroglobulin and haptoglobin have all been found to be stable for a period in stains. Although α_1-antitrypsin was not stable, the large variation of this protein within the population suggests that it may still be of use. Albumin and β_1C/A-globulin appear to be unsuitable proteins. Further work is necessary to establish whether any more suitable plasma proteins could be of use.

Single chain glycoproteins may prove to be the most stable proteins in blood stains.

Further work is required before confidence could be placed in a profiling system for matching whole blood samples with stain extracts.

Much work remains to be done to determine the personal and population variations of those proteins already studied, and any others which prove to be of use in a profiling system. When these figures are available, an estimate of the discriminating power can be given and a more thorough appraisal of a plasma protein profiling system made. Work currently in progress in the Chemistry Department at Loughborough University of Technology may make such an analysis possible in the near future.

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APPENDIX 1.

Amounts of Plasma Proteins Extracted from Blood Stains.

a) Prealbumin.

Age of stain/days	Amounts of Prealbumin Extracted form Stains/µg Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	0.64	0.78	1.05	0.62	-	-
0	0.58	0.69	0.92	1.08	-	-
1	-	-	-	-	1.08	0.93
2	-	-	0.84	0.69	-	-
4	-	-	-	-	1.00	0.87
5	0.41	0.53	-	-	-	-
6	-	-	0.80	0.75	-	-
8	0.59	0.70	-	-	0.98	0.88
9	-	-	0.93	0.79	-	-
11	-	-	-	-	0.82	0.63
13	-	-	0.97	0.82	-	-
15	0.64	0.68	-	-	0.92	0.87
18	-	-	-	-	0.77	0.67
19	-	-	0.81	0.72	-	-
22	0.65	0.72	0.64	0.52	0.75	0.65
25	-	-	-	-	0.91	0.82
27	-	-	0.92	0.81	-	-
28	-	-	-	-	-	-
29	-	-	-	-	0.83	0.78
31	-	-	-	-	0.93	0.87
33	-	-	-	-	-	-
35	0.50	0.65	-	-	-	-
36	-	-	1.08	0.87	0.91	0.86
39	-	-	-	-	1.21	1.12
41	-	-	1.01	0.96	-	-
42	0.60	0.64	-	-	-	-
43	-	-	-	-	0.79	0.77
46	-	-	-	-	1.02	0.87
47	-	-	0.91	0.73	-	-
49	0.59	0.69	----	----	-	-
51	-	-			-	-
52	-	-			1.02	0.81

"-" : not measured. "----" : not detectable. "Sub." : Subject

b) Albumin.

Age of stain/days	Amounts of Albumin Extracted from Stains/μg Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	102.0	114.0	109.7	107.5	-	-
0	137.0	129.4	109.8	113.6	-	-
1	-	-	-	-	121.0	118.0
2	-	-	116.8	113.0	-	-
4	-	-	-	-	100.1	115.6
5	99.0	107.3	-	-	-	-
6	-	-	101.7	113.9	-	-
8	88.4	106.0	-	-	118.7	119.6
9	-	-	113.7	103.3	-	-
11	-	-	-	-	110.6	115.9
13	-	-	109.8	95.4	-	-
15	93.7	98.6	-	-	103.0	96.7
18	-	-	-	-	103.3	106.2
19	-	-	108.9	94.5	-	-
22	87.0	96.3	120.6	97.0	102.6	106.0
25	-	-	-	-	103.1	108.0
27	-	-	111.9	106.9	-	-
28	91.6	93.8	-	-	-	-
29	-	-	-	-	106.3	95.6
31	-	-	-	-	96.9	90.1
33	-	-	106.2	96.1	-	-
35	88.7	84.8	-	-	-	-
36	-	-	107.8	95.6	101.1	100.6
39	-	-	-	-	94.1	92.4
41	-	-	91.8	93.2	-	-
42	79.9	80.7	-	-	-	-
43	-	-	-	-	87.2	77.0
46	-	-	-	-	86.2	83.1
47	-	-	106.7	107.8	-	-
49	75.04	84.8	-	-	-	-
51	-	-	90.9	89.3	-	-
52	-	-	-	-	89.6	87.5

"-" : not measured.

"Sub." : Subject.

c) Orosomucoid.

Age of stain/days	Amount of Orosomucoid Extracted from Stains/μg Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	1.60	2.32	1.91	1.88	-	-
0	1.50	1.91	1.50	1.47	-	-
1	-	-	-	-	2.07	1.71
2	-	-	1.79	1.67	-	-
4	-	-	-	-	2.66	2.25
5	1.60	2.10	-	-	-	-
6	-	-	2.10	1.73	-	-
8	-	-	-	-	2.22	1.83
9	-	-	1.88	1.74	-	-
11	-	-	-	-	2.05	1.75
13	-	-	2.43	2.16	-	-
15	1.43	1.91	-	-	2.40	1.91
18	-	-	-	-	2.40	1.96
19	-	-	2.12	1.95	-	-
22	1.68	1.93	2.36	1.85	2.32	1.96
25	-	-	-	-	2.37	1.95
27	-	-	2.49	2.36	-	-
28	1.66	2.04	-	-	-	-
29	-	-	-	-	2.36	1.98
31	-	-	-	-	2.65	2.23
33	-	-	2.07	1.95	-	-
35	1.72	2.33	-	-	-	-
36	-	-	2.48	2.33	2.45	2.16
39	-	-	-	-	2.46	1.92
41	-	-	2.17	1.98	-	-
42	1.57	1.88	-	-	-	-
43	-	-	-	-	2.41	2.10
46	-	-	-	-	2.35	1.89
47	-	-	-	2.46	-	-
49	1.44	2.62	-	-	-	-
51	-	-	2.58	2.22	-	-
52	-	-	-	-	2.45	1.77

"-" : not measured.

"Sub." : Subject.

d) Haptoglobin.

Age of stains/days	Amounts of Haptoglobin Extracted from Stains/ug Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	3.28	5.04	7.36	7.14	-	-
0	3.11	4.97	6.25	7.30	-	-
1	-	-	-	-	4.54	4.40
2	-	-	5.08	5.37	-	-
4	-	-	-	-	5.00	5.00
5	3.98	6.19	-	-	-	-
6	-	-	5.05	5.59	-	-
8	4.95	7.50	-	-	5.09	4.70
9	-	-	6.85	7.09	-	-
11	-	-	-	-	4.64	4.48
13	-	-	6.81	8.31	-	-
15	4.99	9.04	-	-	4.40	4.55
18	-	-	-	-	4.58	4.51
19	-	-	8.91	9.60	-	-
22	-	-	8.00	8.85	4.39	4.63
25	-	-	-	-	3.96	4.25
27	-	-	11.35	12.04	-	-
28	7.73	10.81	-	-	-	-
29	-	-	-	-	4.75	4.66
31	-	-	-	-	7.32	8.24
33	-	-	13.43	13.88	-	-
35	----	----	-	-	-	-
36			----	----	7.63	9.21
39					----	----

"-" : not measured.

"----" : not detectable.

"Sub." : Subject.

e) Transferrin.

Age of stains/days	Amounts of Transferrin Extracted from Stains/μg Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	5.20	6.32	9.40	8.58	-	-
0	6.86	6.38	8.95	8.37	-	-
1	-	-	-	-	8.74	8.10
2	-	-	7.88	7.58	-	-
4	-	-	-	-	10.35	10.28
5	7.58	9.15	-	-	-	-
6	-	-	8.14	6.85	-	-
8	4.81	6.21	-	-	11.25	11.17
9	-	-	10.92	9.56	-	-
11	-	-	-	-	10.17	10.08
13	-	-	11.44	10.77	-	-
15	6.15	8.82	-	-	9.54	9.49
18	-	-	-	-	9.26	8.68
19	-	-	12.27	11.11	-	-
22	5.95	6.35	9.94	8.10	9.90	9.79
25	-	-	-	-	8.97	9.04
27	-	-	10.28	10.00	-	-
28	6.48	7.68	-	-	-	-
29	-	-	-	-	11.02	10.90
31	-	-	-	-	9.87	9.67
33	-	-	11.03	10.88	-	-
35	6.65	8.31	-	-	-	-
36	-	-	11.26	11.82	9.99	10.68
39	-	-	-	-	8.99	9.85
41	-	-	11.47	11.21	-	-
42	6.50	7.48	-	-	-	-
43	-	-	-	-	9.77	9.54
46	-	-	-	-	9.09	8.50
47	-	-	10.99	10.35	-	-
49	6.14	11.25	-	-	-	-
51	-	-	10.72	8.52	-	-
52	-	-	-	-	9.40	8.62

"-" : not measured.

"Sub." : Subject.

f) α_2-Macroglobulin.

Age of stain/days	Amounts of α_2-Macroglobulin Extracted from Stains/µg Experiment 1 Sub 1	Experiment 1 Sub 2	Experiment 2 Sub 2	Experiment 2 Sub 3	Experiment 3 Sub 2	Experiment 3 Sub 3
(Whole blood value)	5.32	3.92	5.44	4.40	-	-
0	5.01	3.49	4.41	4.16	-	-
1	-	-	-	-	5.23	4.38
2	-	-	4.73	4.62	-	-
4	-	-	-	-	5.05	3.58
5	4.93	3.81	-	-	-	-
6	-	-	4.18	4.35	-	-
8	6.38	4.97	-	-	4.92	4.21
9	-	-	4.47	4.10	-	-
11	-	-	-	-	4.47	3.95
13	-	-	5.03	5.06	-	-
15	5.70	6.17	-	-	4.61	4.24
18	-	-	-	-	4.65	4.22
19	-	-	5.35	5.03	-	-
22	7.88	5.55	7.28	6.15	3.99	3.58
25	-	-	-	-	5.09	4.82
27	-	-	6.11	6.04	-	-
28	-	-	-	-	-	-
29	-	-	-	-	5.73	5.18
31	-	-	-	-	5.86	5.48
33	-	-	6.55	5.89	-	-
35	10.02	5.44	-	-	-	-
36	-	-	-	8.29	6.16	6.43
39	-	-	-	-	7.01	6.63
41	-	-	9.04	7.92	-	-
42	5.91	5.14	-	-	-	-
43	-	-	-	-	6.68	6.05
46	-	-	-	-	5.56	5.16
47	-	-	----	----	-	-
49	----	----			-	-
51					-	-
52					6.96	5.91

"-" : not measured.

"----" : not detectable.

"Sub." : Subject.

g) α_1-Antitrypsin

Amount extracted/µg Experiment 3 Subject 2	Amount extracted/µg Experiment 3 Subject 3	Age of stain/days
6.09	6.70	1
6.62	6.88	4
5.68	5.98	8
6.03	6.64	11
5.63	5.93	15
5.65	6.06	18
5.31	5.84	22
5.77	6.32	25
5.90	6.37	29
5.86	6.36	31
5.92	6.57	36
5.55	6.09	39
5.95	6.32	43
5.07	5.29	46
5.03	5.23	52

h) β_1C/A-Globulin.

Amount extracted/µg Experiment 3 Subject 2	Amount extracted/µg Experiment 3 Subject 3
1.05	0.85
1.05	0.89
1.54	1.35
1.18	1.13
1.57	1.45
1.47	1.36
1.57	1.45
1.62	1.38
2.10	2.03
2.17	1.99
2.26	2.06
1.90	1.76
2.45	2.11
1.89	1.57
2.16	1.57

APPENDIX II.

Ratios of the Amounts of Individual Proteins Extracted to the Amounts of Orosomucoid in the Stain Extracts.

a) Prealbumin.

Age of stain/days	Ratio to Orosomucoid Experiment 1 Sub.1	Experiment 1 Sub.2	Experiment 2 Sub.2	Experiment 2 Sub.3	Experiment 3 Sub.2	Experiment 3 Sub.3
(Whole blood value)	0.40	0.34	0.55	0.33	-	-
0	0.39	0.36	0.62	0.74	-	-
1	-	-	-	-	0.52	0.54
2	-	-	0.47	0.42	-	-
4	-	-	-	-	0.38	0.39
5	0.26	0.25	-	-	-	-
6	-	-	0.38	0.43	-	-
8	-	-	-	-	0.44	0.48
9	-	-	0.50	0.45	-	-
11	-	-	-	-	0.40	0.36
13	-	-	0.40	0.38	-	-
15	0.45	0.36	-	-	0.38	0.46
18	-	-	-	-	0.32	0.34
19	-	-	0.38	0.37	-	-
22	0.39	0.37	0.27	0.28	0.32	0.33
25	-	-	-	-	0.39	0.42
27	-	-	0.37	0.34	-	-
28	-	-	-	-	-	-
29	-	-	-	-	0.35	0.39
31	-	-	-	-	0.35	0.39
33	-	-	-	-	-	-
35	0.29	0.28	-	-	-	-
36	-	-	0.43	0.37	0.37	0.40
39	-	-	-	-	0.49	0.58
41	-	-	0.47	0.48	-	-
42	0.38	0.34	-	-	-	-
43	-	-	-	-	0.33	0.37
46	-	-	-	-	0.44	0.46
47	-	-	0.27	0.30	-	-
49	0.41	0.26	-	-	-	-
51	-	-	----	----	-	-
52	-	-			0.42	0.46

"-" : not measured. "----" : not detectable. "Sub." : Subject.

b) Albumin.

Age of stain/days	Ratio to Orosomucoid Experiment 1 Sub.1	Experiment 1 Sub.2	Experiment 2 Sub.2	Experiment 2 Sub.3	Experiment 3 Sub.2	Experiment 3 Sub.3
(Whole blood value)	63.75	49.13	57.43	57.18	-	-
0	91.33	67.75	73.20	77.28	-	-
1	-	-	-	-	58.57	69.10
2	-	-	65.24	67.63	-	-
4	-	-	-	-	37.92	51.38
5	61.88	51.10	-	-	-	-
6	-	-	48.43	65.81	-	-
8	-	-	-	-	53.56	65.54
9	-	-	60.48	59.37	-	-
11	-	-	-	-	54.02	66.25
13	-	-	45.19	44.17	-	-
15	65.52	51.62	-	-	42.95	50.51
18	-	-	-	-	43.04	54.19
19	-	-	51.37	48.46	-	-
22	51.79	49.90	51.10	52.42	44.22	54.06
25	-	-	-	-	43.58	55.43
27	-	-	44.94	45.30	-	-
28	55.17	45.98	-	-	-	-
29	-	-	-	-	45.13	48.26
31	-	-	-	-	36.58	40.39
33	-	-	51.30	49.27	-	-
35	51.57	36.39	-	-	-	-
36	-	-	43.47	41.04	41.28	46.58
39	-	-	-	-	38.23	48.11
41	-	-	42.30	47.07	-	-
42	50.88	42.91	-	-	-	-
43	-	-	-	-	36.19	36.72
46	-	-	-	-	36.78	43.90
47	-	-	31.02	43.82	-	-
49	52.11	32.38	-	-	-	-
51	-	-	35.23	40.24	-	-
52	-	-	-	-	36.59	49.45

"-" : not measured.

"Sub." : Subject

c) Haptoglobin.

Age of stain/days	Ratio to Orosomucoid Experiment 1 Sub.1	Experiment 1 Sub.2	Experiment 2 Sub.2	Experiment 2 Sub.3	Experiment 3 Sub.2	Experiment 3 Sub.3
(Whole blood value)	2.05	2.17	3.85	3.80	-	-
0	2.07	2.60	4.17	4.97	-	-
1	-	-	-	-	2.20	2.58
2	-	-	2.84	3.22	-	-
4	-	-	-	-	1.88	2.22
5	2.49	2.95	-	-	-	-
6	-	-	2.40	3.23	-	-
8	-	-	-	-	2.30	2.58
9	-	-	3.64	4.07	-	-
11	-	-	-	-	2.27	2.56
13	-	-	3.54	3.85	-	-
15	3.49	4.73	-	-	1.83	2.38
18	-	-	-	-	1.91	2.30
19	-	-	4.20	4.92	-	-
22	4.60	5.60	3.39	3.78	1.89	2.36
25	----	----	-	-	1.67	2.18
27			4.56	5.10	-	-
28			-	-	-	-
29			-	-	2.02	2.35
31			-	-	2.76	3.70
33			6.49	7.12	-	-
35			----	----	-	-
36					3.11	4.26
39					----	----

"-" : not measured.

"----" : not detectable.

"Sub." : Subject

d) Transferrin.

Age of stain/days	Ratio to Orosomucoid Experiment 1 Sub.1	Experiment 1 Sub.2	Experiment 2 Sub.2	Experiment 2 Sub.3	Experiment 3 Sub.2	Experiment 3 Sub.3
(Whole blood value)	3.25	2.72	4.92	4.56	-	-
0	4.57	3.34	5.97	5.65	-	-
1	-	-	-	-	4.23	4.74
2	-	-	4.40	4.54	-	-
4	-	-	-	-	3.89	4.57
5	4.74	4.36	-	-	-	-
6	-	-	3.88	3.96	-	-
8	-	-	-	-	5.08	6.12
9	-	-	5.81	5.49	-	-
11	-	-	-	-	4.97	5.76
13	-	-	4.71	4.99	-	-
15	4.30	4.62	-	-	3.98	4.96
18	-	-	-	-	3.86	4.43
19	-	-	4.60	5.70	-	-
22	3.54	3.29	4.21	4.38	4.27	4.99
25	-	-	-	-	3.79	4.64
27	-	-	4.13	4.24	-	-
28	3.90	3.76	-	-	-	-
29	-	-	-	-	4.68	5.51
31	-	-	-	-	3.72	4.34
33	-	-	5.33	5.58	-	-
35	3.87	3.57	-	-	-	-
36	-	-	4.54	5.07	4.08	4.94
39	-	-	-	-	3.65	5.13
41	-	-	5.29	5.66	-	-
42	4.14	3.98	-	-	-	-
43	-	-	-	-	4.05	4.55
46	-	-	-	-	3.88	4.49
47	-	-	3.19	4.21	-	-
49	4.26	4.29	-	-	-	-
51	-	-	4.16	3.84	-	-
52	-	-	-	-	3.84	4.87

"-" : not measured.

"Sub." : Subject.

e) α_2-Macroglobulin.

Age of stain/days	Ratio to Orosomucoid Experiment 1 Sub.1	Experiment 1 Sub.2	Experiment 2 Sub.2	Experiment 2 Sub.3	Experiment 3 Sub.2	Experiment 3 Sub.3
(whole blood value)	3.33	1.69	2.85	2.34	-	-
0	3.34	1.83	2.94	2.83	-	-
1	-	-	-	-	2.53	2.56
2	-	-	2.64	2.77	-	-
4	-	-	-	-	1.90	1.59
5	3.08	1.81	-	-	-	-
6	-	-	1.99	2.51	-	-
8	-	-	-	-	2.22	2.31
9	-	-	2.38	2.36	-	-
11	-	-	-	-	2.18	2.26
13	-	-	2.18	2.34	-	-
15	4.47	3.23	-	-	1.92	2.22
18	-	-	-	-	1.94	2.15
19	-	-	2.52	2.58	-	-
22	4.69	2.88	3.08	3.32	1.72	1.83
25	-	-	-	-	2.15	2.47
27	-	-	2.45	2.56	-	-
28	-	-	-	-	-	-
29	-	-	-	-	2.43	2.62
31	-	-	-	-	2.21	2.46
33	-	-	3.16	3.02	-	-
35	5.83	2.33	-	-	-	-
36	-	-	4.84	3.56	2.51	2.98
39	-	-	-	-	2.85	3.45
41	-	-	4.17	4.00	-	-
42	3.48	2.73	----	----	-	-
43	----	----			2.77	2.89
46					2.37	2.73
49					-	-
51					-	-
52					2.84	3.34

"-" : not measured.

"----" : not detectable.

"Sub." : Subject.

f) α_1-Antitrypsin.

g) β_1C/A-Globulin.

Ratio to Orosomucoid Experiment 3		Age of stain/days	Ratio to Orosomucoid Experiment 3	
Subject 2	Subject 3		Subject 2	Subject 3
2.95	3.92	1	0.51	0.50
2.49	3.06	4	0.40	0.40
2.56	3.28	8	0.70	0.74
2.94	3.80	11	0.58	0.65
2.35	3.10	15	0.65	0.76
2.35	3.09	18	0.61	0.69
2.29	2.98	22	0.68	0.74
2.44	3.24	25	0.69	0.71
2.51	3.22	29	0.89	1.03
2.21	2.85	31	0.82	0.89
2.42	3.04	36	0.92	0.96
2.26	3.17	39	0.77	0.92
2.47	3.01	43	1.02	1.01
2.16	2.79	46	0.80	0.83
2.05	2.95	52	0.88	0.89

APPENDIX III.

Ratios of the amounts of each protein, relative to orosomucoid, for one subject, to the same quantity for the second subject, in each experiment.

Experiment 1.

Age of stain /days	Pre.	Alb.	Hpt.	Trf.	α_2-M
0	1.08	1.34	0.79	1.36	1.82
5	1.04	1.21	0.84	1.08	1.70
15	1.25	1.26	0.73	0.93	1.38
22	1.05	1.03	0.82	1.07	1.62
28	-	1.19	----	1.03	-
35	1.03	1.39		1.08	2.50
42	1.11	1.18		1.04	1.27
49	1.57	1.60		0.99	----

Experiment 2.

Age of stain /days	Pre.	Alb.	Hpt.	Trf.	α_2-M
0	0.83	0.94	0.83	1.06	1.03
2	1.11	0.96	0.88	0.97	0.95
6	0.88	0.73	0.74	0.98	0.79
9	1.10	1.01	0.89	1.05	1.00
13	1.05	1.02	0.91	0.94	0.93
19	1.02	1.06	0.85	0.81	0.97
22	0.96	0.97	0.70	0.96	0.92
27	1.08	0.99	0.89	0.97	0.95
33	1.28	1.04	0.91	0.96	1.04
36	1.16	1.05	----	0.89	1.35
41	0.97	0.89		0.93	1.04
47	0.92	0.70		0.76	----
51	-	0.87		1.08	

Experiment 3.

Age of stain /days	Protein. Pre	Alb	α_1At	Hpt	Trf	α_2-M	β_1C/A
1	0.96	0.85	0.75-	0.85	0.89	0.99	1.02
4	0.97	0.74	0.81	0.85	0.85	1.19	0.99
8	0.92	0.82	0.78	0.89	0.83	0.96	0.94
11	1.11	0.82	0.77	0.89	0.86	0.96	0.89
15	0.84	0.85	0.76	0.77	0.80	0.87	0.86
18	0.94	0.79	0.76	0.83	0.87	0.90	0.88
22	0.97	0.82	0.77	0.80	0.86	0.94	0.91
25	0.91	0.79	0.75	0.77	0.82	0.87	0.97
29	0.90	0.94	0.78	0.86	0.85	0.93	0.87
31	0.89	0.91	0.78	0.76	0.86	0.90	0.92
36	0.94	0.89	0.80	0.73	0.83	0.84	0.97
39	0.85	0.79	0.71	----	0.71	0.83	0.84
43	0.89	0.99	0.82		0.89	0.96	1.01
46	0.95	0.84	0.77		0.86	0.87	0.97
52	0.91	0.74	0.69		0.79	0.85	1.00

"-" : not measured.

"----" : not detectable.