UNIT IV. DETERMINATION OF SPECIES OF ORIGIN

SECTION 15. OLDER METHODS

15.1 Introduction

If the results of the examination of unknown bloodstains are to have very much probative value, the species of origin must be established. It may be necessary on occasion to carry out a determination of species of origin on a body fluid or secretion other than blood, but since the question arises much less frequently with body fluids than with blood, less attention has been paid to the problem. If immunological tests are to be used, the principle remains the same, of course, regardless of which fluid is under examination. Most current methods in common use for determining species of origin are immunological ones. The basis for these methods was established in the last few years of the 19th Century, and the techniques actually devised and put into practice in 1901. Prior to that time, medicolegal investigators were very concerned about the problem, but the techniques were not very satisfactory.

15.2 Chemical Methods

In 1829, J.-P. Barruel introduced a method for determining the species of origin of bloodstains. Blood or bloodstains would emit a completely characteristic odor, he said, when treated with an excess of concentrated sulfuric acid. He claimed, in addition, that human blood could be differentiated with regard to sex of origin based upon the pungency of the odor. Orfila was apparently convinced of the usefulness of this approach, for in 1835 the technique was used in a homicide case in which he served as the medico-legal expert along with Barruel and Chevallier (see section 3), although no results could be obtained with the exhibits submitted. In 1842, Mandl noted that the method was unreliable, and not suited for use in legal medicine. Schmidt (1848) evaluated Barruel's technique, and said that it was not reliable. In 1853, Tardieu *et al*. re-examined the technique in connection with a case they were working on, and concluded, after a series of blind trial tests on stains of known origin, that the test was not reliable.

In 1848, Casanti reported that the blood of different mammals, including human beings, formed a characteristic residue when dried and treated with concentrated phosphoric acid. He said, too, that menstrual blood could be differentiated from circulating blood in this fashion. The method does not appear to have enjoyed any popularity, doubtless because of its unreliability.

15.3 Micrometric Methods

Micrometry, the measurement of red cells as precisely as possible in smears made from fresh blood as well as in reconstituted stains, came into use as a method for species determination of the 19th Century. There was considerable disagreement among various authorities over the certainty which could be attached to the results of these determinations. The controversy was never actually resolved. It simply became irrelevant shortly after 1900, when the immunological methods were discovered and quickly put into use in medico-legal practice.

For the most part, the technique involved the reconstitution of the erythrocytes from bloodstains, and examination of a fairly large number of cells microscopically. Careful measurements were performed on the size of the cells, and these compared with values for the known sizes of red cells from many different species. Most vertebrate red cells are enucleate, and it was usually not difficult to diagnose invertebrate blood. But, particularly among the mammals, a number of species have red cells which are quite similar in size. The efforts which were made, and the disagreements which existed, must likewise be considered in the context of the accuracy of the methods which were then available. There was never complete agreement that species could be correctly diagnosed using microscopical measurements, or micrometry. A good many authorities came to believe that a proper examination could establish only that blood was of mammalian origin.

The notion that red cell size might be exploited for the determination of species of origin can be traced to some of the earliest discussions of medico-legal blood examinations in France. In 1821, Prevost and Dumas had established that the red blood cells of various species differed in size. In 1827, Dulong is said to have remarked at a meeting of the Royal Academy of Medicine that the size of red cells is a very characteristic feature, even when the blood is old and dried, and that this property could be used to help decide the species of origin of the blood. Orfila (1827), however, was not convinced, having carried out a number of experiments to test the possibility. Orfila's reputation being what it was, the matter does not appear to have come up again for several years.

In 1842, Mandl published an extensive paper on the use of microscopy in medico-legal investigations, and strongly advocated the measurement of the red cells as a means of determining species origin. There was no difficulty in distinguishing the nucleate red cells of birds, reptiles, and so forth, from the enucleate ones of mammals. Mandl said, however, that one could not discriminate among the red cells of mammals by this method. Schmidt (1848) briefly discussed the micrometric method in his book, and indicated that he thought it was more promising than the methods which had been in use up to that time. Robin and Salmon readily distinguished human from duck bloodstains in a case in 1857. The bloodstains at issue, they reported, had all the

microscopic characteristics of human blood and could not have come from a duck as the defendant was claiming. Fleming (1861) discussed the technique, but did not think it was very reliable. Roussin wrote a paper on the subject in 1865, advocating the method strongly. He introduced his solution for the preservation of red cells from bloodstains (see section 5.3) in this paper, but he did not think that the blood of mammals could be differentiated by the measurements. The successful use of the method in two cases was reported. In 1869, Richardson in Philadelphia, who became one of the foremost advocates of the method, published a paper saying that one could, by careful measurement of the red cells, distinguish readily between human, sheep and pig blood. He extended his studies to other species, noting several years later the importance of using high power objectives in carrying out the measurements, so as to obtain sufficiently high magnification to be able to distinguish relatively small differences (Richardson, 1874a, 1874b). He thought that there was no difficulty in distinguishing human blood from the blood of pig, ox, deer, cat, horse, sheep and goat, even in stains that were five years old. Later, he examined blood from a number of people of different ethnic backgrounds, and said that there were no significant differences in the size of the red cells among the different races of humankind (Richardson, 1877). Woodward (1875) took exception to Richardson's claims, noting that the cells of the dog were in fact very similar in size to those of humans. He said that he did not believe there was any method available for the unequivocal diagnosis of human blood. Tidy (1882), while noting with interest Richardson's work, said that in giving testimony to a Court about the species of origin of a bloodstain, ". . . it is better, in the present state of science, at once to confess our inability to give a definite reply." Hemphill (1875) noted that he thought it possible to diagnose mammalian blood in stains, but not to establish which particular species was involved.

In 1875, Gulliver published one of the most extensive single investigations of the sizes and shapes of the red cells of vertebrates. Dozens of species of animals were included in the studies, and extensive tables prepared to summarize the findings. These data became known as "Gulliver's Tables", and were widely quoted and sometimes reproduced in the medico-legal literature for 25 years. The relationships he found in the sizes of red cells of various species were summarized in a figure in his paper, which is reproduced as Fig. 15.1.

In 1873, a committee appointed by the Societé de Médecine Légale in France reported that it thought the organization should adopt the stance that it was possible for a qualified expert to decide by micrometric methods whether or not a bloodstain was of human origin (Mialhe *et al.*, 1873). "Il [referring to the expert] mesurera les globules et pourra ainsi affirmer s'il s'agit ou non de sang humain," they wrote.

The major objections voiced by opponents of the accuracy of the methods were that the differences in size between many mammalian species cells were very small, and fell within the experimental error of the measurements, that the interspecific variations were sometimes exceeded by intraspecific variation, and that the cells underwent irregular changes in size and shape upon drying and reconstitution. Richardson and other adherents of the method did not fully accept these arguments. He thought that red cell size varied only very little within a species, and that the reconstituted cells were exactly the same size as those which had originally dried out.

In 1882, Vibert carried out a number of studies on the technique, proposing two solutions for the conservation of red cell size and shape as well. He did not think that human blood could be differentiated from mammalian blood, however. Masson reviewed the material on this matter extensively in 1885 in a series of papers. He conductd a number of experiments as well. A number of the solutions which had been proposed were tested both with fresh blood and with bloodstains, Vibert's solution being recommended for the former, Virchow's for the latter. A number of experiments were done to try and find out what factors cause the changes in red cells upon drying. Masson thought that the faster the blood dried out, the better the cells were conserved in terms of shape and size. He thought that unknown stains could be assigned to one of several categories of animals, arranged according to the size of the red cells, but he did not think a specific diagnosis was possible with a bloodstain. Formad (1888) in his book reviewed the entire subject extensively and well. Most of the opinions which had been expressed on the accuracy of the method were covered, and the references given. Formad himself was persuaded that Richardson's conclusions were valid in many respects. He thought that there was no difficulty in distinguishing human blood from that of domestic animals in stains, assuming that a large enough number of cells could be examined and measured (500 was recommended), and omitting the guinea pig from consideration. Human blood could not be distinguished from every animal blood, and he noted that the probative value of testimony presented by an expert who had conducted such examinations relied in great part on the way in which the questions were asked of him. He thought it would be misleading for the Court to gain the impression that human blood could not be distinguished from *any* animal bloods by the micrometric method. Ewell (1892a) measured 4000 red cells, and found variations within the same species. He also said that different observers would get different values, and that the variation was large enough to make one doubt the published figures. He did not think red cells in stains could be restored to their *in vivo* proportions. He thought that the micrometric method could be useful in diagnosing blood as being of mammalian origin, but not more.

In 1892, Jones reported on a murder case in Ascension Parish, Louisiana, in which he had examined bloodstain evidence. The body of a man named Simeon Cascio had been discovered September 22, 1892. Suspicion fell on the victim's brother-in-law, one Joseph Polito who had been seen with the victim shortly before the murder, and who had some of the victim's possessions with him at the time of his arrest.

Dr. Jones examined stains on the blade and handle of a knife, on a pocket book, on the collar of the victim's shirt and several stains on paper money bills. The stains on the knife and pocket book, he said in his report, "presented the characteristics of human blood." The stains on the currency were identified as being blood, and he said that the stains on the shirt collar were "due to human blood." Ewell (1892b) took very great exception to Jones' conclusion that he had determined the stains on the collar to be human blood. "In view of the consensus of opinion among microscopists who have examined this question, that in the present state of science it is impossible to identify human blood, as such, it would be interesting to know how Dr. Jones reached a conclusion at variance with the almost unbroken current of authority," he wrote. Jenne (1896) agreed with the view that human and animal blood in stains could not be distinguished by micrometric methods.

Axtell (1895) reported in detail on a murder case in Denver, Colorado, in which he had been able to determine by micrometry that the stains on the suspect's clothing were mammalian in origin, and consistent with human blood. The defendant had claimed that the stains were from the blood of a rabbit, but Axtell was able to exclude several different species of rabbits as being the source of the bloodstains on the basis of differences in red cell sizes. The defendant in the case entered a plea of guilty before Axtell had the opportunity to present his findings before a Court. In 1892, Bell reviewed the micrometric method in some detail, and presented the veiws of most authorities. To illustrate the kinds of tables that were employed, Bell's table of comparative measurements is reproduced as Table 15.1. Corin (1901) gave a brief review of micrometric methods. Several papers by 19th Century French authors, which go into the subject in considerable detail, have been included in the Translations (Unit IX).

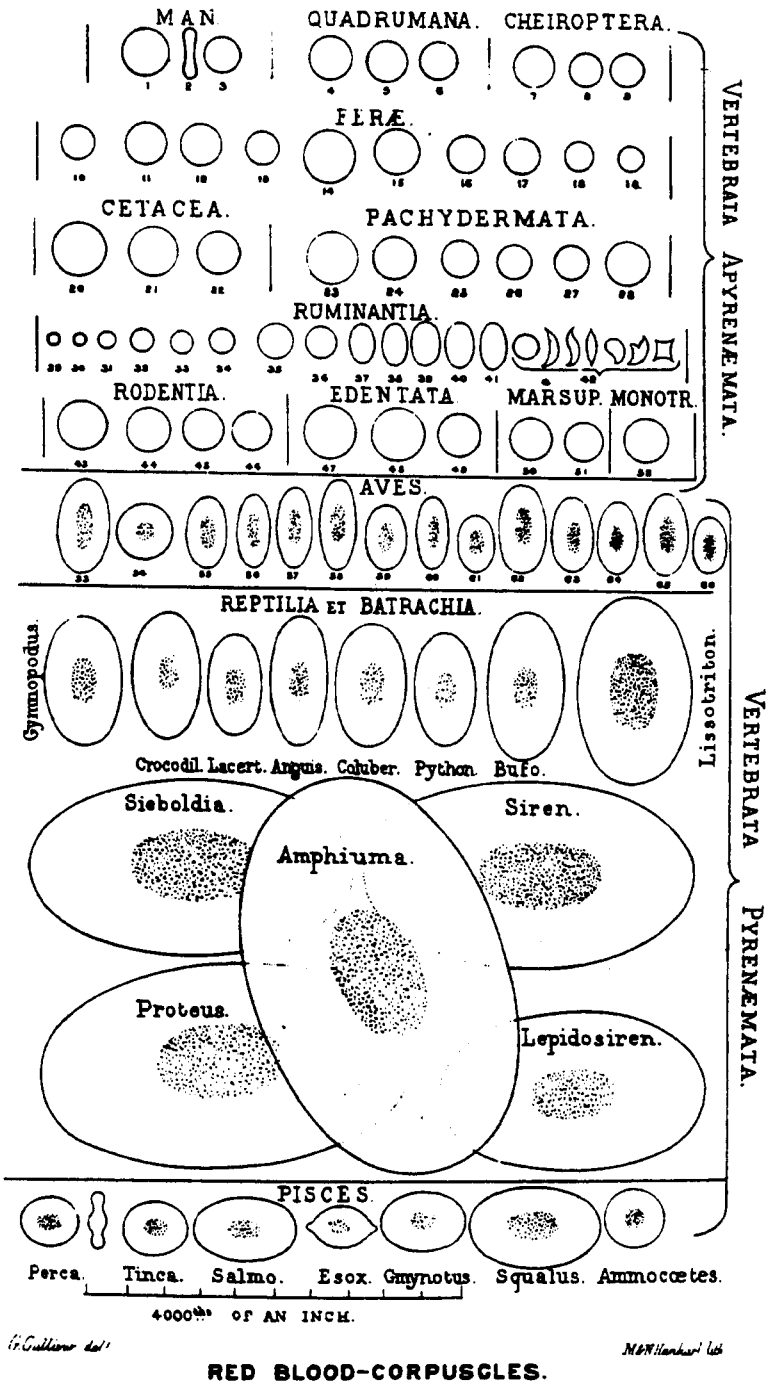

Figure 15.1 Relationships of red cell sizes of many species (after Gulliver, 1875)

All the objects are red blood-corpuscles done to one and the same scale, which is at the foot of the drawing. The whole length of the scale represents $\frac{1}{400}$ of an English inch, and each one of its ten divisions $\frac{1}{4000}$ of an inch, as described at page 475. Only corpuscles of the average sizes and quite regular shapes are given; and they are all magnified to the same, or nearly the same, degree—to wit, about 800 diameters.

VERTEBRATA APYRENÆMATA.

Homo.

1. Corpuscle lying flat.
2. The same on edge.
3. Membranous base of the same, after removal by water of the colouring-matter.

Quadrumana.

4. Simia troglodytes.
5. Ateles ater.
6. Lemur anguanensis.

Cheiroptera.

7. Cynonycteris collaris.
8. Vespertilio noctula.
9. Vespertilio pipistrellus.

Feræ.

10. Sorex tetragonurus.
11. Ursus labiatus.
12. Basaaris astuta.
13. Cercoleptes caudivolvulus.
14. Trichechus rosmarus.
15. Canis dingo.
16. Mustela zorilla.
17. Felis tigris.
18. Paradoxurus pallasii.
19. Paradoxurus bondar.

Cetacea.

20. Balæna boops.
21. Delphinus globiceps.
22. Delphinus phocæna.

Pachydermata.

23. Elephas indicus.
24. Rhinoceros indicus.
25. Tapirus indicus.
26. Equus caballus.
27. Dicotyles torquatus.
28. Hyrax capensis.

Ruminantia.

29. Tragulus javanicus.
30. Tragulus meminna.
31. Tragulus stanleyanus.
32. Cervus nemorivagus.
33. Capra caucasica.
34. Capra hircus.
35. Bos urus.
36. Camelopardalis giraffa.
37. Auchenia vicugna.
38. Auchenia paco.
39. Auchenia glama.
40. Camelus dromedarius.
41. Camelus bactrianus.
42. Cervus mexicanus (see page 483)

Rodentia.

43. Hydrochœrus capybara.
44. Castor fiber.
45. Sciurus cinereus.
46. Mus messorius.

Edentata.

47. Myrmecophaga jubata.
48. Bradypus didactylus.
49. Dasypus villosus.

Marsupialia.

50. Phascolomys wombat.
51. Hypsiprymnus setosus.

Monotremata.

52. Echidna hystrix.

VERTEBRATA PYRENÆMATA.

Aves.

53. Struthio camelus.
54. The same, made round and deprived of colour by water.
55. Vanga destructor.
56. Lanius excubitor.
57. Bubo virginianus.
58. Syrnea nyctea.
59. Columba rufina.
60. Columba migratoria.
61. Dolichonyx oryzivorus.
62. Buceros rhinoceros.
63. Psittacus augustus.
64. Phasianus superbus.
65. Pelecanus onocrotalus.
66. Trochilus, sp.

Reptilia et Batrachia.

Gymnotus ægyptiacus.
Crocodilus acutus.
Lacerta viridis.
Anguis fragilis.
Coluber berus.
Python tigris.
Bufo vulgaris.
Lissotriton vulgaris.
Sieboldia maxima.
Siren lacertina.
Proteus anguinus.
Amphiuma tridactylum.

Pisces.

Perca cernua, one corpuscle lying flat, the other on edge.
Tinca vulgaris.
Salmo fontinalis.
Esox lucius.
Gymnotus electricus.
Squalus acanthias.
Ammocœtes branchialis.
Lepidosiren annectens.

Figure 15.1 Explanation of the red cell size diagram on the facing page from the original article.

COMPARATIVE TABLE OF THE AVERAGE RESULTS OF MEASUREMENTS OF RED BLOOD CORPUSCLES OF MAMMALS.

Each column giving the average size (diameter) of the Corpuscles as obtained by various observers, expressed in fractions of the English inch, side by side with the common expression (roughly) in millimeters.

	Gulliver, 1845 and 1875.		Wormley, 1885.		C. Schmidt, 1848. Mallinin, 1875.		French Medico Legal Society, 1873, and Wilker.		Masson, 1885.		Hans Schmid, 1878.		Woodward, 1875.		Personal Observations.	
	In.	M. M.	In.	M. M	In.	M. M.	In.	M. M.	In.	M. M	In.	M. M	In.	M. M.	In.	M. M.
Elephant	1.2745	0.0092	1.2788	0 0093	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Great Anteater	1.2769	0.0066	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Walrus	1.2769	0.0066	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Sloth	1.2865	0.0066	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Ornithorynchus	1.3000	0.0081	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Whale	1.3099	0.0060	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Opossum	1.3557	0.0071	1 3145	0 0080	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Capybara	1.3190	0.0080	1.3164	0.0080	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Man	1.3200	0.0079	1.3260	0 0078	1.3800	0.0077	1.3257	0 0078	1.3257	0.0078	1 3412	0 0074	1 3092	0 0082	1.3200	0.0079
Seal	1.3281	0.0078	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Beaver	1.3325	0.0076	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Muskrat	1.3550	0.0073	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Porcupine	1.3969	0.0075	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Monkey	1.3419	0.0074	1 3382	0 0076	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Kangaroo	1.3440	0.0074	1 3410	0 0074	.....	.....	.....	.....	1.3800	0 0077	.....	.....	1 3213	0.0079	1.3400	0 0076
Guinea Pig	1.3588	0.0071	1.3223	0.0079	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.3450	0.0074
Wolf	1.3600	0.0070	1 3422	0 0074	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.3580	0.0071
Dog	1.3539	0.0071	1.3561	0 0071	1.3636	0.0070	1.3485	0 0073	1 3577	0.0071	1 3646	0.0066	1 3246	0.0078	1 3603	0.0069
Rabbit	1.3607	0.0070	1.3653	0 0070	1.3966	0.0064	1 3653	0 0069	1 3680	0.0070	.....	.....	.....	.....	.....	.....
Ass	1.4000	0.0063	1.3630	0.0070	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rat	1.3784	0.0066	1 3658	0 0070	1 3908	0.0064	.....	.....	.....	.....	1 5000	0 0051	.....	.....	.....	.....
Bear	1.3698	0.0070	1.3656	0 0066	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mouse	1.3814	0.0067	1 3743	0 0067	1 5000	0 0051	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mule	.....	.....	1.3760	0 0067	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Squirrel	1.4000	0.0064	1.4140	0.0061	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Ox	1.4267	0.0060	1.4319	0.0060	1.4854	0 0058	1.4545	0.0056	1.4257	0.0060	1.4695	0.0054	.....	.....	1 4200	0 0060
Pig	1.4230	0.0060	1.4268	0.0059	1.4098	0 0062	1.4098	0 0062	1.4098	0.0062	1.4098	0.0062	.....	.....	1 4250	0.0060
Horse	1.4000	0.0065	1.4243	0.0059	1.4464	0 0057	1.4545	0 0056	.....	.....	.....	.....	.....	.....	1 4310	0 0059
Cat	1.4404	0.0058	1.4373	0 0058	1.4545	0.0056	1.3922	0.0065	1.4440	0.0057	.....	.....	.....	.....	.....	.....
Sheep	1.5300	0.0048	1.4912	0.0051	1.5649	0 0045	1.5076	0 0050	1.4464	0.0057	1 6000	0.0049	.....	.....	1.5000	0.0051
Goat	1.6366	0.0040	1.6184	0.0041	1.6000	0.0040	1.5385	0.0046	.....	.....	.....	.....	.....	.....	1.6100	0.0043

Table 15.1 Comparative Red Cell Measurements by Several Authorities (after Bell, 1892). References in the table may be found in the reference list, except "Gulliver (1845)" and "Welker". French medico-legal society (1873) is listed as Mialhe et al. (1873) in the reference list.

SECTION 16. IMMUNOLOGICAL TESTS WITH BLOODSTAINS

16.1 Precipitin Test

16.1.1 Development of the precipitin test and its medicolegal application

The development of a specific immunological test for determination of the species of origin of bloodstains was a major event in the evolution of legal medicine. It provided for the first time a technique for unequivocally establishing species of origin in stains. All authorities soon came to agree on the specificity and certainty provided by the new method, Wood (1902a) having said to the Massachusetts Medico-Legal Society that one could finally testify absolutely as to the species of origin of bloodstains, rather than having to say that the results were "consistent with" the stain having originated from a particular species. Precipitin reactions are discussed more generally in section 1.3.4.2. Recognition of precipitating antibodies, and their application to forensic investigations, must be regarded as natural outcomes of the rapid and ever-widening development that was occurring in immunology in the last years of the 19th century, and which continued into the present one. In the years 1901 to 1920, five Nobel prizes for Physiology and Medicine were awarded in immunology: in 1901 to Emil A. von Behring "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths"; in 1905 to Robert Koch "for his investigations and discoveries in relation to tuberculosis"; in 1908 to Elie Metchnikoff and Paul Ehrlich "in recognition of their work on immunity"; in 1913 to Charles R. Richet "in recognition of his work on anaphylaxis"; and in 1919 to Jules Bordet "for his discoveries relating to immunity."

Immunology had gotten its start as a proper scientific field of inquiry because of concern with infectious and highly contagious diseases, epidemics of which affected large segments of the population at times. The recognition, and ultimate acceptance, of the notion that these diseases were caused by specific microorganisms provided a basis for detailed investigations into the subject of immunity and its mechanism.

Behring and Warnecke (1892) made the important discovery that animals injected with diphtheria toxin elaborated specific antitoxins. Pfeiffer (1894) discovered that if animals were treated with bacteria, specific antisubstances form in the serum of the injected animal. If this serum and the bacteria are mixed together and injected into the peritoneal cavity of a healthy animal, the bodies of the bacilli are degraded. If bacteria are mixed with the antiserum *in vitro*, the bacteria are agglutinated, and will settle out of the suspension as a mass. Uhlenhuth (1911) attributed this discovery to Gruber and Durham in 1896. In 1897, Kraus made the important observation that bacterial antibodies have the property of precipitating cell free bacterial extracts. This paper marked the first description of precipitin antibodies, which would shortly play an important role in immunological species differentiation. It was soon realized that bacterial substances were not the only ones to which animals would elaborate antibodies. Bordet (1898) found that guinea pigs produce a potent red cell agglutinating serum against rabbit red blood cells when immunized with defibrinated rabbit blood. Similarly, rabbits which had received several injections of defibrinated hen blood yielded a serum which actively agglutinated (agglutinin) and lysed (hemolysin) hen red cells. In addition, it was found that the antiserum contained a specific precipitin for hen serum. This discovery was made, Bordet said, by Tschistovitch at the Pasteur Institute. In 1899, Tschistovitch found that rabbits elaborated a precipitin antibody to eel serum with which they had been injected. The precipitates were soluble in solutions of strong base. To Tschistovitch, therefore, belongs the credit for the recognition of the fact that animals will form precipitating antibodies to the serum of an animal of another species. Bordet (1898) could show that milk proteins were antigenic in rabbits, giving rise to precipitins for the casein. The serum containing the antibodies was referred to as "lactoserum". Fisch (1900) in this country confirmed Bordet's observations on the antigenicity of casein. Wassermann and Schütze (1900) extended these studies to show that the precipitin test could be used to differentiate the milk of different species of animals immunologically. In 1900, Myers showed that crystalline hen egg albumin was antigenic in rabbits, giving rise to an antiserum which cross reacted to some extent with duck ovalbumin solutions. The antiserum was entirely specific for the ovalbumins, however, and did not react with a number of other animal protein solutions. Independently, Dr. Paul Uhlenhuth, whose name came to be more or less synonymous with the precipitin test in its forensic applications, published a paper in 1900 in which he said that he had successfully differentiated the egg albumin from several species of birds using precipitating antisera prepared in rabbits. In the course of these experiments, he found that rabbits immunized with chicken blood elaborated precipitating antisera for the chicken serum, and this antiserum did not react with the sera of horse, donkey, cow, sheep or pigeon. Further, the antisera prepared against hen blood showed only a slight cross reaction, and this after a long time, with hen ovalbumin, demonstrating that immunological specificity could extend not only to the proteins of different species, but also to proteins from different sources within the

same species. As Uhlenhuth remarked about these experiments some years later in the Harben Lecture (1911), ". . . . *the foundation was laid for the forensic method of distinguishing between different specimens of blood,* for the serum of rabbits treated with the blood of man or of any other animal gave a precipitate only with blood solutions of the corresponding species to that whose blood had previously been injected to the rabbit."

The stage having thus been set, 1901 saw the publication of a number of papers which specifically addressed the immunological differentiation of human from animal bloods using the precipitin test, and recommended that the method be adopted in medico-legal examinations of blood stains. Uhlenhuth (1901a) reported in the February 7 issue of *Deutsche Medizinische Wochenschrift* that he had been able to distinguish cattle blood from horse, donkey, pig, ram, dog, cat, deer, hare, guinea pig, rat, mouse, rabbit, chicken, goose, turkey, pigeon and human bloods using a rabbit immune anti-cow precipitin serum. Similarly, human blood could be differentiated from the others using serum from rabbits which had been immunized with human blood. Normal rabbit serum did not react with saline solutions of any of the bloods. Further, it could be shown that saline extracts of human bloodstains dried on pieces of wood could be distinguished from horse and cattle bloodstains treated similarly. The stains were a month old when tested. A summary of this work appeared in a later issue of *Muenchener Medizinische Wochenschrift* (Uhlenhuth, 1901b). Independently and almost simultaneously, Wasserman and Schütze (1901) arrived at the same conclusions regarding the species specificity of the reaction and its applicability to dried bloodstains, this paper appearing in the February 18 issue of *Berliner Klinische Wochenschrift.* They noted that serum could be used as immunizing antigen in order to obtain antisera lacking antibodies to the cells (the agglutinins and hemolysins). Uhlenhuth (1901c) reported the technique in detail in the medico-legal literature in May, noting that species differentiation was possible in 3 month old bloodstains, in blood frozen for 2 weeks in the snow, and even in putrefied blood, provided a completely clear extract could be obtained for testing. A number of badly putrefied samples reacted readily with the antiserum, and human menstrual blood in a urine specimen could be detected easily. Human urine containing albumin reacted with the antiserum as well (Uhlenhuth, 1901d). This observation was also made by Dieudonné (1901). Further studies on the precipitin test indicated that bloodstained materials from criminal cases, made available from past cases by the judicial authorities, could be reliably diagnosed in every case. Results could even be obtained with bloodstains that had been washed (Uhlenhuth, 1901e). It was noted that cross-reactions occurred between more closely related species, e.g. anti-ram reacted to some extent with goat and cattle blood. Uhlenhuth recognized that the relationship between immunological cross reactivity in the precipitin test and species relatedness was a matter of fundamental biological importance. Anti-human serum had been shown to react with albumin-containing urine, and it also reacted with human semen and with pulmonary mucus from tuberculosis patients to some extent. The antiserum, therefore, detected human albumin, and was not necessarily specific for blood (Uhlenhuth, 1901e; Wassermann and Schütze, 1903). Uhlenhuth noted that antiserum preparation required considerable experience, and that there was variability in the rabbits used to produce it. He said that the use of high quality, carefully tested antisera was of paramount importance in forensic investigations, and suggested that antiserum production be carried out in a few specialized institutes.

Nuttall (1901a) confirmed the specificity and value of the precipitin test. He noted further that the serum used for immunization needn't be fresh in order to elicit antibodies. He showed also that the reaction worked well in dried bloodstains and in putrefied blood. The investigations on the phylogenetic relationships among various animal species, using immunological cross reactivity as a basis, were begun at this time (Nuttall and Dinkelspiel, 1901a and 1901b), and it was noted that anti-human serum cross reacted with the blood of several species of monkeys. Antisera could also be prepared against human pleural fluid, and this serum cross reacted to some extent with human tears and nasal mucus. Medico-legal application of the precipitin method was strongly recommended. Ziemke (1901a and 1901b) confirmed the value of the technique, noting that species could be diagnosed in bloodstains up to six years old as well as on a variety of substrata. Frenkel (1901) and Stern (1901) likewise showed that the precipitin test was fully applicable to bloodstains, and both noted the cross reactions with monkey bloods. The term "antiserum" appears to have been introduced at this time simultaneously and independently by Nuttall (1901a) and by Stern (1901) to apply to the serum containing the antibodies (usually, immune rabbit serum). Corin (1901) noted in his review that the precipitin antisera were more specific if the serum globulins were isolated and used for immunization.

The bulk of evidence thus indicated that the precipitin test was both specific within certain limits and fully applicable to forensic samples, and the use of the test rapidly became widespread. Many authorities confirmed its value and noted its limitations. Kratter (1902) dissented for a time from the current of opinion, saying that he had been unable to confirm a number of Uhlenhuth's observations on specificity, reliability and medico-legal applicability of the test. Uhlenhuth (1902) responded to Kratter's article, giving further detailed experimental justification for the test's reliability and specificity in the diagnosis of species in bloodstains on objects involved in criminal cases. Stonesco (1902) said that because of the known cross reactions of the antisera, it would be better to use the term "probably" in giving evidence on the species of origin of bloodstains in which a positive test had been obtained for human blood. Schulze (1903) more or less agreed with this view, saying that the Court should be made aware of the test's limitations as well as of its great value. Whitney (1902 & 1903) described successful results with the method, and Wood (1902b and 1903) gave an

account of the technique he used, and said that he had been able to identify human blood on an alleged murder weapon (a stone) in a case from New Hampshire. Ewing (1903) and Mallett (1903a and 1903b) both said they thought anti-human sera prepared in chickens were more specific, and gave fewer cross reactions than rabbit antisera. Mallett thought there might be interracial immunological differences which could be detected by means of the precipitin test, this based on a few experiments with serum from Caucasians and Negroes. Bruck (1907) was able to obtain differences in the intensity of reactivity with the sera of people from different races using the complement fixation technique (section 16.6.1), and thought that the method might be useful in this respect. Marshall and Teague (1908), in extensive experiments using both the precipitin and complement fixation techniques, also observed that there were slight differences in reactivity of the sera of persons from different races with antisera prepared against the serum of a member of a particular race. But these differences were so slight that they did not agree with Bruck that racial origin could be diagnosed by immunological techniques.

Some authorities around this time began using the term "humanized serum" to apply to the antiserum. Thus, rabbit immune anti-human serum was referred to as "humanized" rabbit serum. DeLisle (1905) prepared antihuman sera in horses, and recommended this large animal as a source because of the large quantity of antisera which could be obtained. It is now known that horse and a few other animal species often produce precipitin antisera which have somewhat different characteristics from most rabbit antisera. The horse antisera are characterized by a narrower zone of equivalence between antigen and antibody, that is, the antigen-antibody complex is soluble in either excess antigen or excess antibody, and the range over which precipitation will occur is narrower than with rabbit antisera (Maurer, 1971). This matter is discussed in more detail in section 1.3.4.2. In 1903, Hitchens reviewed the highlights in the development of immunology, emphasizing the value of precipitin antisera in medico-legal work. Ewing and Strauss (1903) gave an extensive review of precipitins and their medico-legal applications.

An extremely comprehensive series of studies on the application of the test to forensic materials was reported by Graham-Smith and Sanger in 1903. Many of the test's parameters were explored in detail, and studies were conducted on blood stains on a large number of different materials under different conditions. A few chemicals exerted a deleterious effect on the antiserum, and some stains on leather substrata were not able to be diagnosed. Very high titer antisera could be produced using relatively small quantities of immunizing sera, of the order of 3 to 5 mℓ at each of three to four injections. Many workers had employed considerably larger quantities of immunizing serum.

Robertson (1906) recounted his experiences with the test, and noted that he got positive tests in stains that were 9 years old. In 1908, Gay reported what was probably the first application of the precipitin test in this country in the enforcement of the game laws. Uhlenhuth (1901f) had shown earlier that the method was applicable to the differentiation of meats from different species of animals. Gay distinguished between precipitins against sera (seroprecipitins) and those against meat protein (musculoprecipitins), and attributed this distinction to Vallee and Nicholas. In the case in question, a man in Massachusetts was accused of illegally killing a deer, based on the finding of an animal heart in his possession by the police. The suspect claimed that the heart was from a cow. Dr. Gay prepared antisera to extracts of authentic beef and deer heart muscle as well as to extracts of the suspected heart. It could be shown that the heart found in the man's possession was in fact that of a deer. Clarke (1914) reported a similar case in California, in which he identified deer meat in a poaching case, which went to a jury trial. In 1917, Hunt and Mills reported on a case in which the precipitin test failed to react with several blood-stained objects in a criminal case. Two anti-human sera were used, one prepared by them and the other prepared abroad. These bloodstains were later shown to be of human origin by the complement fixation technique. They cautioned, therefore, that a negative precipitin test does not necessarily exclude the presence of homologous antigen (blood) in a stain.

In 1909, Uhlenhuth and Weidanz published a monograph which reviewed the subject of forensic immunology to the time, and provided very detailed experimental procedures and methods for every aspect of the precipitin technique. This book was a standard reference for a number of years. In 1911, Uhlenhuth delivered the Harben lecture in England, recounting many of the experiments that had led to the development of the precipitin test, and giving his own recollections. This paper was the only one of his many contributions which appeared in English.

In 1914, Hektoen discussed the production of antisera to human and animal sera, finding that a single injection of 30 mℓ immunizing serum was about as effective as three successive ones of 5, 10 and 15 mℓ, respectively. In 1917, Hektoen carried out experiments to determine whether antibody production in rabbits was enhanced if animals were used which had previously been employed for antibody production (not necessarily with the same antigen). There were no differences between such animals and immunologically virgin rabbits, and Hektoen suggested that antisera were therefore best prepared in the latter. Ewing (1903) and Mallott (1903a and 1903b) had recommended the production of antisera in fowl. Sutherland (1914) preferred to prepare his antisera in domestic fowl, but these antisera exhibited some peculiar characteristics. Sutherland and Mitra (1914) noted that too rapid thawing of frozen antisera caused it to exhibit interspecific cross reactions. These could sometimes be eliminated by allowing the serum to sit in the dark for a time, or by dilution with normal nonimmune fowl serum. Hektoen (1918a) found that fowl were more reliable, liberal producers of antisera than were rabbits. Good, high titer antisera could be obtained in most cases in 10–12 days after a single intraperitoneal injection of 20 mℓ defibrinated blood or of serum. He noticed some of the same peculiarities which

Sutherland and Mitra had earlier observed, and in another paper (1918b) suggested that it would probably be best to use rabbits for making antisera. Hektoen and McNally discussed the precipitin test in detail, including the preparation and evaluation of antisera in their review of medico-legal examination of blood stains in 1923. Fujiwara (1922a) said that heat-coagulated serum elicited antibody production as well as did fresh serum. Schmidt (1921), working with egg albumin, had shown that antibodies obtained with denatured protein could react with the denatured protein as well as with the native molecules. Blumenthal (1927) reviewed in detail the methods for making antisera, and gave what he regarded as the best protocol for immunization. Proom (1943) was able to obtain potent antisera in over 80% of the rabbits used with alum-precipitated antigen. Over the years, many investigators have looked into the preparation of antisera for the precipitin test. Many of these studies are discussed in some detail in Schleyer's (1962) review.

At present, many laboratories obtain antisera from commercial sources. Schleyer (1962) said that antisera should meet certain criteria before being employed in medico-legal work. They should be sterile, free from turbidity, of high potency and species-specific. By high potency was meant that the antiserum should have a minimum titer of $1{:}10^4$ to $1{:}2 \times 10^4$ against homologous antigen, and quite obviously the antiserum must not cross react with the sera of other animals to be exlcuded in the tests at the dilutions at which the test is performed. Otto and Somogyi (1974) said that most of the cross reactions of antihuman sera with non-human animal sera were accounted for by antibodies to IgG and to α-globulins.

It is worthy of mention that the use of adjuvants in immunizing animals to obtain antisera has permitted better yields of higher titer antisera in many cases. Since the work of Freund (see, for example, Freund and McDermott, 1942), many immunologists have employed adjuvants in the preparation of antisera. Herbert (1973) discussed the use of water-in-oil adjuvants, giving a review of the technique as well as some of the experimental details.

16.1.2 More recent developments—gel methods

Another important development has been the evolution of methods which use gel media for detection of the antigen-antibody reaction. Before gel media were introduced, the precipitin test was carried out in tubes. Often, antiserum, which is more dense than the usual aqueous (e.g. saline) antigen solutions, was placed into a tube and antigen solution carefully layered over the top so as to form an interface at which the precipitate could then form. Alternatively, the antigen solution could be placed in the tube first, and the antiserum solution layered underneath it by means of a capillary pipette. This procedure is sometimes called a "ring test". The earlier workers all employed this sort of method, in many cases using what would be considered by present standards excessive quantities of reagents, on the order of one to several mℓ. Material can be conserved by carrying out the test in small capillaries. The use of capillary tubes was first recommended by Hauser in 1904. Schoenherr (1952) gave a technique for the precipitin test in Pasteur pipettes. The ring test is still in use in many laboratories (see, for example, Boyd, 1946 and Hunt *et al.*, 1960). The test can be carried out on a microscope slide and the precipitate observed in the microscope under dark field illumination (Marx, 1920).

The first immunodiffusion experiment was done by Bechhold in 1905, although his primary interest was not in antigen-antibody reactions. He allowed goat serum to diffuse into a gelatin medium into which had been incorporated rabbit antiserum to the goat serum. The experiment was done to obtain a precipitate different from those obtained when inorganic chemicals were allowed to diffuse together in gels and form precipitates. One can, for example, put a drop of $Na_2Cr_2O_7$ onto a gel into which has been incorporated $AgNO_3$, and a series of concentric rings of $AgCr_2O_7$ precipitate will form. These are called "Liesegang rings", and Bechhold attributed their discovery to R. E. Liesegang in 1898. No notice appears to have been taken of Bechhold's experiments by the immunologists of the time. In 1920, Nicolle *et al.* devised a single immunodiffusion technique for the identification of bacteria. Bacteria were grown in agar medium containing specific antisera for particular bacterial species. If the bacterial cell wall antigens were homologous to the antiserum, a halo formed in the gel around the colony. None of the early workers properly appreciated the meaning and potential applicability of multiple precipitin band formations, which were occasionally observed, but thought they were similar to the Liesegang phenomenon. The possibility that they might represent more than one antigen-antibody system, present in the same test system, was overlooked.

In 1946, Oudin published his first paper on immunodiffusion. This work represented the beginning of the development of the technique as a powerful immunological tool. Antiserum containing gels were overlayed with homologous antigen solutions and the behavior of the precipitin band which formed in the gel was studied. The precipitin band appears to diffuse through the gel in proportion to the initial concentration of antigen, its diffusion coefficient, and inversely to the antibody concentration. Multiple antigens cause the formation of multiple bands, which migrate independently. In succeeding years Oudin developed the method further and devised a theoretical basis for the observations (Oudin, 1947, 1948 and 1949). A review of the principles underlying Oudin's technique and immunodiffusion generally may be found in Oudin (1971), Aladjem (1971) and Oudin and Williams (1971).

Oudin's technique is called single immunodiffusion. It is discussed in connection with the principles of immunodiffusion in section 2.2.

The late 1940's witnessed the development of techniques for double diffusion analysis of antigen-antibody reactions in gels by Elek in England and Ouchterlony in Sweden. The method was developed primarily for assessing the diphtheria bacillus toxin reaction with homologous antitoxin. Elek pub-

lished his preliminary findings in 1948, a more detailed account following in 1949. Ouchterlony published his first account in 1948 as well (Ouchterlony, 1948a and 1948b). The theoretical basis was laid down in a series of papers in the following year (Ouchterlony, 1949a, 1949b and 1949c). Elek's interest in the subject did not continue, but Ouchterlony has continued to work in the field, one of the best single references being his monograph, published in 1968. This work covers not only the agar gel method, but discusses some of the other support media such as cellulose acetate membranes. Detailed descriptions of various methods are given, as well as the underlying theoretical basis for the variations which have been described. Crowle's book also gives a very complete treatment of the subject (Crowle, 1973). Oakley and Fulthorpe (1953) developed the double diffusion technique for use in tubes, with special reference to its application to the analysis of bacterial toxins. Double diffusion is discussed in section 2.2.2.

Muller *et al.* (1950) applied Oudin's technique to medicolegal practice. Gum acacia was tried as a medium but was abandoned in favor of agar and agar-gelatin mixtures (Muller & Michaux, 1952). In 1958, Muller *et al.* noted the applicability of the double diffusion methods. Species determination could be carried out using a microdiffusion technique in agar originally devised by Hartmann and Toillez in 1957 (Muller *et al.*, 1959). Mansi (1958) devised a microimmunodiffusion technique which was used and recommended by Fiori (1963). Gajos and Brzecka (1968) said that threads from bloodstained fabrics could be incorporated directly into the gel for species determination, thus avoiding not only the time required for extraction, but the concomitant loss of material. Maresch and Wehrschütz (1963) described a precipitin test for species determination carried out on microscope slides in 2 mm thick agar gels. Feinberg (1961) devised a "microspot" double diffusion test, which could be carried out on microscope slide cover slips. Katsura (1976) utilized this method to diagnose the species of origin of very small amounts of blood using anti-human Hb antisera (see section 7.1). The precipitin lines were visualized using a phase contrast microscope, and even stains 48 years old were said to react within 3 hrs at 30°. Feinberg (1962) published a modification of his spot test for use on cellulose acetate membranes. El-Guindi (1972) reported successful results with an ordinary macroimmunodiffusion procedure in agar gels. He used several arrangements of sample and antiserum wells.

In 1959, Bussard suggested taking advantage of the electroendosmotic properties of agar in carrying out immunoelectrophoretic analysis of antigen-antibody reactions. A system could be devised in which the antigen and the antibody migrated toward one another, a precipitate forming at the point of their interaction. This technique, he called electrosyneresis. It has also been called electroprecipitation and crossed over electrophoresis or crossing over electrophoresis (see section 2.4.2). Culliford (1964 and 1967) devised a crossed over electrophoretic technique for species determination in forensic casework which allowed a large number of samples and controls to be run simultaneously in a short period of time, using only a few $\mu\ell$ of material for each test. In this method, small wells about 1.5 mm in diameter are punched in an agar gel about 5 mm apart. The stain extract (antigen) is placed in the cathodic well of a neighboring pair, and the antiserum in the anodic one. The γ-globulin antibodies migrate cathodically because of electroendosmosis, while the other serum proteins migrate anodically. The net result is a precipitin reaction occurring about midway between the paired wells. The test was performed in veronal buffers, pH 8.6, containing calcium lactate. Čarny reported a similar technique in 1971. Grunbaum (1972) noted that crossed over electrophoresis could be done on cellulose acetate membranes as well. Although it is usual practice to prepare antisera against whole human or animal sera, Tran Van Ky *et al.* (1968) recommended the use of antisera prepared specifically against the γ-globulin fraction.

Lincoln (1975) reported on a very interesting series of cases involving alleged witchcraft, illustrating how the events were unraveled by determining the species of origin of the articles involved.

16.1.3 Effects of some external influences

The age of a stain alone apparently does not prevent a positive precipitin test. Haseeb (1972) got a positive test on a 12 year old stain kept at room temperature. Linoli (1971) examined the "flesh and blood" from the eucharistic miracle that is said to have occurred at Lanciano, Italy, in the 7th Century A.D. The blood, which was about 1200 years old at the time of examination, gave a positive precipitin test with anti-human serum. Smith and Glaister (1939) reported a positive precipitin test on extracts from mummified tissue many thousands of years old. It has been known for a long time, however, that old blood stains do not easily yield serum proteins to mild, aqueous extracting solvents, like water or saline. Dorrill and Whitehead (1979) said that 5% (v/v) 0.880 ammonia was a much more effective extraction medium for older stains than water for purposes of extracting serum protein for species determination in older stains.

Muller *et al.* (1966) showed that a positive precipitin test could be obtained on stained garments which had been dry cleaned. There are circumstances, however, under which the precipitin test may fail. Since antisera are almost universally prepared against serum proteins, serum protein must be present in, and extractable from, the stain if a reaction is to be expected. There is not a necessary relationship between the amount of hemoglobin and the amount of serum protein in a stain extract. One cannot judge how much serum protein may be present on the basis of the amount of pigment present, as was pointed out by Okamoto in 1902. With fairly freshly dried stains there is a strong likelihood that considerable blood pigment will be extracted, but sometimes without sufficient serum protein to give a positive precipitin test, particularly if extraction time is short (Mueller, 1934). 24 hour extraction times are recommended in such cases. Mezger *et al.* (1933) noted that with a blood stain on wood,

the dissolved superficial layers of stain did not give a precipitin test. It was necessary to extract the wood substratum, the serum protein having soaked into the wood more deeply than the red portion of the stain. Olbrycht (1950) showed that extracts of dried human blood mixed with earth can fail to give a positive precipitin test with anti-human serum, presumably because of the absorptive properties of the soil. In all cases in which the precipitin test failed, the extract did not contain detectable protein. The same results were obtained with dried blood mixed with aluminum oxide or finely pulverized iron ore. Some bloodstained earth samples did yield precipitin-positive extracts, as did bloodstained limestone and bloodstained sand. A few chemicals caused nonspecific precipitation of the antiserum, and of normal control serum as well. These included aluminum and iron chlorates, aluminum chromate, tannic acid and an extract of spruce bark. Some organic solvents, dilute acids and bases, and peroxide cause non-specific precipitation of the antiserum (Vollmer, 1949).

Exposure of bloodstains to lengthy steeping in cold water does not prevent their giving a positive precipitin test. Older stains yield less protein to the steeping fluid than do fresher ones, and older bloodstains retained more serum protein in 50° water than in cold water (Smith and Glaister, 1939). Detergents do interfere with the precipitin test. Some detergents, even in dilutions up to $1:10^4$, will give a flocculent precipitate with antiserum as well as with normal serum (Burger, 1956; Klose, 1961). Burger (1956) thought these reactions were caused by alkyl- and arylsulfonates or sulfates in the detergents. Water rinsing does not entirely remove these chemicals, but rinsing stained fabric with a solution of methanol-ethanol-amyl alcohol-trichloroethylene prior to saline extraction does rid the substratum of the interfering substances, and allows the test to be performed. Burger noted that these observations underscored the importance of running cloth controls. Schoenherr (1957) noted that detergent residue on glass can cause precipitation of a test serum, a point well worth noting if the test is done in glassware that is washed and reused.

Exposure of bloodstains to heat causes the precipitin reaction to become weaker, and reaction time longer. There is an upper limit of exposure, beyond which the reaction will be negative. The limit is a function of both the temperature and the time of exposure. Smith and Glaister (1939) could still get a positive test on stains exposed to 130° or 150° for 15 min, or to 200° for 2 min. Exposure to heat also tends to fix the stains and render the protein increasingly insoluble. This phenomenon was observed very early by Katayama (1888) and Hammerl (1892) with regard to hematin compounds. Stains which have been ironed may be extremely insoluble (Schech, 1930). Much longer extraction times are required for stains that have been exposed to heat than under ordinary circumstances (Schech, 1930; Schleyer, 1948 and 1962). It is sometimes necessary to use 0.1N NaOH for extraction in these cases since saline will not extract the proteins even after extended periods of steeping (Schleyer, 1948).

Lee and DeForest (1976) devised an interesting variation of the precipitin test, applicable to stains which contain species-specific antigens, but which do not readily yield them up to extraction media. They could show that a substantial fraction of the species-specific serum protein (saline-extractable protein) was lost upon steeping bloodstained cloth in water or detergent solutions. The loss of protein positively correlated with the temperature of the washing water from 25° to 100°. This result was not in accord with that of Smith and Glaister (1939), who observed that hotter water extracted less protein from stained fabric than room temperature water, and thought that the hotter water tended to fix the stain, and prevent protein loss. Losses were greater in Lee and DeForest's experiments if a cold water rinse step was included following the washing step. The results indicated that only a very weak precipitin test at best could be obtained from bloodstains exposed to washing water at temperatures of 75° or hotter, without agitation. It was thought that some species-specific protein might still be present in the stains, however, notwithstanding the fact that it could not be extracted with saline in quantities sufficient to give a precipitin test. An inhibition test was therefore devised in which the washed stained material was incubated with relatively low titer antihuman serum (1:16) for 24 hr at 4°. Aliquots of the antiserum were then removed and tested in an Ouchterlony double diffusion system with fresh human serum to see whether the stained material had substantially reduced the titer of the antiserum. Positive results were obtained, and it could be shown that washed control bloodstains made from cow, pig, horse, sheep, duck, chicken, rabbit, dog and cat bloods had no inhibitory effect on the antihuman serum. It was noted that this procedure could be applied equally well in situations in which the bloodstain contained a soluble substance which interfered with the ordinary precipitin test. The interfering substance could be washed out, and the stain then subjected to the inhibition procedure.

Itoh (1979) devised a somewhat similar kind of inhibition test for species determination using anti-human serum. He said that the procedure was applicable to bloodstains and to muscle tissue. Here, anti-human serum of appropriate titer was incubated with the material to be tested. The absorbed antiserum was then titrated with latex particles, coated with serum protein antigens (see also in section 16.5).

At various times, there has been interest among immunologists in the preparation and characteristics of antisera against heat-denatured serum proteins, particularly the comparison of reactions of such antisera with native and denatured antigen (see, for example, Schmidt, 1908). These experiments were of significance in advancing immunological knowledge, but not of much practical significance for medico-legal investigations (Schleyer, 1962). It is clear that moderate exposure to heating, e.g., 10 min at 55–70°, causes denaturation in serum proteins leading to changes in the shape of the molecules and giving rise to aggregation, but such proteins retain their antigenicity (Peeters *et al.*, 1970).

16.1.4 Tests with anti-human hemoglobin antisera

Preparation and use of anti-human hemoglobin sera has been discussed in section 7.1. These antisera can be used for the determination of species of origin as well as for the identification of the presence of blood. Anti-hemoglobin sera are generally not as potent as anti-serum sera, because hemoglobin is not as antigenic as most of the serum proteins. The same considerations apply to the use of anti-human hemoglobin as do to the use of anti-human serum reagents. Assuming species specificity has been established for an anti-human Hb serum, however, its reaction with a bloodstain extract may be regarded both as an indication of the presence of hemoglobin (presence of blood) and a diagnosis of human origin.

16.1.5 The antigen-antibody reaction. Optimization of reactant concentrations

No complete review of the theory of antigen-antibody reactions would be appropriate here. A good deal of work has been done on the subject and some discussion is given in section 1.3.4.2. Munoz and Becker (1950) and Becker *et al.* (1951) gave a detailed treatment of the reaction as it takes place in Oudin's single diffusion system. The precipitin reaction in solution was discussed by Kendall (1942). The effect of salt concentration was studied in detail (Aladjem and Lieberman (1952) and Lieberman and Aladjem (1952). A very good review by many specialists may be found in Chapters 13 and 14 of Williams and Chase (1971).

With some variations, depending upon the system under study, antigen-antibody reactions have certain characteristics in common. It has been known for a long time that, if one titrates a fixed quantity of antigen with decreasing amounts of antibody, the antigen-antibody reaction does not occur at extreme excesses of either component. In many precipitin systems, for example, no precipitation is observed at the excess antigen or the excess antibody extremes of the titration series. It is important, therefore, to obtain some idea of where the optimum precipitating concentrations lie in order to insure that the conditions chosen will not be characterized by an excess of either component. In carrying out the precipitin test for determination of species, it is common practice to make dilutions of the serum, or of the stain extract. The antiserum is often employed undiluted or at a particular constant dilution. Since there is considerable variation in the concentration of proteins in bloodstains, as well as variation in the efficiency of extracting them, some attention has been paid to determining the protein concentration of stain extracts. The extract can then be diluted if necessary to optimize the protein concentration prior to performing the test. Allison and Morton (1953) devised a simple test for approximating the amount of protein in a stain extract. Drops of progressively doubly diluted blood solutions were placed onto a filter paper and dried. A dried drop of stain extract was treated similarly, and the concentration of protein estimated by eye on the basis of color. If a more accurate estimate is wanted, the dried spots could be stained with a protein stain, the paper background destained, and the color of the extract spot compared with the colors of the "standard" spots. Lynch (1928) mentioned three other methods for estimating the protein concentration in stain extracts. The so-called "foam test" is based on the fact that 1:1000 dilutions of serum in saline, which have air bubbled through them, will form bubbles at the surface which persist for about 10 min while in greater dilutions of serum, the bubbles disappear almost at once. Nitric acid precipitates protein, and extract can be layered over a nitric acid solution to see whether an opalescent precipitate forms at the interface. In 1:1000 dilutions of serum, a faint precipitate will be observed, while in greater dilutions, no precipitate will form. A similar test is based on the protein precipitating power of sulfosalicylic acid. The opalescence caused by addition of $\frac{1}{20}$ of a volume of 50% sulfosalicylic acid to bloodstain extract may be compared to that in a series of standards prepared from dilutions of whole serum. Schleyer (1962) discussed the sulfosalicylic acid test in his review. It is usually said that a 1:1000 dilution of whole serum or its equivalent in a stain extract is optimal for carrying out the precipitin test.

It may also be mentioned that the pH of stain extracts should be in the neighborhood of neutrality for best results with precipitin reactions, and to diminish the possibility of pseudoreactions. Normally, there will be no problem because saline is usually used for extraction, but if 0.1N NaOH or ammonia were required as an extraction medium, some neutralization would be necessary.

16.2 Antihuman Globulin Serum Inhibition

Antihuman globulin serum (AHG serum, Coombs' serum) is an antiserum prepared against human serum globulin. Since the antibodies of serum are in the globulin fraction, AHG serum contains antibodies to the other antibodies, a fact whose significance will become clear in the discussion below, and see section 1.3.4.1.

In 1949, Wiener *et al.* suggested a new serological test for the determination of human serum globulin. The serum globulin is human specific, and the application of the method to species determination is based on this property. There are certain kinds of antibodies to the Rh_o (D) receptor of human red cells which do not agglutinate Rh+ cells in saline. These so-called "incomplete" or "blocking" antibodies do, however, combine with the antigenic receptor and sensitize the Rh+ cells (see sections 1.3.4.1 and 22.4). The presence of such antibodies on red cells can be detected by using anti-human globulin serum. The AHG antibodies are capable of combining with the anti-Rh antibody which is, in turn, bound to the red cell. If AHG serum is added to red cells with incomplete antibody bound to their Rh_o (D) receptor, agglutination will occur. Cells with incomplete antibodies attached to their receptors are said to be "sensitized". This principle is illustrated in Fig. 16.1. A bloodstain contains the human serum globulin proteins, and, if incubated with AHG serum, will bind the antibodies and reduce the titer of the AHG serum. An inhibition test can therefore be devised, in

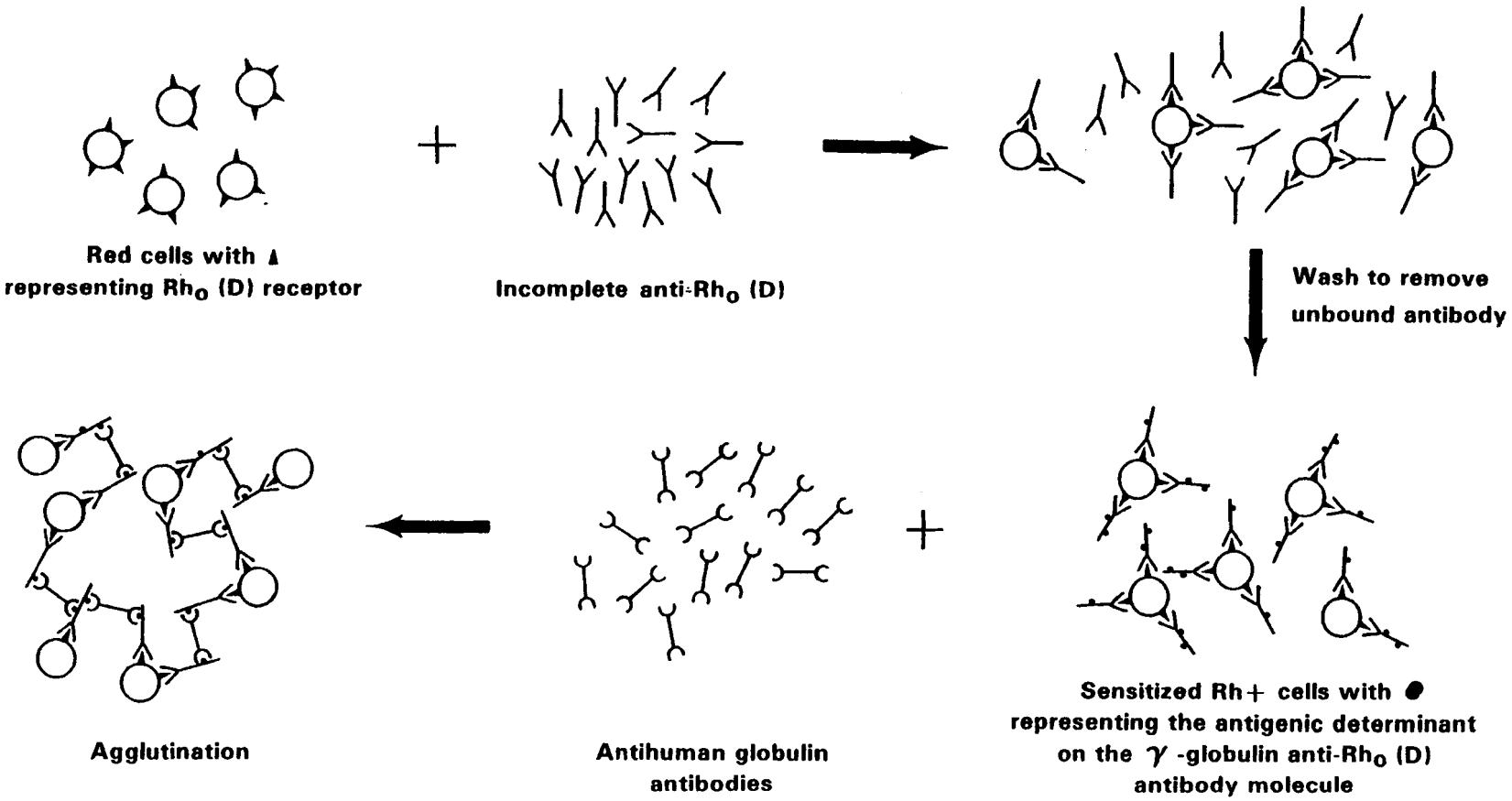

Figure 16.1 Scheme of Agglutination of Sensitized Rh+ Cells by Antihuman Globulin Serum

which bloodstained material is incubated with AHG serum, and the serum then tested to see whether it will agglutinate sensitized Rh+ test cells. If no agglutination occurs, the bloodstained material has removed the antibodies from the AHG serum, and it is, therefore, of human origin. If agglutination does occur, the stain did not reduce the combining power of the AHG serum and it is, therefore, not of human origin. The test for human origin is illustrated schematically in Fig. 16.2. In practice, the test is slightly more involved, in that appropriate dilutions of the AGH serum must be chosen so that the reduction in titer brought about by the serum globulin in a human bloodstain can be detected by the test system. Wiener *et al.* utilized a doubling dilution titration technique, and could quantitate the serum globulin in the test sample. Urine, semen, cerebrospinal fluid, saliva, and the sera of ox, horse, rabbit and Rhesus monkey did not inhibit the AHG reaction, and it was suggested that the method might find a medico-legal application.

Anderson (1952) reported that the method was more specific than the precipitin test, and could be applied to the detection of any animal serum, provided specific anti-globulin serum was available. The work was extended in 1954, and he could show that the technique was applicable to bloodstains. Human blood could not be distinguished from chimpanzee blood, but could be differentiated from all other animal bloods tested. Allison and Morton (1953) confirmed Anderson's (1952) findings. They showed that the test worked well with bloodstains on different substrata. It also gave positive results with alkali-denatured serum protein, although the inhibitory effect was reduced, and a positive test for human blood was obtained on cadaveric material from a mummy 5000 years old.

The value of the test was soon confirmed by a number of other laboratories. Several different techniques were proposed, some calling for titration, while others arranged the dilutions of the reagents in such a way that a one-step "all-or-none" procedure could be followed. Vacher *et al.* (1955) gave their method and said that semen, saliva, urine, meconium and fecal matter did not give inhibition. A variety of natural and synthetic textile materials were found not to interfere. Ruffié and Ducos (1956) published their method for carrying out the test, and Dérobert *et al.* (1957) enlarged upon their earlier (Vacher *et al.*, 1955) studies.

Jungwirth (1956) in Germany, Dell'Erba (1957) in Italy and Liberska and Smigielska (1958) in Poland all confirmed the specificity and sensitivity of the method. Ducos (1958a) described the techniques for the AHG serum inhibition test, as well as for the precipitin test and the passive hemagglutination test (see Section 16.3) in detail. He noted that if all three tests were performed on a sample, interpretation is simple if all results agree, but that it becomes very difficult if different tests give conflicting results. Cramp (1959) described a relatively simple version of the test which he said had been found to be quite satisfactory in 170 cases in New South Wales. Mosinger *et al.* (1960) described a two dimensional titration protocol for the AHG serum and the anti-Rh_o serum which, it was said, should be carried out with the reagents in order to select the optimal concentrations for actually performing the test. Schleyer (1962) discussed this technique in his review. Grobellar *et al.* (1970a) said that they selected their antisera for the test based on the criteria established by Proom (1943) for precipitin sera, namely that human serum should react at dilutions of 1:8000 while other animal sera should not react at dilutions of 1:50 or greater. It is important to include proper controls in this test, including the usual cloth control, and controls on the various reagents used in the test. Cramp (1959) said that he routinely included a positive human bloodstain control and two negative animal bloodstain controls as well. Hunt *et al.* (1960) showed that the antihuman globulin inhibition test was possible with a 1 cm^2 piece of cloth containing no more than 0.1 $\mu\ell$ of blood. Saliva and tears caused reduction as well if they were not too dilute. They recommended an AHG serum which, when diluted 1:400, gave a titer of 1:8 against Rh_o+ (D+) cells strongly sensitized with anti-D (anti-Rh_o).

Klose (1962) indicated that the presence of detergents in test samples will interfere with the test, unless a separation procedure is employed to get the globulin away from the surfactant material. The procedure using paper chromatography, described in section 7.2, was found to be satisfactory, and the detergent material could be located by its fluorescence under UV light.

Patzelt *et al.* (1977) said that the test could be done using human γ-globulin-coated latex particles. These are available commercially for use in testing for Rheumatoid Arthritis Factor in serum. Rheumatoid Arthritis Factor behaves as an antibody to human γ-globulin. Patzelt *et al.* said that false positive reactions could be gotten with the blood of higher mammals, and false negative results with sera from people with high concentrations of Rheumatoid Arthritis Factor. The false negatives were not seen, however, in bloodstain tests.

16.3 Passive Hemagglutination Techniques

In 1951, Boyden observed that certain preparations of inulin could render red cells able to absorb proteins onto their surfaces from solution. Inulin is a storage polysaccharide in certain plants (e.g. the Jerusalem artichoke) and is a polymer analogous to starch except that it is made up of fructose units instead of glucose units. Boyden found that preparations of inulin which rendered the cells able to absorb protein could, at high dilution, agglutinate the cells. Inulin preparations not having the property did not agglutinate the cells. It had been known since the work of Reiner and Fischer (1929) that dilute tannic acid solution could agglutinate red cells, and it was decided to test tannic acid solution for their ability to render red cells protein-absorbing. It was found that sheep red blood cells treated with 1:20,000 dilutions of tannic acid readily absorbed proteins from saline solutions. The cells could then be washed, and an antiserum homologous to the absorbed proteins would readily cause agglutination of the cells. Red cells which have been treated

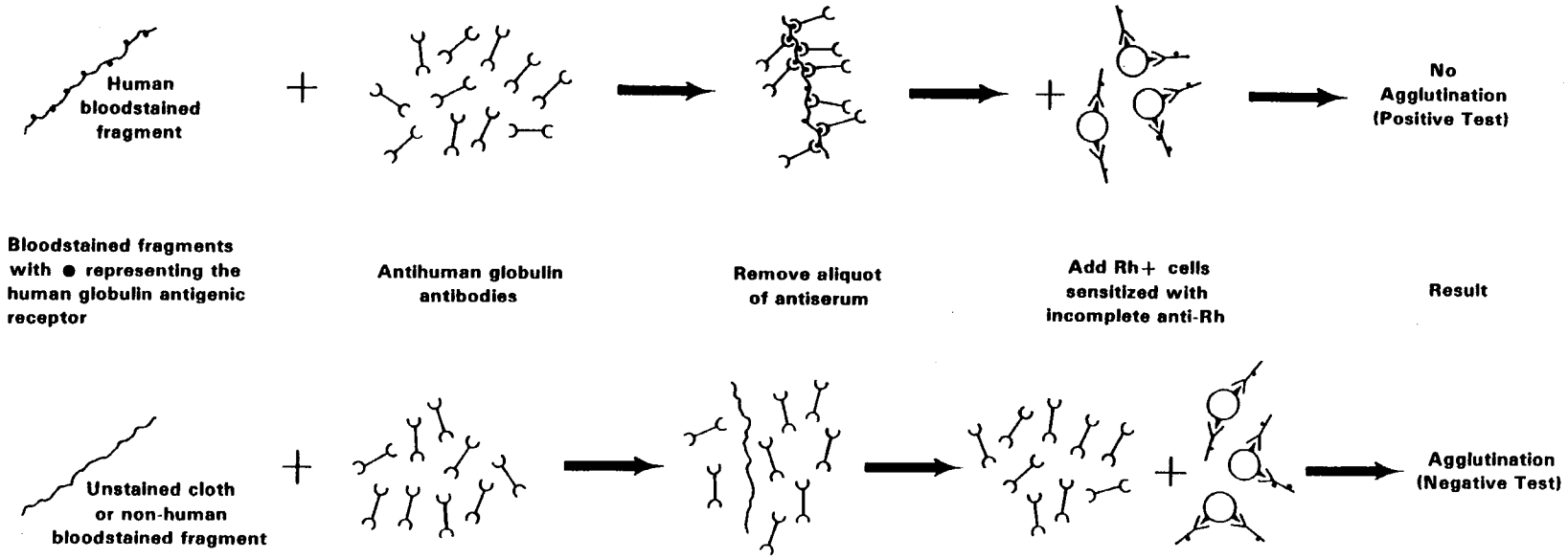

Figure 16.2 Scheme of Testing Bloodstain for Human Origin by the Antihuman Globulin Inhibition Method

with dilute solutions of tannic acid to render them protein-absorbing are referred to as "tanned cells".

Coombs *et al.* (1952) used a somewhat different technique to achieve essentially the same result, namely to devise an agglutination method for the assay of soluble antigens. Using benzidine tetrazonium chloride as a conjugating agent, egg albumin and chicken globulin proteins could be conjugated to incomplete Rh antibody molecules without affecting the latters' ability to combine with the Rh receptor on the red cell. Red cells could then be sensitized with anti-Rh antibodies to which had been conjugated either ovalbumin or chicken globulin protein. Antisera to either of these proteins would then bring about agglutination of the cells having the homologous antigen present.

Ducos (1956) put the tanned red cell technique to use in medico-legal species determination. Using a modified tanned red cell preparation method described by Dausset *et al.* (1955), he incubated bloodstain extracts with the tanned red cells. The cells were then washed, and tested for agglutination with AB serum, a series of blood group antibodies, and AHG serum. Of greatest interest was the result obtained with AHG serum. Only cells which had been exposed, after tanning, to human bloodstain extract, and which had therefore absorbed human globulin, were agglutinated by the AHG serum. The reaction was very specific and Ducos noted that it had the advantage over the AHG-inhibition method (section 16.2) that agglutination in this case represented a positive test, while a positive test in the former case was represented by the absence of agglutination. Ducos extended these studies (1958a, 1958b) showing that, while this technique was somewhat less sensitive than the precipitin and AHG-inhibition tests, it was more specific. In cases of exposing tanned red cells to human bloodstain extracts in doubling dilutions, and testing with AHG serum, agglutination could be observed at dilutions of 1:5000. In 1960, Ducos again described the method, suggesting that in medico-legal cases, several of the techniques should be used in order to be more certain about the species of origin. Hara *et al.* (1969) incubated stain extract with anti-human serum, washed the samples, then eluted the bound antibody, which was detected using tanned red cells to which had been absorbed human serum protein.

16.4 Mixed Antiglobulin Technique

In 1963, Styles *et al.* described a mixed agglutination technique for species determination based on the species-specific serum globulin present in bloodstains. The principle underlying mixed agglutination is as follows: a complete antibody to an antigen contained in the bloodstained fibril is allowed to react with the receptor in the stain, and the excess antibody is then washed away. Red cells containing the same antigen are then incubated with the "sensitized" stain fiber, and will be bound by the remaining combining site of the antibody, resulting in the cells arranging themselves in an orderly fashion along the fiber. The test cells are in effect "agglutinated" to the fiber rather than being agglutinated to one another.

A test for human species origin of a bloodstain was devised using this principle, and the technique called the "mixed antiglobulin reaction" by its originators. A bloodstained thread, which, if human blood is present, contains human globulin, is incubated with AHG serum. The test material is then washed to remove excess, unbound antibodies. Test cells are Rh positive and have been sensitized with an incomplete anti-Rh antibody. The mixed agglutination (mixed antiglobulin) reaction occurs if any AHG has been bound to the fiber, i.e., if the fiber contined a human bloodstain. The principle is illustrated in Fig. 16.3. Hara *et al.* (1969) employed the method, although they did not use AHG serum. An anti-human serum serum, which had been absorbed with monkey serum, was used. The test cells were tanned erythrocytes, to which had been absorbed human serum proteins. They said the technique was more sensitive than the precipitin test either in tubes or in agar gels. They referred to the technique as the "double combination method", a term which the Japanese workers have usually preferred to "mixed agglutination" (see section 1.3.4.1).

16.5 Sensitized Particle Techniques

16.5.1 Sensitized colloidon particles

In 1925, Freund established the groundwork for particle agglutination techniques in his physico-chemical studies on the agglutination of tubercle bacilli, and of protein-lipid extracts from these cells. The cells and the lipoprotein particles derived from them are maintained in a dispersion by a surface potential difference. When the potential is reduced below a threshold value, the dispersion is destabilized and the particles aggregate. All sera have the effect of reducing the potential, but if the particles are coated with an antigen, specific antiserum reduces it more effectively to a subthreshold value, and aggregation occurs. Jones (1927) applied this principle to the agglutination of colloidon particles, to which had been absorbed ovalbumin, by a specific anti-albumin serum. In 1940, Cannon and Marshall described a technique for the accurate determination of the titer of precipitin antisera using sensitized colloidon particles. Antigen was absorbed onto the particles, which were then washed to rid the suspension of unabsorbed antigen. The antisera could then be titrated with accuracy using these "indicator" particles.

Giaccone (1958) applied this principle to the determination of species of origin of bloodstains. Bloodstain extracts were incubated with washed colloidon suspension for 12–24 hrs at 4°. The particles were then thoroughly washed and resuspended in saline. A small aliquot of the particles was incubated with an equal volume of antiserum, the mixture centrifuged, shaken lightly, and read for agglutination either macroscopically or microscopically. Positive, specific reactions were obtained with undiluted and with 1:10 diluted bloodstain extracts. Concentrated control sera incubated

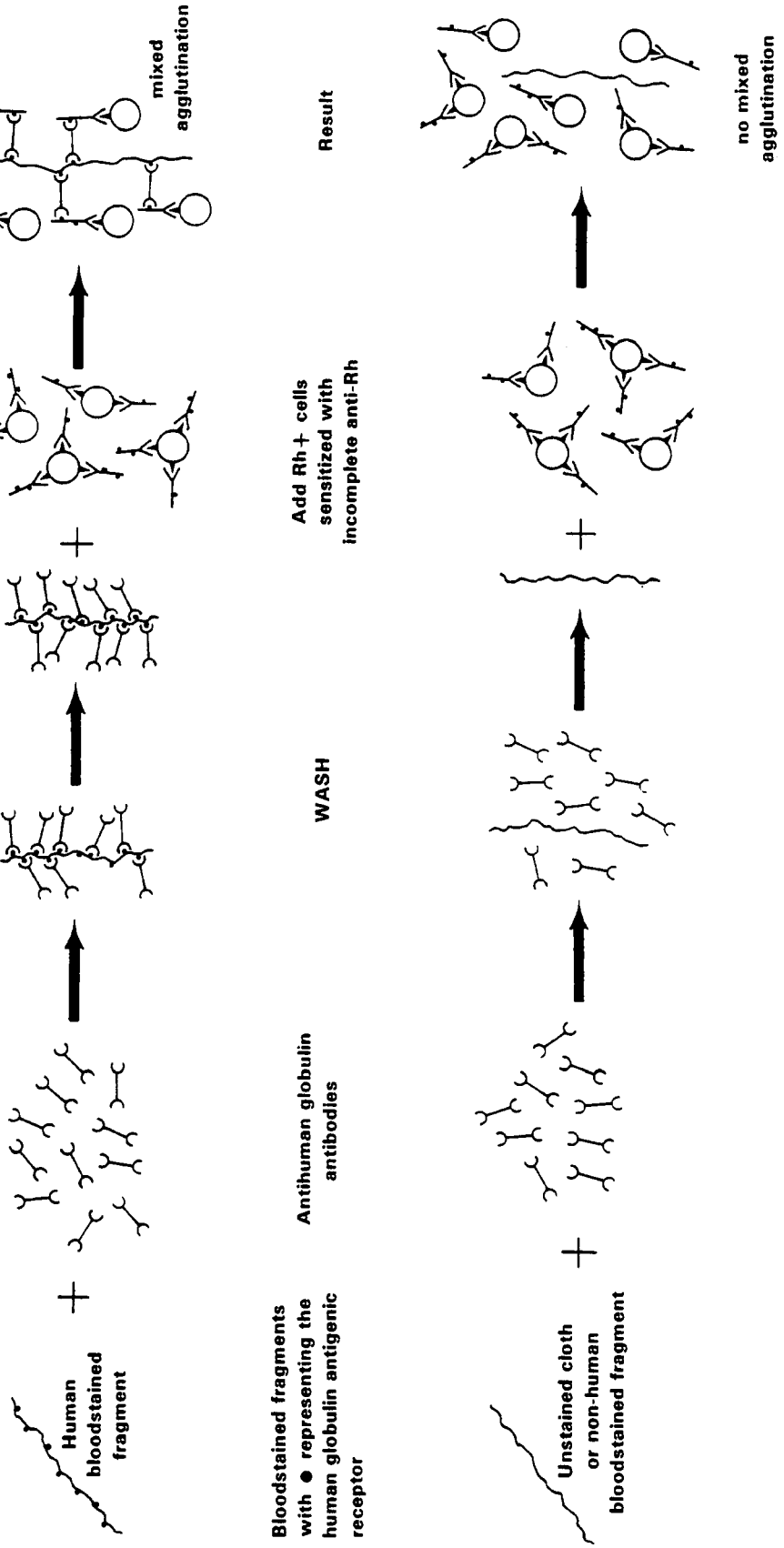

Figure 16.3 Scheme for Testing Bloodstains for Human Origin by Mixed Antiglobulin Technique

with the colloidon mixture instead of bloodstain extract sometimes caused spontaneous agglutination. Spontaneous agglutination also sometimes occurred when stain extract was added, but did not survive the washing steps.

Schleyer (1962) reported that he had conducted experiments on this method in collaboration with Hilgermann in 1960. The method was found to be specific and useful, and no spontaneous agglutination was observed in the course of these studies. Human serum in dilutions of 1:8000 gave positive, though weak reactions, whereas the precipitin test could not be obtained at dilutions of human serum greater than 1:4000.

16.5.2 Sensitized latex particles

In 1956, Singer and Plotz applied the particle agglutination principle to the serological diagnosis of rheumatoid arthritis, using sensitized latex particles. Robbins *et al.* (1962) showed that latex particles coated with human chorionic gonadotropin (see section 8.2.1) were agglutinated by a specific anti-HCG serum, and suggested that the technique might well lend itself to the development of a pregnancy test. In 1973, Cayzer and Whitehead applied the sensitized latex particle technique to medico-legal species diagnosis. The latex particles were sensitized with immunoglobulins, isolated from a sheep immune anti-human serum, in glycine buffered saline at pH 8.2. Protein concentrations of between 1 and 2 mg/mℓ with equal volumes of 2% latex suspensions were routinely employed for sensitization, but these conditions varied depending upon the particular globulin preparation. Dilutions of serum or of stain extract were mixed with the sensitized latex particles on a glass plate, the mixture rocked gently for 2 min, and agglutination read macroscopically against a dark background. The test was positive with human serum dilutions as high as 1:10,000. Cross reactions with the sera of closely related primates were positive at similar dilutions. All other animal sera tested gave negative results at serum dilutions greater than 1:100, and in some cases at dilutions greater than 1:40. Extracts of 2 year old human and primate bloodstains gave strongly positive reactions while extracts from stains of turkey, rabbit, hamster, rat, cat, mouse, dog, sheep, horse, cow, pig and donkey bloods gave negative reactions. Neat human saliva gave a strongly positive reaction but the reaction diminished rapidly at saliva dilutions greater than 1:100. Neat human semen gave a weak positive reaction. These studies were extended to animal bloodstains (Whitehead *et al.*, 1974) and latex particles were sensitized with the immunoglobulins of antisera against deer, cow, sheep, dog, cat, horse, mouse, hen and guinea pig as well as human sera. Some cross reactions were seen in the experiments with sera, but with the bloodstain extracts, the only cross reaction was a weak reaction of cow bloodstain extract with anti-deer particles. A few of the anti-animal particles reacted with human rheumatoid sera, but not at dilutions greater than 1:500. A number of other substances were tested for non-specific reactions with the anti-human particles. Soap solutions and fabric softener gave weak reactions, the only strong reaction (to a dilution of 1:1600) being given by milk. It was found, however, that milk agglutinated the unsensitized latex particles as well. Whitehead and Brech (1974) indicated that the latex particle technique could be applied to an extract from a single thread, which could then be employed for blood grouping by the elution technique since the species-specific antigens are water soluble whereas the blood group antigens are not.

16.6 Other Immunological Tests

16.6.1 Complement fixation

Complement (or alexin) is a multi-component system of serum proteins, and participates in the action of lytic antibodies (see section 1.3.5). Antibody must be present on a cell, bound to its receptor, before complement can combine with that cell causing lysis. In most antigen-antibody reactions, the resulting complex will combine with complement if it is present. Such combination is known as complement fixation. For purposes of the present discussion, complement fixation can serve as an indicator that an antigen-antibody reaction has occurred (see section 1.3.5.3). Since complement participates in the lysis of red cells by specific hemolysins, and is in fact required for hemolysis, hemolytic systems are convenient tools for the detection of the presence or absence of complement. All sera contain complement, but the amount varies even among members of the same species. Guinea pig serum is a common source of complement in complement fixation assays. If an antigen and its antibody are allowed to react in the presence of complement, some complement will be taken up, or fixed. The amount of complement added to the system can be arranged so that all of it is fixed. If the solution is now tested with a hemolytic system, its hemolytic power will be found to have been lessened or abolished. A common hemolytic system used in these assays consists of sheep red blood cells which have been sensitized with an anti-sheep red cell hemolysin prepared in rabbits. If such test cells are incubated with a test solution, and hemolysis occurs, complement was still present in the test solution indicating that it has not been fixed, i.e. that the antigen-antibody reaction which was being tested has not taken place. Conversely, if no hemolysis occurs, complement has been fixed and the antigen-antibody reaction may be inferred to have occurred. The complement in the serum containing the hemolysin is inactivated by heating prior to sensitizing the cells.

It may be noted that a number of the early studies on complement, and its ability to be "fixed" in antigen-antibody reactions, were carried out by the Nobel laureate immunologist Jules Bordet and his collaborators. Bordet showed in 1898 that an anti-rabbit erythrocyte hemolysin, prepared in guinea pigs, would first clump and then dissolve (hemolyze) rabbit red cells. He could show further that both antibody and complement were required for the lysis to take place, and that complement, but not antibody, was inactivated by heating for about 30 min at 60°. Since it was not clear at the time that one and the same antibody molecule

could bring about a variety of different effects when the antigen-antibody reaction occurred (such as agglutination, lysis, fixation of complement, etc.), antibodies were given descriptive names, based upon their action in a particular system (e.g. agglutinin, precipitin, lysin, etc.). Antibodies which "sensitized" cells so that they could subsequently be lysed in the presence of complement were called "sensibilisatrices" by the French workers and "amboceptors" by the German workers. Gengou (1902) established that complement could be fixed in precipitin reactions as well as in lytic ones. This fact, he demonstrated using rabbit antiserum to cow milk proteins and to ovalbumin, as well as in several other precipitating antigen-antibody systems. These studies were enlarged upon by the work of Gay (1905a and 1905b) and of Bordet and Gay (1906).

The complement fixation test for the medico-legal diagnosis of species of orgin was introduced by Neisser and Sachs (1905 and 1906a). They were prompted to carry out their experiments by the studies of Moreschi (1905) on the anti-complementary properties of certain sera. Neisser and Sachs used a hemolytic system consisting of rabbit immune anti-ox erythrocyte serum, known to be hemolytic for sheep red cells, and the sheep red cells were sensitized with this antiserum. Fresh guinea pig serum served as the source of complement. The antiserum had had its complement inactivated. 1 mℓ of a 5% suspension of sheep cells was readily hemolyzed in the presence of 5 $\mu\ell$ antiserum and 50 $\mu\ell$ guinea pig serum. Anti-human serum serum, prepared in rabbits, did not interfere with the hemolytic reaction. However, if 0.1 $\mu\ell$ human serum or 1 $\mu\ell$ monkey serum and 0.1 $\mu\ell$ anti-human serum was incubated with the complement, and the indicator system then added, no hemolysis occurred. Rat, pig, goat, rabbit, horse and ox sera had no such inhibiting effect on the hemolytic reaction. Saline extracts of 3 month old human bloodstains, diluted in some cases to the extent that a precipitin test could not be obtained, inhibited hemolysis in the system just as had human serum. Saline extracts of similarly aged bloodstains from sheep, fowl, rabbit, guinea pig, ox and horse did not inhibit hemolysis. They recommended that the test be employed as a control on the basis of the fact that a hemolytic reaction is easier to read than a weak precipitin reaction, that hemolytic sera are easier to obtain than precipitin sera, and that opalescence of the antiserum, which is a problem in a precipitin tube test, presented no difficulties in this test.

In another series of experiments Neisser and Sachs showed that with normal rabbit serum as the source of complement, hemolysis was inhibited by as little as 10 nℓ fresh human serum incubated with 10 $\mu\ell$ anti-human serum. They said that an antiserum which would inhibit hemolysis with 0.1 $\mu\ell$ fresh human serum, i.e. a very high titer antiserum, should be used in carrying out forensic tests. Friedberger (1906) conducted a number of experiments on the technique, and thought that its sensitivity might be a drawback. He obtained a "human" reaction with a saline extract of a stain of human perspiration and chicken blood. He was using a very high titer antiserum, however, and appears to have overlooked the fact that sweat may contain serum proteins which can react with an anti-human serum serum (Sutherland, 1907). Ehrnrooth (1906) got inhibition of hemolysis with 1:80,000 dilute human serum in a system which used rabbit serum as the source of complement and a goat red cell-rabbit immune anti-goat erythrocyte antiserum as the detection device. Muir and Martin (1906) showed that complement fixation occurred with 10 nℓ human serum incubated with 50 $\mu\ell$ of a potent rabbit anti-human serum. 1 $\mu\ell$ of human serum was required to obtain a definite precipitin test, indicating that the complement fixation test was about 100 times more sensitive than the precipitin test. They suggested, as had Friedberger (1906), that only very potent antisera, which could bring about complement fixation with 10 nℓ human serum, should be used in medico-legal investigations.

Uhlenhuth (1906a) tested a number of articles in his collection by complement fixation and obtained a number of false positive reactions. Some materials contained substances which could bring about the non-specific fixation of complement. A number of other materials, such as cotton-wool, pasteboard and gauze, which could be substrata for bloodstains, were negative. He got a complement fixation test in a case (Uhlenhuth, 1906a) from a bloodstain on a sack, when the precipitin test was negative and where examination of the bloodstain showed quite unequivocally that it was of avian origin. He was inclined not to put too much confidence in the test, therefore, and did not think it should be trusted as a check on the precipitin test (Uhlenhuth, 1906b). Neisser and Sachs replied (1906b) that adventitious substances could sometimes bring about non-specific complement fixation, and that a boiled bloodstain extract control should be run, since boiling would destroy the specific reaction but not the non-specific one. Another means of detecting such non-specific reactions is the running of a control in which stain extract is incubated with saline or non-immune serum (Sutherland, 1907). Schütze (1906) found the complement fixation test to be specific and more sensitive than the precipitin test. Graetz (1910 and 1912) carried out a number of studies on the method. Sutherland (1907) discussed the test, and his own experiments on its applicability. Stockis (1910) reviewed this method along with the other major tests for species determination. Complement fixation was reviewed in detail by Pfeiffer in 1938. In 1914, Sutherland mentioned that the test was not done routinely in India because sufficiently potent antisera could not be obtained. Olbrycht (1950) noted that the test is employed only infrequently in medico-legal practice, because of the experience required to carry it out properly, the controls that are necessary, and the fact that the precipitin test is usually more convenient and simpler. In the older literature, complement fixation is sometimes referred to as "complement deviation".

16.6.2 Anaphylaxis (Hypersensitivity)

The principle of anaphylaxis as a means of detecting antigen-antibody reactions was discussed briefly in sec-

tion 10.4.2, and more generally in section 1.3.6. Anaphylaxis as an immunological device for the medico-legal diagnosis of species of origin was explored by the earlier workers, but the technique is too time-consuming and cumbersome to be of any real value in practice, when simpler, equally effective alternatives are available. The anaphylaxis test is also sometimes called the hypersensitivity test (Überempfindlichkeitsprobe).

A number of workers appear to have suggested that the anaphylactic test be applied to medico-legal problems around the same time, and independently of one another. Thomsen wrote a full paper on the subject in 1909, which appeared in the March 25 issue of the *Zeitschrift für Immunitätsforschung und Experimentelle Therapie.* Shortly thereafter, Sleeswijk (1909) published a paper on serum hypersensitivity. On the page preceding the title page of this article, he made a point of noting that he had discussed the forensic applicability of the technique at an Academy proceeding in Amsterdam on March 27, 1909, and that his work had been done independently. Pfeiffer's paper appeared in 1910, and had been delivered at a September, 1909, meeting of the German medico-legal organization. Uhlenhuth took credit for having been the first to note the medico-legal applicability of the anaphylaxis method (Uhlenhuth and Weidanz, 1909). This claim is in fact true. Although he did not publish a full paper on the subject at the time, he did make the point clearly in a discussion which took place at the Berliner Militärärztliche Gesellschaft on December 14, 1908 (see in *Dtsch. Militaeraerztl. Z.* **38,** Vereinsbeilage, pp. 3–4, 1909).

In principle, the test is simple, but time consuming. An animal, usually a guinea pig since this animal tends to exhibit relatively consistent and characteristic symptoms of anaphylactic shock, is injected with sensitizing antigen. Extremely small doses are required to bring about the subsequent hypersensitivity, which becomes evident after about 10 to 14 days. A second, usually much larger dose of homologous antigen will then bring about anaphylactic shock in most of the animals. They normally go into convulsions, while displaying a characteristic set of symptoms, and die fairly soon. Some sensitized animals do not show the characteristic shock reaction, however. Sera to be used for sensitization must be heated to 55–60° for 30 min so that it does not cause a reaction in the animals. And the animals must be given injections which are as close to their body temperature as possible, or the symptoms very similar to anaphylaxis may result and mislead the observer (Sutherland, 1910). Rosenau and Anderson (1907, 1908 and 1909) conducted extensive experiments on the anaphylaxis, hypersusceptibility phenomenon. Among other things, they could show that a variety of proteinaceous substances would cause anaphylaxis in guinea pigs, that maternal hypersensitivity was transferred to the fetus whether sensitization had occurred prior to or after conception, that the active principle in horse serum causing sensitization was inactivated by heating to 100° for 1 hr, that exceedingly small doses (0.1 $\mu\ell$) of sensitizing antigen were required if administered intracranially, and that dessicated proteinaceous matter worked equally as well in inducing hypersensitivity as the fresh substances.

Thomsen (1909) could show that bloodstain extracts were completely suitable as sensitizing antigens. The reaction was not completely specific, however, in that some monkey sera could cause the reaction in animals sensitized with human serum. Pfeiffer (1909) showed that guinea pigs which have been hypersensitized to an antigen show a marked decrease in body temperature upon administration of the shocking injection, even if the other symptoms of anaphylaxis are not present. This temperature change, he believed, should be the sole criterion for judging the presence of the reaction in an animal in medico-legal work (Pfeiffer, 1910). Uhlenhuth and Haendel (in Uhlenhuth and Weidanz, 1909) showed that even old material could cause hypersensitivity in test animals. A fourteen year old, decomposed bloodstain, which would not give a precipitin reaction, gave a positive anaphylaxis test. Sutherland (1910) pointed out that, were it possible to hypersensitize the test animals in advance and have them sitting around at the ready, and then simply test bloodstain extracts as they were received in the laboratory, some of the objections to the test's inconvenience would be answered. Unfortunately, the shocking dose must be quite large, and there is seldom enough bloodstain to produce a sufficiently large amount of extract for the purpose. Since the sensitizing dose can be very minute, the bloodstain extract must normally be used in this way, necessitating the 10 to 14 day waiting period. Minet and Leclercq (1911) conducted extensive experiments on the technique with bloodstained materials. Stains on a variety of substrates were tested, and a number of chemicals, including ammonia, potassium permanganate and phenol, were mixed with bloodstains before testing. In all cases, the reaction took place. Stains 12 and 20 years old gave results that were indistinguishable from fresh blood, while a 28 year old stain gave a somewhat less intense reaction. They recommended that Pfeiffer's temperature decrease criterion be used in determining the presence or absence of the reaction. The technique was recommended for medico-legal investigations, and particularly in situations where the precipitin and complement fixation tests would not give a result. Pfeiffer (1938) discussed the method in some detail in his review, and concluded that the technique should be used where other, simpler ones would not suffice. Olbrycht (1950) noted in his review that the method was prohibitively cumbersome, and also said that the judgment of the presence of the anaphylactic reaction was too subjective, even if the body temperature decrease is used as the criterion.

16.6.3 Hemolysins

Hemolysins are antibodies which bring about the destructive lysis of the red cells which contain the homologous antigen. In 1869, Creite observed that if an animal received a transfusion of foreign blood, it developed hemoglobinuria. He also noted that the animal's red cells were dissolved (lysed) by foreign serum, but he apparently did not appreciate the relationship between the two observations. Landois

(1875) carried out extensive experiments on the transfusion of blood from one animal to another. Transfusions between unrelated species were always accompanied by hemoglobinuria in the recipient animal, and Landois recognized that this was the result of hemolysis brought about by the donor serum. In transfusions between closely related species, these results were not seen. In 1898, Belfanti and Carbone showed that artificial hemolysins could be produced by immunization of an animal with the blood of another animal, and that they were specific for the red cells of the animal whose blood had been used for immunization. Artificially produced hemolysins are used in the complement fixation assays described in sections 16.6.1 and 1.3.5.3. In 1901, Deutsch proposed the direct use of hemolytic antisera for the diagnosis of species of origin of bloodstains. The method relied on the lysis of the red cells in the bloodstain by the antiserum. Hemolysins could be prepared against any desired animal or human red cell, and complete hemolysis was obtained within 24 hrs of incubation of homologous bloodstain with antiserum. Normal serum, used as a control did not bring about hemolysis. The difficulty with such a method is in judging the degree of hemolysis which has occurred in a dried bloodstain. In a red cell suspension, it is relatively easy to assess hemolysis, but in bloodstains, which may already be partially hemolyzed, there are many problems in interpretation. Nuttall (1904) noted that he thought there were many potential sources of error in this method, and that it would be better to use the more reliable precipitin test. The subject was briefly reviewed by Sutherland (1907) and does not appear to have been pursued.

16.6.4 Agglutinins

When the serum of an animal is mixed with the red cells of another, not too closely related animal, the cells are agglutinated. Sutherland (1907) attributed the initial observation of this phenomenon to Landois in 1890. In 1904, Marx and Ehrnrooth attempted to devise a species test for bloodstains based upon this principle. A saline extract of a nonhuman, mammalian bloodstain would cause fresh human cells to be agglutinated, while with an extract of a human bloodstain, there was either no effect or else rouleaux formation occurred. They thought that the isoagglutinins of human serum did not persist in an active state in bloodstains longer than 1 month. In cases of bloodstains less than a month old, they said that a drop of saline diluted human serum, obtained by allowing a saline diluted drop of human blood to settle for 24 hrs, should be added to the text mixture. This addition would cause the clumping by homologous isoagglutinins to become more marked, while it would tend to break up the aggregates formed by heterologous agglutinins. They said that the test would be a useful preliminary one, to be carried out prior to doing the precipitin test. Pfeiffer (1904) obtained positive results with the test on bloodstains on wood and linen that were 37 and 24 years old, respectively, and felt that it was a good preliminary test. De Domincis (1904) found that heating bloodstains to 150° prevented the reaction, and he obtained false positive reactions with a number of body fluid substances other than blood. Carrara (1904) looked into the technique. He found that the agglutinins were inactivated by a few minutes exposure to 70°. He also said that saturated borax solutions or Paccini's solution (see Section 5.3) could be used to extract agglutinins from bloodstains that had become insoluble. Martin (1905) carried out extensive experiments on the test with a number of different animal bloods, and concluded that it was not reliable and should not be used in forensic practice. Sutherland (1907), having gotten agglutination of fresh human cells with an extract of human menstrual blood, and having failed to get it with a number of animal bloodstain extracts, agreed with Martin.

Leers, in his 1910 monograph, described his own procedure for carrying out the test, but emphasized that it was not to be regarded as a substitute for the precipitin test, and that it was necessary to use proper controls. Most authorities agreed that a negative test was noninterpretable, because it could not be known whether agglutination had failed to occur because the blood was human, or because the agglutinins had become inactive in a stain that was in fact of animal origin. Baecchi (1910a and 1910b) took a look at the issue of the presence of human isoagglutinins in the stains. Marx and Ehrnrooth had originally suggested that the isoagglutinins of human blood deteriorate faster in bloodstains than do the heteroagglutinins in animal bloodstains. Further, they suggested that the two could be distinguished on the basis of differences in agglutination behavior. Baecchi recognized the problem, and said that one should carry out the test with a number of examples of human red cells. If these were all agglutinated uniformly, he said, the probability of the stain having contained heteroagglutinins, and therefore, of being of animal origin, would be very much increased. Lattes (1913) took up the problem of distinguishing between the isoagglutinins and the heteroagglutinins in this procedure, and appears to have been the first investigator to have suggested a simple method for it based on the principles governing isoagglutination in human blood (see section 19 for full discussion). His objection to Baecchi's approach was that it was "blind", in that no account was taken of the blood group of the test cells used. False positive results in the test, caused by the presence of human isoagglutinins in human bloodstains, could be avoided, Lattes said, by the very simple expedient of using group O test cells. Under these conditions, he noted, the test could be of value provided it gave positive results. Negative results could not be interpreted. He felt that the test could be used to screen samples for a subsequent precipitin test, and in addition, could serve to indicate possible human-animal blood mixtures in the stain in cases where the agglutination test was positive and the precipitin test with anti-human serum was also positive.

16.6.5 Serum-hemoglobin precipitation

In 1967, Kimura published a series of papers on the precipitin reaction of human hemoglobin with human serum. The reaction was first observed in Ouchterlony gels, and was noted to be independent of the ABO group (1967a). A series

of experiments was conducted with a number of animal sera and hemoglobins as well as with human serum and Hb. The only homologous reaction besides the human one which occurred was that of monkey hemoglobin with monkey serum, and it was weaker than the human reaction. Human serum reacted weakly with monkey and rabbit hemoglobins (1967b). It could be shown by immunoelectrophoresis that hemoglobin was reacting with the albumin of the serum (1967c). A medico-legal application of the phenomenon was proposed (1967d) in which human serum was used as test reagent, the bloodstain serving as the source of hemoglobin. Extracts of 6 months old human bloodstains gave the test with serum dilutions of up to 1:16.

In 1971, Hartmann and Oepen said that they had tested many combinations of sera and hemoglobin, and could not confirm Kimura's findings. They said that the precipitin reactions between serum or albumin and hemoglobin were nonspecific and could be avoided in immunodiffusion tests by keeping the distance between wells less than 6 mm and the temperature lower than 20°. Human serum would react with a number of animal hemoglobins in their experiments, and they said that the method was of no value in diagnosing species of origin.

16.6.6 Phytoprecipitin and phytagglutination methods

That the seeds of certain plants contain agglutinins for red cells has been known for a long time. In 1888, Stillmark found that aqueous extracts of *Ricinus communis* were hemagglutinating (Gold and Balding, 1975). Plant extracts which agglutinate red cells are usually called lectins, or phytagglutinins. More recently, the term "receptor specific proteins" has been introduced, and includes agglutinins from animal sources (protectins) as well. This subject is now quite complicated and its literature immense. More is said about lectins in section 19 (ABO system). Some plant extracts can also cause precipitation of animal sera. These are called phytoprecipitins.

In 1963 Haferland reported that aqueous extracts of *Bryophyllum diagremontienum* contained a phytoprecipitin specific for human serum. There was a weak reaction with some monkey sera. In 1964, he showed that the precipitin detected human serum in bloodstains up to 5 years old on a number of substrata by the agar gel diffusion test. In one instance, the phytagglutinin reacted when anti-human serum did not. Haferland recommended the phytoprecipitin for medico-legal species diagnosis.

In 1963, Raszeja reported that the phytagglutinin from the mushroom *Laccharia laccata Berk* was specifically inhibited by human serum, as well as by human saliva, semen and milk. The sera of 35 animals showed no inhibition. This principle was proposed as the basis of a forensic species diagnosis test, and an extract of a 36 year old human bloodstain showed the inhibition reaction. The lectin has an anti-H specificity, and it was found in subsequent studies that "anthropomorphic" monkey (e.g. chimpanzee) sera also cause inhibition, but that the sera of "zoomorphic" monkeys (e.g. Rhesus monkey) do not (Raszeja, 1966).

In 1974, Bhatia conducted a number of experiments on phytagglutinins from a number of plants with the blood of a number of different animals. Three lectins were specific for guinea pig blood, while five others gave a combination of reactions, the patterns of which were distinctive for cow, chicken, sheep, rabbit, frog and rat bloods. Bloodstains were not studied in these experiments.

Tumosa (1976) looked at the reactions of the red cells of 37 different species with the "anti-A_1" lectin from *Dolichos biflorus,* and found that only those of the barasinga, chimpanzee, rhinoceros and wallaroo reacted. It has been appreciated for some time that the ABH blood group receptors of human red cells are actually widely distributed surface structures in many different living things. The matter is discussed in more detail in appropriate parts of section 19. In this respect, Tumosa (1977) has found that a variety of animal bloods are fully reactive with anti-A, anti-B and with anti-H.

Chowdhuri *et al.* (1975) looked at the phytoprecipitin activity in 50 different plant seed extracts against human and a variety of animal sera. 25 plant extracts showed phytoprecipitin activity, and many of these were nonspecific. Two extracts were specific for horse serum, one for chicken serum, one for goat serum and another for dog serum. Four lectins reacted with human and monkey sera.

16.6.7 Gamma-globulin deviation

In 1957, Ambrosi attempted to take advantage of the fact that antibodies are present in the γ-globulin fraction of serum, proposing that a reduction in the γ-globulin fraction of the antiserum after incubation with homologous antigen could serve as an indication that the antigen-antibody reaction had occurred. The quantities of γ-globulin present before and after the reaction were detected by paper electrophoresis. Although this method is not, strictly speaking, purely immunological, it is no less appropriately presented in this section than in a separate one dealing with electrophoresis. Ambrosi said that a consistent reduction in γ-globulin was observed when small aliquots of antisera were tested following incubation with 1:500 dilutions of homologous bloodstain extracts. No reduction was seen with heterologous antigen. The absolute value of the reduction varied in the homologous case, but was never less than 14.7%. Schleyer (1962) said that Ambrosi's paper electrophoresis experiments had not been carried out very rigorously, and that Hilgermann, working in his (Schleyer's) laboratory in 1960, had utterly failed to confirm Ambrosi's results.

16.6.8 Fluorescent antibody technique

In 1968, Gajos reported that he had conducted exploratory experiments on the applicability of fluorescent-labelled antibody technique to medico-legal immunology. The results were not very satisfactory, one of the problems being that many fabrics contain fluorescent "brightening" substances which have similar emission maxima. He believed further work would be required to render the technique applicable to

forensic problems, such as that of species of origin determination.

16.7 Immunoelectrophoresis

Immunoelectrophoresis was first described by Grabar and Williams in 1953, who applied the technique to the separation and characterization of human serum proteins. Since that time, it has become a standard method of immunological investigation (see Grabar and Burtin, 1964 and section 2.4). An important development was Scheidegger's (1955) introduction of a micro-immunoelectrophoretic method, which was widely adopted and modified by many workers. Immunoelectrophoresis has not been widely employed as a means of species determination. Muller and Fontaine (1960) explored the possibility, but said that the method was not inherently superior to other gel methods, and that the specificity of the test was a function of the specificity of the antiserum. Schleyer (1962) gave a detailed description of a method which he had devised in collaboration with Schneider. He agreed with Muller and Fontaine in noting that the specificity of the antiserum was the critical feature in determining the specificity of the test.

16.8 Cross Reactions of Antisera—The Problem of Closely Related Species

It has been recognized since the earliest experiments with precipitating antibodies that antisera to a particular protein cross-reacted with closely related proteins. This fact was clear from the studies on the reactions of anti-hen ovalbumin with duck ovalbumin (Myers, 1900; Uhlenhuth, 1900). Very soon after the precipitin test for medico-legal diagnosis of species was proposed, it was clear that antisera to a particular species cross reacted with the sera of closely related species, as for example, anti-ram sera with goat and cow blood (Uhlenhuth, 1901e) and anti-human serum with monkey bloods (Nuttall and Dinkelspiel, 1901a and 1901b; Frenkel, 1901; Stern, 1901). The cross reactions of anti-human serum have been a matter of particular concern to medico-legal investigators ever since that time. In some countries, which have indigenous monkey and other primate species, and where these bloods may be present in exhibits submitted for examination, the problem is a serious one. In this country and in Europe, it is improbable that such cross reacting primate bloods would be encountered.

A number of approaches have been taken to find ways of differentiating the bloods of closely related species, particularly of humans and primates. Among these are selective absorption of antisera with the cross-reacting antigens (Weichardt, 1905), so-called cross-immunization ("kreuzweise immunisierung") (Uhlenhuth, 1905), and various techniques for carefully estimating the quantity of precipitate obtained. By cross immunization is meant the preparation of antibodies to the proteins of a particular species by immunization of a member of the closely-related, cross-reacting species.

Welsh and Chapman (1910) indicated that they could immunologically differentiate the ovalbumins of closely related species by carefully determining the weight of the precipitates under carefully controlled conditions. Berkeley (1913) could not confirm Uhlenhuth's contention that specific precipitins to human proteins could be prepared in monkeys. They were unable to raise precipitin antisera to humans in two species of monkeys, although it is now known that this can certainly be done. Fujiwara (1922b) said that human and monkey bloods could be differentiated by the complement fixation test using anti-human serum absorbed with monkey blood.

In more recent times, a good deal of work was done on the problem by the South African forensic scientists, the differentiation of human and primate bloods being a serious problem in their practice. In 1952, Taylor published a detailed paper on the subject, in which the standards of potency and specificity for antisera as stated by many authorities were reviewed. In addition, the various techniques for attempting to render antisera specific for a single species were reviewed, and a number of experiments conducted. He had some success with anti-human sera prepared in baboons and vervet monkeys, but none of the antisera could differentiate human blood from that of the chimpanzee, the most closely related primate. Shapiro (1954) flatly stated that Taylor's experiments had conclusively shown that human blood could not be differentiated from that of the most closely related primates. Gradwohl (1956) tested anti-human serum with the sera of several monkey species and a small baboon species with negative results. The antiserum did react to chimpanzee, gorilla and orangutan sera though. An anti-chimpanzee reacted with gorilla, orangutan and human sera, as did an anti-orangutan preparation. He did not think there was any way to distinguish these species by means of the precipitin test. Vagnina (1955) discussed the problem of the differentiation of a closely related species, and said that Dr. Boyden had told him that it was indeed possible to distinguish closely related species by precipitin tests, but not using the methods employed by most medico-legal investigators. A photoelectric turbidity measuring system had to be employed, and antisera selected with very great care. Boyden and DeFalco (1943) and Boyden *et al.* (1947) described their technique in detail. An antigen was titrated to its endpoint with very carefully selected antiserum, the turbidity being measured in each dilution from the prozone, through the equivalence zone, and into the postzone with the Libby photronreflectometer. Curves can then be constructed in which tube number (in order of increasing antigen concentration) was plotted against turbidity units. The areas under these curves, which vary both in area and in shape with homologous vs heterologous antigens, are proportional to the summated turbidities in the titration series. The summed turbidity value is sometimes referred to as "relative area". This type of analysis is sensitive to very small differences in antigen structure, and could differentiate between closely related antigens, including human and chimpanzee serum. A representative set of results from Boyden

(1958) is shown in Fig. 16.4. The turbidity determinations were shown to correlate well with other methods of quantitative determination of precipitin reactions, such as gravimetry and total antibody nitrogen. Gravimetry, the accurate measurement of the mass of the precipitate, suffers from the disadvantage that combining valencies of antibodies may differ. Determination of total antibody nitrogen is accurate, but more complicated than turbidimetry (Kwapinski, 1972).

In 1955, Coetzee proposed an interesting, if complex technique which was capable of differentiating human from chimpanzee serum. The method employs sensitized tanned red cells as indicator cells (see section 16.3), and is based on the principle that homologous antigen should be able to annul its antiserum, while heterologous antigen (even if closely related) should not be able to do so to the same extent. Another way of putting it is that the failure of species X proteins to annul anti-species Y antiserum shows that X and Y are, in fact, different. The test demonstrates non-identity, although it could be used to establish identity if enough experiments were carred out. The test was arranged as follows: serial dilutions of human serum in one set of tubes, and serial dilutions of chimpanzee serum in another, were incubated with serial dilutions of anti-human globulin (AHG) serum. After a time, tanned red cells, sensitized with human serum globulin, were added to all the tubes, and agglutination read after a suitable interval. A set of results is shown in Table 16.1. In a similar way, beef and horse meat extracts could be readily distinguished. In 1958, Coetzee showed that the technique was applicable to bloodstain extracts, and that human and chimpanzee bloodstains could be differentiated from one another (i.e., chimpanzee serum could be shown to be non-identical with human) as had been done in the case of the sera.

Lichter and Dray's studies (1964) indicated that anti-human sera prepared in chimpanzees and in Rhesus monkeys could distinguish a number of human-specific serum proteins. Gempel *et al.* (1960) could differentiate human and monkey sera using an anti-human serum prepared in the monkey. The antiserum was apparently of very low potency, however, and the reaction was very slow. Using anti-human and anti-monkey sera prepared in rabbits, and absorbed with heterologous serum, it was possible to differentiate between human and monkey sera on the basis of the number and structure of precipitin lines in Ouchterlony gels (see section 2.2.2). Bloodstains were not studied in the course of these experiments. Sivaram *et al.* (1975) indicated that monkey blood could be distinguished from human blood using a very similar sort of analysis of the Ouchterlony diffusion precipitin bands, but employing a very potent anti-human globulin serum.

Grobellar *et al.* (1970b) utilized the AHG inhibition test (section 16.2) to differentiate between human and primate sera. AHG serum was prepared in goats, and with selected examples of the reagent, no inhibition was observed with primate sera at dilutions greater than 1:50 while human serum gave inhibition at dilutions of 1:1000. The test was

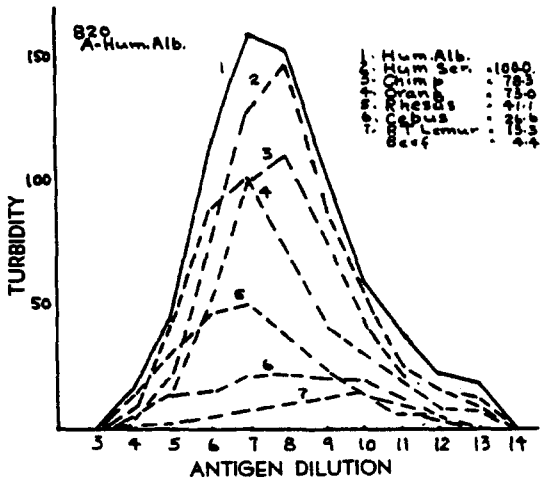

Figure 16.4 Quantitative Precipitin Analysis by Reflectrometry. Reactions of an anti-human albumin serum with a series of primate sera. From "Comparative Serology: Aims, Methods and Results" by Alan Boyden, in: *Serological and Biochemical Comparisons of Proteins,* ed. by William H. Cole. Copyright © 1958 by Rutgers, The State University. Reprinted by permission of Rutgers University Press.

Table 16.1 Results of the Application of the Coetzee Method to Human and Chimpanzee Sera (after Coetzee, 1955)

Dilutions of human/chimpanzee sera (reciprocal)	Dilutions of anti-human globulin serum (reciprocal)											
	3	6	12	24	48	96	192	384	768	1536	3072	C
4	c	c	c	c	c	c	c	—	—	—	—	—
8	+	c	c	c	c	c	c	—	—	—	—	—
16	+	+	c	c	c	c	c	c	—	—	—	—
32	+	+	c	c	c	c	c	c	—	—	—	—
64	+	+	+	c	c	c	c	c	c	—	—	—
128	+	+	+	+	c	c	c	c	c	c	—	—
512	+	+	+	+	+	+	c	c	c	c	c	—
1024	+	+	+	+	+	+	+	c	c	c	c	—
2048	+	+	+	+	+	+	+	+	c	c	c	—
C	+	+	+	+	+	+	+	+	+	+	+	—

Key to abbreviations in figure: + = agglutination with both human and chimpanzee sera; c = agglutination only with chimpanzee serum; — = no agglutination; C = control (normal rabbit serum).

recommended for medico-legal species diagnosis in South Africa.

Čarny (1969) recommended immunoelectrophoresis as a means of discriminating between the bloods of closely related animals.

16.9 Serum Protein Structure and Phylogeny—Taxonomic Serology and Immunology

Structural similarities in the proteins of different species, and their corresponding immunological cross reactivity are a reflection of phylogeny. This fact was recognized almost simultaneously with the discovery of precipitins for blood proteins. The undisputed pioneer in this field was G. H. F. Nuttall. In 1901, he was preparing antisera to scores of animal bloods, and recognized the fundamental biological importance of immunological cross reactivity as a delicate measure of protein structural similarities (Nuttall, 1901b). In the course of several years time, antisera were prepared by Nuttall and his collaborators against hundreds of animal bloods. These were tested with homologous and heterologous sera in a systematic fashion. The results were obviously complex, and took up dozens of tables. They cannot be readily summarized. All the material is collected in Nuttall's classic work, *Blood Immunity and Blood Relationship,* which appeared in 1904. The book additionally provided a detailed review of immunological investigations up to the time, and included a chapter on medico-legal species differentiation by the precipitin test.

More recently, quantitative techniques have been employed in comparative immunology and serology. These were reviewed by Boyden (1958). The reflectometric procedure for assessing the quantity of precipitate formed in the precipitin reaction was discussed in the foregoing section. Boyden said that with appropriate quantitative precipitin technique and careful selection of antisera, there was no reason for being unable to differentiate very closely related species in forensic medicine, including human and chimpanzee blood. He said that the usual technique employed in medico-legal investigations, the use of antiserum against whole serum rather than a specific protein, and the use of a single concentration of antigen and antibody for the test, was quite crude. He did not find particularly astonishing the fact that a number of medico-legal authorities had reported their inability to differentiate closely related species by the precipitin test. The use of quantitative immunological reactions to establish taxonomic relationships was discussed in the review as well, and some data presented. Readers interested in this subject are also directed to Leone (1964), which contains articles by many specialists on all the varied aspects of taxonomic serology and immunology.

Sensabaugh (1975) pointed out the intimate connection between immunological cross reactivity of those proteins which have evolved rather slowly, and the medico-legal differentiation of species of origin. Depending on the discriminatory capability of the technique being employed, the phylogenetic relationships between related species impose intrinsic limits on the ability to differentiate related bloods. Medico-legal tests would be far more satisfactory in these terms, he said, if monospecific antisera to the more rapidly evolving proteins were employed, thereby taking advantage of greater differences in the structure of similar proteins, even in very closely related species.

In the full paper on the subject, Sensabaugh (1976) noted that cross reactions between the proteins of closely related species are an inevitable consequence of evolution. Cross reactivity can be turned to advantage in medico-legal investigations, because of the known relationship between degree of cross reactivity and species relatedness. Proteins must differ from one another in sequence by about 40% of their residues in order to be completely non-cross reactive to an antiserum to one or the other. In discriminating between primates, anti-Ig gamma chain and anti-Ig kappa chain sera were found to be much better than antisera to albumin or transferrin. Sensabaugh noted further that different examples of commercial "anti-human" serum contained antibodies against different serum proteins, and that unless such characteristics of an antiserum are known, it is difficult to make the most productive use of a species test or indeed to select the most suitable antiserum.

Bauer (1969, 1970a, 1970b and 1970c) has carried out extensive studies of human and animal serum proteins using immunological methods with the objective of determining phylogenetic relationships among the species. Particular proteins could be placed into what he called "immunological evolution groups" based upon their cross reactivity, a direct measure of the speed with which they have evolved.

SECTION 17. Other Methods for Species Determination of Blood and Bloodstains, Body Fluids and Tissues

17.1 Differential Denaturation of Hemoglobin with Alkali

Although the absorption spectrum of hemoglobin in various species does not differ greatly, the rate at which the molecule denatures in alkali is decidedly species-dependent. Krüger observed this phenomenon in 1887. Magnanimi (1898) applied the principle to bloodstains, and thought it had forensic applicability. Zeimke (1901c) confirmed many of the previous findings, likewise recommending the method for medico-legal examinations. Krüger had another extensive paper on the subject in 1925, although he had no interest in forensic blood investigations. Schleyer (1962) quoted the Russian authors Blumenfeld and Krasovickaja as having said in 1955 that human blood could be readily distinguished from a number of animal bloods by following the rate of alkali denaturation of hemoglobin spectrophotometrically. Analogous results were obtained with bloodstain extracts. Their observations were generally in agreement with those of the earlier investigators. Human hemoglobin denatured at the fastest rate (1–2 min), followed by cat Hb (6–7 min) and dog Hb (12–14 min). Fowl, goat, ox and sheep hemoglobins do not significantly denature in alkali after an hour. Schleyer conducted a number of experiments with this technique in 1961, and summarized his results in the 1962 review. It could be confirmed that human Hb denatured faster than that of any animal hemoglobin tested. He said, in addition, that the pattern of denaturation might actually be more characteristic of the species than the rate. Schleyer thought that the technique was primarily applicable to fresh blood or to very fresh bloodstains. The alkali denaturation curves are characteristic for whole blood dilutions, but not for pure solutions of hemoglobin. Human hemoglobin is not denaturable by alkali at blood dilutions greater than 1:950.

It must be noted that fetal hemoglobin (Hb F) is quite resistant to alkali denaturation. This fact was used as a basis for differentiating Hb F from adult hemoglobin (Hb A) prior to the development of electrophoretic techniques (see section 8.3.1). The presence of Hb F in a bloodstain could cause a grave error if the alkali denaturation technique for species differentiation were being applied. Hb F is further discussed in section 38.

17.2 The fibrin plate method

The fibrinolytic system was discussed in section 8.1.2 (and see Figure 8.1). The work of Mullertz and Lassen (1953) on the fibrinolytic system had indicated that human serum contains substantially larger amounts of proactivator than most animal sera. On the strength of this observation, Szöllösy and Rengei in 1959 and 1960 devised a species of origin test based on fibrinolysis (Rengei and Szöllösy, 1959; Rengei, 1960; Szöllösy and Rengei, 1960). A fibrin plate was prepared using bovine fibrinogen in buffer in the presence of a small amount of thrombin. This mixture was allowed to clot and the surface then dried in a 37° incubator. A small quantity of streptokinase was mixed with a buffer extract of a bloodstain, and a drop of this mixture applied to the surface of the fibrin plate. Streptokinase is an artificial activator of proactivator, and its addition to the serum in the bloodstain sets in motion the fibrinolytic machinery. After a suitable incubation period, a marked lytic area develops in the fibrin plate if the extract was from a bloodstain of human origin. The authors claimed absolute specificity for the method, as well as a sensitivity of 0.3 μg human serum protein. Positive results were obtained with bloodstains on various substrata, including iron and glass, which were up to 8 months old, even ones which had been kept at 56°. It was noted that human milk and tears would give the fibrinolytic reaction as well. In 1962, Kumano utilized the technique, and said that it was sensitive and specific. He got a positive result with a 30 year old bloodstain, and on stains which had been exposed to 100° heat for an hour, or washed. Putrefied liquid blood could not be diagnosed satisfactorily by this method.

Schleyer (1962) presented a description of a method which he had found to work well. The method was found not to be species-specific, however, if non-human sera were less dilute than 1:100. At serum dilutions of 1:1000, only human blood gave a positive reaction, and Schleyer noted that with this method, as with the precipitin test, it was important to employ dilute (1:1000) bloodstain extracts to insure specificity. Mohri (1963) confirmed the results of previous workers, and Morioka (1965) said that putrefaction destroyed the fibrinolytic activity of blood steadily over the course of a month. Mikami *et al.* (1966) recommended the technique for species determination in small fibers of bloodstained material, to which they said they could consecutively apply a catalytic test, the fibrin plate test, and a mixed agglutination technique for the determination of the blood group. Akaishi (1965) noted that the test was sensitive to a whole blood dilution of 1:25,600. Hirose (1976), in his studies of the effects of rust on dried blood (see section 5.1 and Table 5.2), said that the precipitin test remained positive for 51 days after the mixing of the blood with the iron only if pH 9.4 buffer solution was used for extraction. The fibrin plate test was barely positive after 51 days using pH 9.4 buffer as extraction medium, and it became negative at 7 days if saline was used.

17.3 Hemoglobin Separation by Chromatographic and Electrophoretic Methods

Some efforts have been made to apply paper chromatography or gel electrophoresis to the diagnosis of species by separation of the hemoglobins. Fine *et al.* (1956) got separation of the hemoglobins from distantly related species using gel electrophoresis, but the hemoglobins of most mammals tended to migrate about the same distance. These observations were largely confirmed by Depieds *et al.* (1960). Fiori (1957) tried paper chromatography of hemoglobins, and tested a large number of different solvent systems. He could not get satisfactory differentiation of human hemoglobin from that of other species. Vidoni and Marenghi (1957) tried to distinguish blood species on the basis of the patterns obtained by paper chromatography. Schleyer (1962) said that he and Schneider were unable to reproduce these results. Santini (1960) applied circular paper chromatography to the separation of human and animal hemoglobins. He said that a specific, reproducible R_f of 0.72 could be obtained for human hemoglobin using this technique. Berg (1967) said that the hemoglobin electrophoresis and chromatographic methods for species determination were unreliable, and that other techniques should be used.

17.4 Isoenzyme Patterns

In 1962, Samico *et al.* reported that aqueous extracts of stains of human, cat, ox, sheep, goat, pig, turkey, chicken, rat, monkey, possum and toad bloods could be differentiated on the basis of their esterase profiles following starch gel electrophoresis and specific staining for esterase activity. Thick starch gels were employed for the separations, and staining effected with α-naphthyl acetate or α-naphthyl butyrate and Fast Blue RR salt. The banding patterns were different with each animal tested, and the results were better with the butyrate ester. The single band from human bloodstains was due to cholinesterase activity. This band persisted in bloodstains for 210 days if extraction was carried out for 30 min with water, but if extraction were done at 37° for 24 hrs, the band persisted to 310 days.

Madiwale *et al.* (1972a) tested blood samples from bullock, buffalo, sheep, goat, rabbit and human sources for lactic dehydrogenase and malic dehydrogenase activity band patterns by polyacrylamide disc gel electrophoresis. The patterns were reproducible, and different for the various animals. Bloodstain extracts gave similar results (1972b), and the method was considered very sensitive, only about 1 $\mu\ell$ of blood being required.

Some of the isoenzymes which are under the genetic control of polymorphic loci in human beings are found in certain animal bloods. The various human isoenzymes are discussed in Unit VI. In cases where animal blood isoenzyme patterns are consistent, and differentiable from human patterns, electrophoretic enzyme determination might offer a method of helping to identify the species of origin of bloods. Gallango and Suinaga (1979) studied the red cell enzyme UMPK (see in section 37.12) in a series of vertebrate bloods. Herr (1979) showed that a number of differences existed in the red cell acid phosphatase (see in section 29) enzymes of several animal species as compared with the human isozymes. Herr and Konzak (1980) suggested that these ACP patterns, as well as those of red cell PGM and AK (see in sections 27 and 28), might be helpful in differentiating species of origin.

17.5 Species Diagnosis in Other Body Fluids and in Tissues

It is not often necessary to carry out species tests on body fluids other than blood. In theory, the immunological tests could be applied for the purpose to any body fluid in the same way as they are used for blood, provided that specific antisera were available. The preparation and use of antisera to semen has been discussed in a previous section (10.3), as has the preparation and use of antisera to saliva (11.4). There do not seem to have been extensive studies on the species-specificity of these antisera. Most of the concern was with cross reactions with other body fluids, since the objective of the immunological tests was primarily identification of the body fluid.

Popielski *et al.* (1963) said that the precipitin test could be carried out on extracts of urine stains provided these were subjected to a protein concentrating step in advance. Pathologically albuminous urine can be tested more easily.

Tissue samples are sometimes encountered, and must be tested for species of origin. The species of muscle tissue may be conveniently determined by the precipitin test, as has been known since the early work of Uhlenhuth (1901f).

The subject of tissue antigens is complex, and beyond the scope of the present discussion. The species of origin of tissues can be determined based on their presence, however, if appropriate antisera and techniques are available. Milgrom and Campbell (1970) reported on a case in which a piece of intestine and a piece of mesentery, found in a sewer in Niagara Falls, N.Y., were submitted for species determination. It was known from previous work (Milgrom *et al.*, 1964a and 1964b) that tissue contains species-specific antigens which were called "BE antigens", because of their resistance to boiling in water, and their solubility in ethanol. A rather complicated mixed agglutination technique, previously described by Tönder *et al.* (1964), for determination of these antigens was employed in the case. A rabbit antibody to the tissue antigen is first attached to the tissue antigen. Test cells are then prepared by sensitizing sheep red cells with rabbit anti-sheep erythrocyte antibodies. These cells are then treated with a goat anti-rabbit γ-globulin antibody, and the agglutinates which form are broken up. The test cells then have, in effect, one free end of the goat anti-rabbit γ-globulin antibody which can combine with the rabbit antibody attached to the tissue antigen, giving the mixed agglutination reaction. The tissue submitted in the case was determined to be pig intestine using the technique.

It may be noted in conclusion that the entire subject of species of origin determination was reviewed by Pfeiffer (1938), by Boyd (1946) and by Schleyer (1962).

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