SCHECTER, ROGER NORMAN. The Detection of Ceruloplasmin in Avian Plasma and It's Validity as a Biochemical Taxonomic Index (1971). Directed by: Dr. Herbert T. Hendrickson. Pp. 29

It was the purpose of this study to detect the copper-carrying protein ceruloplasmin by specific staining techniques after electrophoretic separation in polyacrylamide gel and then to assess its validity as a biochemical taxonomic index pertaining to avian classification.

Several procedures for the electrophoretic separation of this plasma protein were attempted as were several techniques for ceruloplasmin specific staining. The best procedure and technique of those attempted was derived with the use of human plasma. Avian plasma samples were then treated in the same manner.

It was the conclusion of this study, even though some positive results were recorded, that ceruloplasmin is an unusually elusive protein an avian plasma. Therefore in the present context, it cannot be used as a taxonomic index. Potentials do exist, however, for ceruloplasmins use as a taxonomic index in conjunction with other plasma proteins.

THE DETECTION OF CERULOPLASMIN IN AVIAN PLASMA AND ITS VALIDITY AS A BIOCHEMICAL TAXONOMIC INDEX

by

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A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree

Master of Arts

Greensboro
1971

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Acknowledgments

Sincere appreciation is expressed to Dr. Herbert T. Hendrickson whose guidance and encouragement greatly benefited the author. Appreciation is also extended to Dr. Bruce Eberhart and Dr. William Bates who shared with the author their knowledge and thoughts. Thanks is given to Dr. Andrew Long for his help with statistical suggestions.

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INTRODUCTION

The Validity of Biochemical Taxonomy

Taxonomy is a rather broad field of biology which attempts to classify organisms and arrange them into orderly groups dependent upon the type and degree of the relationships among them. In the classification of vertebrates, particularly birds, many aspects of the birds' entire make-up have been used as taxonomic traits and thus used to show relationships among the avian species. The study of the gross morphology of embryo and adult, paleontology, comparative physiology, comparative behavior, and comparative ecology has given information useful in attempting to classify birds. These areas point to the importance of function and form in taxonomy. Recently there has emerged a method of such classification utilizing information from yet another source: the comparative biochemical properties which an organism possesses. Biochemical taxonomy, or biochemical systematics as it is sometimes called, is a growing and productive discipline.

Crick (1958) was quoted as saying, "biologists should realize that before long we shall have a subject which might be called 'protein taxonomy'." As the result of today's progress in protein biochemistry, most studies in biochemical taxonomy have been done with proteins. The reasons for choosing proteins are logical and simple: (1) the information they carry (2) their intimate relationship with development and phenotypic expression. Proteins are recognized as a medium of information transfer between a genetic message encoded in

nuclear DNA and the processes involved in morphogenesis and function in an organism (Sibley, 1962). Genes, being the units of heredity, are passed through reproduction from individual to individual of the same species. Species are the basic units of taxonomy, and those species which are related morphologically are therefore related genetically (Mayr, 1963). In short, the phenotype is an observed expression of the genotype or genetic make-up of an organism. The rapid development in understanding the structural and functional basis of heredity and the significance of gene-protein relationships is the basis of the importance of studying protein molecules in taxonomy (Sibley, 1962).

The first reason for choosing proteins in the study of biochemical taxonomy is the information they carry. The genetic message is carried from generation to generation encoded in the sequence of genetic coding units, the nucleotides, of DNA. During the development of an organism, the specific sequence of these genetic coding units is translated into specific amino acid sequences of the thousands of differing proteins that determined the form and carry out the functions of an organism (Sibley, 1967). A more detailed discussion of this process than is necessary here can be found in Christian Anfinson's "Genes as Determinants of Protein Structure" and in Yanofsky's "Gene Structure and Protein Structure."

The second reason for choosing proteins is not mutually exclusive from the first--that reason being their intimate relationship with development and phenotypic expression. A gene is a particle of molecular dimensions located in a chromosome in a cell nucleus (or cytoplasm). Gene action therefore starts with intracellular processes which may subsequently be translated into chains of reactions

which culminate in the appearance of visible traits (Dobzhansky, 1951). Proteins are the principal morphological units of the animal body at the molecular level since specific proteins, formed from specific sequences of amino acids, in turn, are complexly joined to form muscles, bones, feathers, blood, organs, and other functional structures (Sibley, 1962). Again, detailed discussion of this process is not necessary for the present study.

Electrophoresis

The ideal method for measuring comparative properties for use in classification would be to actually determine the sequences of amino acids of homologous proteins from the different species being compared. However, current research indicates that available techniques for sequence determination are too slow, laborious and expensive to be practical in comparative studies involving many species (Sibley, 1962). Therefore, studies of whole proteins and protein systems by electrophoresis may be a more logical and informative approach. The primary structure of proteins is based upon the sequence of amino acids. The amino acid sequence for homologous proteins would be different in different species. This difference, even if it is just a difference in placement of two or three amino acids, can be determined by the use of electrophoresis (Sibley, 1967). Electrophoresis has been used extensively for the comparative study of protein systems of egg white (Sibley, 1960; Hendrickson, 1969) and blood plasma or serum (Sibley and Johnsgard, 1959; Smithies 1959a; Baker and Manwell, 1966; Baker and Hanson, 1966; Sibley and Hendrickson, 1970).

The principle of electrophoresis is basically simple; namely, that an electrically charged group will migrate toward one of the

electrodes when placed in an electric field. The net charge carried by a protein in a buffered solution is a function of the nature of the protein, referring to the charges on the amino acid groups, and the pH of the solution. The degree of this charge and the size and shape of the molecule determine the relative mobility of the protein through the electrophoretic media (Smithies, 1959b). Homologous proteins from different species theoretically having different net charges and therefore having different mobilities, can be separated in the media by electrophoresis and their respective mobilities can be compared. By using different combinations of buffers and ranges of pH, optimum conditions can be met for migration and separation.

Until recently, starch gel as an electrophoretic media had been more frequently used, but the use of polyacrylamide gels has become popular. Acrylamide gels give better resolution (Raymond and Wang, 1960), have a more uniform pore size than previously used media (Racusen and Calvanici, 1964), may be stored after staining for longer periods of time (Tombs, 1965), and are generally easier to prepare and work with than starch gels.

Ceruloplasmin

Plasma proteins comprise an interesting assemblage of molecules. They constitute an assemblage due to their common biosynthetic origin, their participation in common processes, and the fact that they occur as the major extracellular components in the circulatory system. Their functions as a group range from maintenance of colloid osmotic pressure, pH and electrolytic balance, to the transport of metallic ions. Early research interest in the plasma proteins was generated in response to disease; the proteins themselves fluctuate according

to body physiology (Putnam, 1965).

Studies of these proteins are not without problems. Their high molecular weights and complex structures make objective study difficult.

Ceruloplasmin is a cuproprotein enzymatically classified as a dehydrogenase (oxidoreductase), as it accepts hydrogen ions (H^+) (Mahler and Cordes, 1966). It is found in the α_2-globulin bands (Putnam, 1965), just behind the front running albumin (in relation to) gel electrophoresis. Its molecular weight has been estimated to be 151,000. Although it exists in multiple forms (Mason, 1965) eight copper atoms per mole are associated with this protein suggesting eight subunits linked together by disulfide bonds with each subunit containing an atom of copper (Kasper and Deutsch, 1963a). It has an isoelectric point of 4.4 (Putnam, 1965) and the major amino acids associated in the protein are aspartic acid, glutamic acid, threonine, glycine, and proline (Kasper and Deutsch, 1963a).

Since most of the copper in the body is incorporated in this copper protein complex names ceruloplasmin, much work has been done in describing the copper in the protein (Kasper and Deutsch, 1963b; Morell et al, 1966; Morell, 1968; Kasper, 1968). Copper exists in two valances within the ceruloplasmin complex, cupric (Cu^{++}) and cuprous (Cu^+). Cupric and cuprous concentrations are equimolar and since ceruloplasmin strongly absorbs light at the 260mμ level, four of the eight copper atoms are considered to be cupric (Blumberg and Eisinger, 1962) and thusly give the molecule its chromophoric properties (Mason, 1965). The stability of this cupric-cuprous complex has a great deal to do with the proper functioning of ceruloplasmin.

The cupric-cuprous atoms are held very precisely in the molecule in a geometric configuration between planar and tetrahedral, so no change of geometry is required during rapid electron transfer (Mason, 1965), a necessity in the transport of metal ions. The eight copper atoms of ceruloplasmin are confined to a very small part of the molecule in cupric-bridge-cuprous pairs which converge toward a common acceptor site and which also may be structurally retracted from the protein surface (Mason, 1965), in which case no activity will be observed. The cupric atoms of the pair are associated with the catalytic processes while the cuprous atoms probably function with some sort of substrate binding capacity (Kasper, 1968).

The stability of the molecule is of extreme importance in the techniques used to locate and stain ceruloplasmin. Buffers (Tris, EDTA) are able to effect changes in the molecule, particularly on its electrophoretic properties, i.e., mobility (Kasper and Deutsch, 1963a). The use of relatively old preparations of ceruloplasmin raises questions as to whether the properties recorded are those of native ceruloplasmin (Kasper and Deutsch, 1963a).

Purpose

As previously mentioned, most of the work with proteins in avian taxonomy has been done with protein systems, i.e., blood plasma, egg-white protein. It was thought to be of interest in the present study to determine if a single specific plasma protein, ceruloplasmin, could be used as a taxonomic index within itself, as has been done with hemoglobin (Sibley, et al, in prep.).

In determining if this were valid, the best techniques and procedures of those studied, for detecting ceruloplasmin after

electrophoretic separation were selected using human plasma, and then these procedures were applied to avian plasma. The results obtained from avian plasma were used to assess ceruloplasmin's usefulness as a taxonomic index. Therefore, the purpose of this study was twofold: (1) to describe the best method of those examined for detecting ceruloplasmin in avian plasma, and (2) to summarize ceruloplasmin's taxonomic significance.

Materials and Methods

Avian specimens used in this study were collected locally by the use of tiered, nylon mist nets at several stations around Greensboro. Gallus gallus specimens were donated by the Natural Science Center. Human blood was donated by the author and Dr. Herbert T. Hendrickson. Whole blood samples (1 to 5 cc according to body size) were taken with a syringe by heart puncture (except for human material), using EDTA (initially) or heparin (in later samples taken) as an anticoagulant. Whole blood samples were centrifuged at 2000 rpm's for 10 minutes. The supernatant (plasma and anticoagulant) was decanted, placed in a vial, labeled (collection number, date, collection location, species) and frozen immediately in boxes according to the specimen's respective Family. Red cells were washed with 1% NaCl solution and re-centrifuged at least three times for use in another study.

Vertical gel electrophoretic separation of the plasma proteins in a continuous buffer system was carried out in polyacrylamide gel with the use of an EC474 Vertical Gel Electrophoresis Apparatus from the E-C Apparatus Corporation. All technical specifications and running procedures for electrophoretic separation can be found in E-C Apparatus Corporation Technical Bulletin 128.

A total of 51 gels were run using different procedures to determine the best method for detecting ceruloplasmin in human and avian plasma. The gels differed in concentration (5% and 7%--see Appendix I), amount of plasma sample run (20 λ's to 40 λ's, 1 λ = 1 microliter), buffers and

pH's used (Tris-Na_2EDTA-Boric Acid pH 8.4; Tris Glycine, pH 9.3; Acetate, pH 5.7; see Appendix II), and staining techniques employed (Alizarin Blue S, Rubeanic Acid, and O-dianisidine, see Appendix III).

Spot tests were made to determine if Rubeanic Acid and O-dianisidine stains were reacting with ceruloplasmin outside the gel. These spot tests consisted of placing Rubeanic acid and O-dianisidine stain in a spot test plate and then adding plasma samples (20λ's and 40λ's). The reaction was positive if the correct colors were observed (greenish black for Rubeanic Acid and brown for O-dianisidine).

Analysis of the bands resultant from specific staining was made by using Rf values for visible components. These values are defined as:

$$Rf = \frac{\text{Distance of ceruloplasmin from application point}}{\text{Distance of Marker dye from application point}} \times 100.$$

Plasma samples contained Bromphenol Blue as standard indicator of mobility and it was also used as the Marker dye in the computation of Rf values.

The gels, after destaining, were wrapped in Saran Wrap, numbered, and stored at room temperature for later reference.

RESULTS

Human Plasma: Rubeanic Acid Stain

No positive results were recorded using Rubeanic Acid Stain (see Table I). Although bands did appear, they were faint brown, contrary to the greenish black bands stipulated (Decleir, 1961 as reported in Wieme, 1965).

Neither the buffer used, the pH of the buffer, the time of the run, nor the amount of sample had any effect on the results. All of these samples were collected using EDTA as an anticoagulant.

Human Plasma: O-dianisidine Stain

With the use of O-dianisidine Stain positive results were obtained (see Tables II and III). Some results were more clear and more reproducible than others.

The results are sub-divided according to the two staining procedures used: Method 1--Owen and Smith, 1968; Method 2--Jensen, 1963 (Appendix III).

Method 1 gave positive results but the bands were faint and not easily detected (see Table II). Acetate buffer, pH 5.7 with 5% gels and 4 hours running time gave the best results. The plasma used in these runs, depicted by Table II, was collected using either heparin or EDTA as an anticoagulant.

Method 2 gave the best results (see Table III). Again, Acetate buffer, pH 5.7 with 5% gels proved to be the best procedure for the run. The bands were dark and easily detected, positive results were

Buffer, pH	Gel Number	Gel Concentration	Run Time	Band Description	Comments
Tris-Na_2EDTA-Boric Acid, pH 8.4	A-92	5%	2 hrs.	(-) Faint Brown	40 λ Samples
	A-93	"	"	"	
Acetate, pH 5.7	A-118	5%	2½ hrs.	(-) Very Faint Brown	
	A-119	"	4½ hrs.	(-) Faint Brown	
Tris Glycine, pH 9.3	A-193	5%	2 hrs.	(-) No bands	Increased concentration of stain

TABLE I

Summary of Results from Human Plasma: Rubeanic Acid

Buffer, pH	Gel Number	Gel Concentration	Run Time	Band Description	Comments
Tris-Na_2EDTA - Boric Acid, pH 8.4	A-183	7%	2 hrs.	(-) No Bands	40% Samples
	A-184	"	"	"	40% Samples; increased concentration stain
	A-186	5%	"	(+) Faint Brown	Stain at application point only
	A-187	"	"	(-) No Bands	
	A-188	"	"	"	
Acetate, pH 5.7	A-200	5%	4 hrs.	(+) Very Faint Brown	Gel sliced; heparinized plasma
	A-201	"	"	"	"
	A-202	"	"	"	"
Tris Glycine, pH 9.3	A-197	5%	3 hrs.	(-) No Bands	Heparinized plasma

TABLE II

Summary of Results from Human Plasma: O-dianisidine, Method 1

Buffer, pH	Gel Number	Gel Concentration	Run Time	Band Description	Comments
Tris-Na_2EDTA-Boric Acid, pH 8.4	A-155	7%	2 hrs.	(-) No Band	Questionable due to position
	A-156	"	"	"	"
	A-157	"	"	(+) Faint Brown	"
	A-158	"	"	"	"
	A-159	"	3 3/4 hrs.	(+) Red Brown	Speckled bands in right position
	A-160	"	"	"	"
	A-161	"	2 hrs.	(+) Faint Brown	Mixed samples human/ Gallus plasma
	A-162	"	2½ hrs.	"	"
	A-163	"	2 hrs.	(-) No Bands	Increased concentration stain
	A-164	"	"	"	40λ Samples
Acetate, pH 5.7	A-199	5%	4 hrs.	(+) Orange Brown	Gel sliced, heparinized plasma
	A-200	"	"	"	"
	A-201	"	"	"	"
	A-202	"	"	"	Heparinized plasma
	A-205	"	2 hrs.	(+) Dark Orange Brown	"
	A-206	"	"	"	"
	A-207	"	"	"	"
	A-210	"	"	"	"
Tris Glycine, pH 9.3	A-197	5%	3 hrs.	(-) No Bands	Heparinized plasma
	A-198	"	"	"	"

TABLE III

Summary of Results from Human Plasma: O-dianisidine, Method 2

reproducible (A-199-A-210). The plasma used in the runs depicted in Table III was collected using heparin as an anticoagulant.

Human plasma was used to determine the best procedure and technique for detecting ceruloplasmin in electrophoretic separation because the staining procedures described in Appendix III were designed to detect human plasma.

According to the results collected in this study, the best method for detecting ceruloplasmin in human plasma samples is as follows:

> Acetate buffer, pH 5.7, 5% gel with a run time of 2 to 4 hours (200 volts, 4° to 5° C), using plasma collected in heparin; O-dianisidine staining procedures as described by Jensen (1963), (see Appendix III).

Now that the best method had been found, it was applied to avian plasma samples.

Avian Plasma: Rubeanic Acid

No positive results were recorded with Rubeanic Acid Stain and avian plasma. Runs were made with exactly the same procedures used with human plasma. (Refer to Table I). EDTA was used as an anticoagulant.

Avian Plasma: O-dianisidine

No positive results were recorded with O-dianisidine Stain (Method I) and avian plasma. Runs were made with exactly the same procedures used with human plasma (Refer to Tables II and III). EDTA and heparin were used as anticoagulants.

Positive results were recorded with O-dianisidine Stain (Method 2) and avian plasma. Gallus gallus specimen appeared as a streak and three other species, Zenaidura macroura, Colaptes auratus, and Toxos-

toma rufum, stained with very faint, light brown bands (see Table IV). The specimen were fresh (1 to 10 days old) and were collected with heparin. Only the previously mentioned species reacted to the stain although several other species were run, and the positive results were not always reproducible, i.e., sometimes they would not stain.

Many species of birds were run (see Appendix IV), but the only positive results recorded were those described above and in Table IV.

Spot Tests

Spot tests using Rubeanic Acid Stain and avian plasma were carried out with no conclusive results. Neither increasing the concentrations of Rubeanic Acid nor increasing the amount of plasma sample had any observable effect.

Spot tests using O-dianisidine (Method 1 and Method 2) and avian plasma were also attempted, again with no positive results. Neither increasing the concentration of O-dianisidine nor increasing the concentration of plasma sample had any detected effect.

The samples used in these spot tests contained Bromphenol Blue which may have disguised any positive reaction.

Due to the lack of positive results, Rf values could not be calculated, although there appeared to be no significant difference in ceruloplasmin mobilities among the four avian specimens that did stain.

Buffer, pH	Gel Number	Gel Concentration	Run Time	Band Description	Comments
Acetate, pH 5.7	A-201	5%	4 hrs.	Streak	only Gallus gallus, heparinized plasma
	A-202	"	2 hrs.	"	"
	A-205	"	2 hrs.	Very faint light brown	T. rufum (+), C. auratus (+), Z. macroura (+), Gallus run but did not stain
	A-206	"	"	"	T. rufum (+), Gallus did not stain
	A-207	"	"	"	T. rufum (+), C. auratus (+), Z. macroura (+)
	A-210	"	"	"	Gallus (+); T. rufum, C. auratus, Z. macroura did not stain

TABLE IV

Summary of Results from Avian Plasma: O-dianisidine, Method 2

Discussion and Conclusions

Baker and Hanson (1966) and Baker and Manwell (1966) attempted to identify ceruloplasmin in geese and pheasant sera respectively. In the former paper it was concluded that "ceruloplasmin could not be detected in geese sera by the o-dianisidine method." In the later paper it was reported that "occasionally ceruloplasmin cannot be found in pheasant sera, and even in the most favorable samples, the o-dianisidine oxidase activity of pheasant sera is much less than that of most mammalian sera." No attempt was made by these authors to outline possible explanations for their negative results.

Levine and Peisach (1963) reported that micromolar concentrations of EDTA, a chelating agent, inhibited ceruloplasmin oxidase activity. Consequently, the use of EDTA as an anticoagulant was abandoned in favor of heparin. A look at the difference in results of Table II and III shows that best results were obtained when heparin was used in human plasma but heparin had little effect on the reaction of avian plasma.

Kasper and Deutsch (1963a) reported that the buffers containing Tris were able to affect changes in ceruloplasmin, particularly in its electrophoretic properties. Therefore, Tris-Na_2EDTA-Boric Acid buffer was abandoned in favor of acetate. Again, reactions involving human plasma were more clear but the buffer change had little effect on avian results.

It might be pointed out, however, that the only avian plasma that

did give positive results was with the use of heparin and acetate buffer. The only deterant was the fact that these results were not reproducible.

Several authors (Raymond and Wang, 1960; Fantes and Furminger, 1967; and Brewer, 1967), have found that ammonium persulfate (AP), used to polymerize acrylamide gels, can cause a destruction of catalytic activity and increased heterogeniety of proteins during electrophoresis. The use of AP in this study did not significantly effect results with human plasma, but it could have been a factor in the results with avian plasma. Riboflavin can be used in place of AP as a polymerization agent without the above effects (Fantes and Furminger, 1967), but it was not used in this study.

H. Ravin (1961) perhaps gives the strongest clue as to the possible reason ceruloplasmin is difficult to detect in avian plasma. In his paper, Ravin describes a method of quantitatively determining the amount of ceruloplasmin contained in a milliliter of plasma. According to this procedure, there is a large difference between the Ravins Values for human and Gallus plasma, 32.2mg% and 2.5 to 4.5 mg% respectively. This difference in concentration of ceruloplasmin in conjunction with one or more of the above effects could possibly explain why ceruloplasmin can be detected easily in human plasma, but only with much difficulty in avian plasma.

In order to get over this obstacle of low concentration of ceruloplasmin in avian plasma, it was thought feasible to purify avian plasma samples so a higher concentration could be obtained. Methods of purification as described by Curzon and Vallet (1960) and Deutsch (1960) were reviewed, but in each method the samples being purified were pooled samples. Obviously, if comparisons are to be made between

species for species specific differences in ceruloplasmin, the samples cannot be pooled. If numerous samples of the same species were pooled, this would eliminate any individual differences and the increased heterogeneity would confuse any results.

Since avian ceruloplasmin has proved to be an unusually elusive object in relation to gel electrophoresis, it cannot be regarded as a significant taxonomic trait within itself.

It may be of some importance when used in conjunction with other plasma proteins.

Perhaps with a further refining of the techniques and procedures described in this study, and more studies carried out on the physical properties of avian ceruloplasmin, this copper-carrying plasma protein could be used as a taxonomic trait at the present time, however, it is of little use.

Appendix I: Gel Media for Electrophoresis

	5%	7%	5% Stock	7% Stock
Cyanogum-41	10.0 gms.	14.0 gms.	40.0 gms.	56.0 gms.
Buffer to	200.0 ml.	200.0 ml.	800.0 ml.	800.0 ml.
TIMED	0.2 ml.	0.2 ml.	0.8 ml.	0.8 ml.
AP	0.2 gms.	0.2 gms.		

Stock solutions may be prepared containing all ingredients up to AP. The AP is added immediately before pouring the gel solution into the cell.

As acrylamide gel is rather flexible at concentrations or 5% or below and cannot support itself, pre-cut sponges are incorporated in the bottom of the gel slab between the cooling plates for added support.

Complete polymerization requires 20-30 minutes.

Appendix II: Buffer Systems

A. Tris - Na_2EDTA - Boric Acid (0.18M), pH 8.4 (Peacock, 1965).

Tris	43.1 gms.
Na_2EDTA	3.7 gms.
Boric Acid	22.0 gms.
Water To	4 liters

Add buffer to cell and pre-run for 15-30 minutes, without samples, using same conditions to be employed in actual separation.

Normal conditions for separation: 300 volts, 2 hours, 3° to 5° C.

B. Tris Glycine (0.4M), pH 9.3 (E-C Technical Bulletin 128).

Tris	138.2 gms.
Glycine	21.0 gms.
Water To	4 liters

Add buffer to cell and pre-run for 15-30 minutes without samples, using same conditions to be employed in actual separation.

Normal conditions for separation: 300 volts, 3 hours, 3° to 5° C.

C. Acetate (0.1M), pH 5.7 (Jensen, 1963 as reported in Wieme, 1965).

Stock Solution:

A. 0.2M solution of Acetic Acid (11.55 ml. in 1000 ml. H_2O)
B. 0.2M solution of Sodium Acetate (27.2 gsm. $C_2H_4O_2Na$: $3H_2O$ in 1000 ml. H_2O)

X ml. of A + Y ml. of B, diluted to a total of 1000 ml.
where X = 48, Y = 452

Add buffer to cell and pre-run for 15-30 minutes without samples, using same conditions to be employed in actual separation.

Normal conditions for separation: 200 volts, 4 hours, 3° to 5° C.

Appendix III: Staining Procedures

A. Alizarin Blue S stain (Uriel, 1969 as reported in Wieme, 1965).

Copper (Cu^{++}) is revealed by the blue complex formed between cupric ions and Alizarin Blue S.

Reagents:

1) Alizarin Blue S stock solution:
prepare a saturated solution of Alizarin Blue S in concentrated acetic acid

2) Acetate buffer, pH 5.2:

0.4M sodium acetate	500 ml.
0.1M acetic acid	500 ml.

3) 70% acetic acid

Procedure:

Fix the plate for 14-16 hours in a solution containing equal volumes of absolute ethanol and solution 2; dilute solution 1 in a ratio 1/10 with solution 3; place plate in this solution for 30 minutes; wash for 30 minutes in solution 3.

Note: This procedure was attempted once with no results and was abandoned because of the time involved.

B. Rubeanic Acid stain (Decleir, 1961 as reported in Wieme, 1965).

Copper (Cu^{++}) is revealed by the precipitation by Rubeanic Acid as a greenish black stain. Sensitive to 0.1 µg. Cu^{++} per ml.

Reagents:

Rubeanic Acid solution: mix just before use	
Acetic acid	5 ml.
0.2% Rubeanic acid in ethanol	25 ml.
94% ethanol	70 ml.

Procedure:

Place fresh plates in the solution for 1 hour, wash in 5% acetic acid.

Note: Rubeanic acid stain was used with plates run with 5% and 7% gel concentrations; Tris-Na_2EDTA-Boric Acid, Tris Glycine, and Acetate buffers; with running times ranging from 2 to 5 hours. Also, some plates were stained with increased concentrations of rubeanic acid, 0.2 gms, in 25 ml. ethanol instead of 0.05 gms. Some plates remained in the staining solution for up to 12 hours. Plasma samples with EDTA and heparin as anticoagulants were stained by this method.

C. O-dianisidine

Ceruloplasmin detected by o-dianisidine due to its oxidase activity. Sensitive to 0.15 µg. copper per ml.

Method 1 (Owen and Smith, 1961).

Reagents:

O-dianisidine	0.2 gm.
HCl (conc.)	
H_2O to	20.0 ml.
0.1M Na Acetate - pH 5.7	20.0 ml.
Ethanol	60.0 ml.

Procedure:

Add o-dianisidine to 10 ml. H_2O; add HCl until O-dianisidine just dissolves; make up to 20 ml. with H_2O. Add remaining ingredients and incubate gel (37° C) in this for 1 hour. Wash in 5% acetic acid. Dark orange-red bands indicate ceruloplasmin.

Note: Owen and Smith's method was used with plates run with 5% and 7% gel concentrations; Tris-Na_2EDTA-Boric Acid, Tris Glycine, and Acetate buffers; with running times ranging from 2 to 4 hours. Also, some plates remained in staining solution for up to 12 hours. Plasma samples with EDTA and heparin as anticoagulants were stained by this method.

Method 2 (Jensen, 1963).

Reagents:

1) Acetate buffer (o.1M, pH 5.7) see Appendix II, C.
2) Ethanol p.a.
3) O-dianisidine solution:
   o-dianisidine 1.0 gm.
   distilled H_2O 50 ml.

   Add sufficient concentrated hydrochloric acid to clear the solution; add distilled H_2O up to 100.0 ml.; this solution

stable at room temperature for at least 3 months.

4) Sodium azide 3% in water

Procedure:

Prepare the substrate solution by mixing together: solution 1, solution 2, and solution 3 in the ratio 6/3/1; place plate in the substrate solution for 90 minutes; transfer plate to solution 4 for 5 minutes; wash in water for 2 hours. Red-brown bands indicate ceruloplasmin.

Note: Jensen's method was used with plates run with 5% and 7% gel concentrations; Tris-Na_2EDTA-Boric Acid, Tris Glycine, and Acetate buffers; with running times ranging from 2 to 4 hours. Some plates remained in staining solution for up to 12 hours. Plasma samples with EDTA and heparin as anticoagulants were stained by this method.

It was found that 25-30 drops of concentrated hydrochloric acid were necessary in step 3.

Solution 4 was left out because sodium azide destroys ceruloplasmin activity (Uriel, 1958), and it was thought that a clearer reaction (band) would result without the addition of sodium azide.

Appendix IV

Avian Specimen (by Families According to Peterson, 1947)

Phasianidae

Gallus gallus
Colinus virginianus

Larinae

Larus delawarensis

Columbidae

Columba livia
Zenaidura macroura

Picidae

Colaptes auratus

Corvidae

Cyanocitta cristata

Mimidae

Mimus polyglottos
Dumetella carolinensis
Toxostoma rufum

Turdidae

Turdus migratorius
Hylocichla mustelina
Hylocichla guttata
Hylocichla ustulata
Hylocichla minima
Hylocichla fuscescens

Parulidae

Helmitheros vermivorus
Dendroica magnolia
Dendroica striata
Dendroica palmarum
Seiurus noveboraensis
Geothlypis trichas
Wilsonia canadensis
Setophaga ruticilla

Icteridae

Quiscalus quiscula

Fringillidae

Richmondena cardinalis
Pipilo erythropthalmus

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