hydroxy-20α-(5′-methyl-2′-pyridyl)-5-pregnene in 150 ml. of ethanol was added a solution of 5.0 g. of potassium hydroxide in 10 ml. of water. After 5 hours at reflux temperature, the solution was concentrated to give a residue which was diluted with water. The precipitate was collected by filtration, washed with water and dried. Recrystallization from absolute ethanol afforded 1.1 g., m.p. 281–282°, $[\alpha]_D$ −77°.

Anal. Calcd. for $C_{27}H_{39}NO_2$ (409.59): C, 79.17; H, 9.60; N, 3.42. Found: C, 79.39; H, 9.61; N, 3.42.

3β,20β-Dihydroxy-20α-(5′-methyl-2′-piperidyl)-5-pregnene (XXVIIa).—To a solution of 1.02 g. (0.0025 mole) of 3β, 20β-dihydroxy-20α-(5′-methyl-2′-pyridyl)-pregnene (XXVI) in 150 ml. of *n*-propyl alcohol was added, in 4 portions over 0.5 hour, a total of 11.5 g. of sodium. After the metal had dissolved entirely, the solution was concentrated. The residue was diluted with water to give a precipitate which was extracted with ether. The organic phase was washed with water and was concentrated to give a remainder which was treated with 12 ml. of a 5% solution of picric acid in absolute ethanol. After 15 hours at 0°, the yellow precipitate of the **picrate of XXVIIa** was collected by filtration to give 750 mg. (46%), m.p. 240–250°. Three recrystallizations from absolute ethanol afforded the analytical sample as golden yellow needles, m.p. 248–250°.

Anal. Calcd. for $C_{33}H_{43}N_4O_9$ (644.75): C, 61.47; H, 7.50; N, 8.69. Found: C, 61.38; H, 7.60; N, 8.82.

A suspension of the picrate in water was treated with dilute aqueous lithium hydroxide and was extracted repeatedly with ether. The organic phase was washed with water and was concentrated. Recrystallization of the dried residue from methanol gave 100 mg., m.p. 207–208°, $[\alpha]_D$ −55°.

Anal. Calcd. for $C_{27}H_{45}NO_2$ (415.64): C, 78.02; H, 10.91; N, 3.37. Found: C, 78.15; H, 10.92; N, 3.64.

The hydrochloride of XXVIIa was recrystallized from absolute ethanol; m.p. 294–296°.

Anal. Calcd. for $C_{27}H_{46}NO_2Cl$(452.11): C, 71.72; H, 10.26; N, 3.10. Found: C, 71.28; H, 10.20; N, 3.04.

Treatment of a 10% aqueous acetic acid solution of XXVIIa with 10% aqueous sodium nitrite gave the **N-nitroso derivative of XXVIIa** which was recrystallized from methanol to give hexagonal plates, m.p. 210–218°.

Acetylation of XXVIIa with acetic anhydride in pyridine, followed by treatment with dilute alkali, afforded the **N-acetyl derivative of XXVIIa** which was recrystallized from methanol to give small plates, m.p. 246–250°.

3β,20β-Dihydroxy-20α-(5′-methyl-2′-piperidyl)-5-pregnene (XXVIIb).—The mother liquor from the picrate of XXVIIa was concentrated to a small volume to give, after 15 hours at 0°, a deposit which was collected and was recrystallized from a mixture of methanol and benzene to afford orange-yellow needles of the **picrate of XXVIIb**, m.p. 238–243°.

Anal. Calcd. for $C_{33}H_{43}N_4O_9$ (644.75): C, 61.47; H, 7.50; N, 8.69. Found: C, 61.74; H, 7.31; N, 8.75.

A solution of the picrate in ethanol was treated with dilute aqueous lithium hydroxide and was diluted with water to give a precipitate which was extracted with ether. The organic phase was washed with aqueous lithium hydroxide and with water and was concentrated. The dried residue was recrystallized from methanol to give needles, m.p. 202–205°, $[\alpha]_D$ −62°.

Anal. Calcd. for $C_{27}H_{45}NO_2$ (415.64): C, 78.02; H, 10.91; N, 3.37. Found: C, 77.89; H, 10.92; N, 3.48.

An intimately ground mixture of XXVIIa and XXVIIb melted at 197–208°. The infrared spectra of the isomers, which displayed quite uneventful fingerprint regions, were difficult to distinguish.

The hydrochloride of XXVIIb, from absolute ethanol, melted at 320–330°.

Treatment of a 10% aqueous acetic acid solution of XXVIIb with dilute aqueous sodium nitrite, gave, after recrystallization from a mixture of dichloromethane and methanol, short, thin needles of the **N-nitroso derivative of XXVIIb**, m.p. 252–258°.

Acetylation of XXVIIb with acetic anhydride in pyridine, followed by treatment with dilute alkali, gave, after recrystallization from methanol, the **N-acetyl derivative of XXVIIb**, m.p. 185–190°.

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The Relation between the Minor Components of Whole Normal Human Adult Hemoglobin as Isolated by Chromatography and Starch Block Electrophoresis

BY A. G. SCHNEK AND W. A. SCHROEDER

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Three hemoglobin components may be separated from hemolysates of normal adult red blood cells by starch block electrophoresis: the main component, A_1, moves at a position intermediate between the most slowly moving component, A_2, and the most rapidly moving component, A_3. By chromatography, eight hemoglobin components may be detected: five (A_{1a}, A_{1b}, A_{1c}, A_{1d} and A_{1e}) move down the column more rapidly than the main component (A_{II}) and two move more slowly (A_{IIIa} and A_{IIIb}). Non-heme containing proteins are also present. The present investigation has determined the correspondence between the components as isolated by the two methods: A_1 contains A_{1c}, A_{1d} and A_{1e}, and A_{II}; A_2 contains A_{IIIb} and non-heme protein(s); A_3 contains A_{Ia}, A_{Ib} and non-heme protein(s).

Introduction

Inasmuch as the pertinent literature relating to the heterogeneity of whole normal human hemoglobin was reviewed in some detail by Clegg and Schroeder,[1] it is necessary here to mention only data that were not discussed by them. To the

(1) M. D. Clegg and W. A. Schroeder, THIS JOURNAL, **81**, 6065 (1959).

three components that Kunkel and Wallenius[2] detected by starch block electrophoresis, another, A_4, has been added in an investigation of Masri, Josephson and Singer[3] by the same method. Although Berry and Chanutin[4] have reported the presence of two hemoglobin components in stored blood by moving boundary electrophoresis, there is no obvious relation between their results and others that deal with the heterogeneity of hemoglobin. Clegg and Schroeder[1] have succeeded in detecting six minor components in whole normal human hemoglobin by chromatography. Quantitatively, starch block electrophoreses suggest that the minor components comprise 5 to 10% of the total hemoglobin whereas chromatographic methods have shown that they make up about 20%.

What may be the source, structure and significance of the minor components in whole hemoglobin is still largely open to question. Of all the components, the best characterized and most thoroughly studied is the component termed A_2 (more slowly moving than the main component on starch block electrophoresis). Hemoglobin A_2 seems to be implicated in the problem of thalassemia as shown by the investigations of Kunkel and collaborators,[5] Gerald and Diamond[6] and Cohen and collaborators.[7]

Because minor components may be detected both by starch block electrophoresis and chromatography, the question naturally arises as to the correspondence between the components as isolated by the two methods. This question does not seem to have been definitely answered although Morrison[8] has commented briefly on the possible correlation. It has, therefore, seemed to be of considerable value to determine definitely the correspondence between the minor components as isolated by the two methods. This correlation has been achieved in the present investigation which has also led to improvement in chromatographic procedure and to the development of a preparative method for isolating and purifying hemoglobin A_2 by a combination of chromatography and electrophoresis.

Very recently, Huisman and Meyering[9] have described a correlation of the components as isolated by starch block electrophoresis, chromatography on carboxymethylcellulose and a type of chromatography on IRC-50. They have adopted two procedures from Allen, Schroeder and Balog[10]: the use of the maximum at 415 mμ in the spectrum of hemoglobin as a more sensitive measure for the detection of minor components and the addition of cyanide to the chromatographic developers to prevent the formation of extraneous zones because of possible partial conversion to ferrihemoglobin. Their results will be mentioned in more detail below.

The obvious components that may be isolated by starch block electrophoresis have been designated as A_1, A_2 and A_3. These designations refer to the main, the most slowly moving, and the most rapidly moving components, respectively. Allen, Schroeder and Balog[10] applied an arbitrary nomenclature (A_{Ia}, A_{Ib}, A_{Ic} and A_{II}) to the components that were isolated by chromatography because the correspondence to A_1, A_2 and A_3 was not determined. This nomenclature was extended by Clegg and Schroeder[1] when more minor components were detected chromatographically. The present investigation has shown that A_1 and A_3 each may be separated chromatographically into several heme-containing components whereas A_2 contains one heme protein and also non-heme protein(s). A definitive system of nomenclature is necessary but probably should be devised by consultation of interested workers as was the system of nomenclature for the abnormal hemoglobins.[11] It has seemed advisable, therefore, to retain the two systems of nomenclature in this paper rather than to extend either system or propose a new one.

Experimental

Preparation of Hemoglobin Solutions.—The procedure for the preparation of the hemoglobin solutions departed only slightly from that of Clegg and Schroeder.[1] Thus, the oxyhemoglobin was converted to carbonmonoxyhemoglobin at the time that the cells were first washed with isotonic saline solution. Thereafter in the preparation, all reagents and dialyzing media were saturated with carbon monoxide. Sometimes, a third centrifugation of the final solution of hemoglobin was carried out at 25,000 *g* in order to remove traces of cell debris which according to Kunkel, *et al.*,[5] interfere with starch block electrophoresis. Prior to chromatography or electrophoresis, the hemoglobin solution was dialyzed against the appropriate carbon monoxide-saturated buffer for at least 15 hr. with two or three changes of buffer.

Procedures for Starch Block Electrophoresis.—The procedures of starch block electrophoresis were based upon those described by Kunkel and collaborators[2,5,12] and by Masri, Josephson and Singer.[3] The first experiments were made in buffer of 0.1 ionic strength but the best and most rapid separations used buffer of 0.05 ionic strength. The latter buffer was prepared by dissolving 93.6 g. of sodium barbital in 2 l. of water bringing the solution to a boil and adding 13.2 g. of barbital and 12 ml. of 6 *N* hydrochloric acid. After dilution to 12.1, the *p*H was adjusted to 8.6 with sodium hydroxide.

Commercial potato starch was purified as follows. The starch was mixed with three times its volume of water. The mixture was stirred vigorously and the particles were allowed to settle for 90 min. after which time the supernatant liquid with suspended impurities and fine particles was removed. This procedure was repeated at least seven times during which the period of settling was decreased to 30 min.

Finally, the starch was filtered, slurried twice with three volumes of warm buffer (50°), refiltered and stored slightly moist at 6° until used.

Starch blocks of two sizes were used: 40 × 15 cm. and 45 × 38 cm. with a thickness of 7 to 12 mm. Electrophoresis was in the direction of the long dimension of each. The block was poured from a homogeneous paste that was pre-

(2) H. G. Kunkel and G. Wallenius, *Science*, **122**, 288 (1955).

(3) M. S. Masri, A. M. Josephson and K. Singer, *Blood*, **13**, 533 (1958).

(4) E. R. Berry and A. Chanutin, *J. Clin. Investigation*, **36**, 225 (1957).

(5) H. G. Kunkel, R. Ceppellini, U. Müller-Eberhard and J. Wolf, *ibid.*, **36**, 1615 (1957).

(6) P. S. Gerald and L. K. Diamond, *Blood*, **13**, 61 (1958).

(7) F. Cohen, W. W. Zuelzer, J. V. Neel and A. R. Robinson, *ibid.*, **14**, 816 (1959).

(8) M. Morrison, Conference on Hemoglobin, Natl. Acad. Sci., Natl. Res. Council, Washington, D. C., Publication No. 557, 1958, p. 218.

(9) T. H. J. Huisman and C. A. Meyering, *Clin. Chim. Acta*, **5**, 103 (1960); "Protides of the Biological Fluids," 7th Colloqium, 1959, edited by H. Peeters, Elsevier Publishing Company, 1960, p. 71.

(10) D. W. Allen, W. A. Schroeder and J. Balog, THIS JOURNAL, **80**, 1628 (1958).

(11) Anonymous: Statement Concerning a System of Nomenclature for the Varieties of Human Hemoglobin, *Blood*, **8**, 386 (1953).

(12) H. G. Kunkel, "Methods in Biochemical Analysis," Vol. I, Interscience Publishers, Inc., New York, N. Y., p. 141.

pared from one volume of moist starch and one volume of buffer. The preparation of the block followed the general procedure of Kunkel and collaborators[2,5,12] and Masri, *et al.*[3] As they have pointed out, the degree to which excess water must be removed by sponge or blotting paper in order to prepare a satisfactory block can be learned only by experience.

The technique of Kunkel, *et al.*,[5] for applying the sample has been the most satisfactory; that is, the sample was injected in small portions into many small slits in the block. Samples of 0.1 to 0.2 ml. were placed in 0.5-cm. slits about 2 cm. apart. A 0.5-ml. tuberculin syringe with a 23-gauge needle was convenient to use in this procedure. After application of the samples, the block was placed in contact with the electrode vessels and allowed to equilibrate thermally and hydrostatically for half an hour. The technique of Masri, *et al.*,[3] in which the sample is slurried with starch and then placed in a rectangular slot in the starch block did not give good results in our hands and required a second electrophoresis in order to obtain pure components.

For the electrophoresis, contact between the block and buffer vessels was made with linen cloths that were saturated with buffer. Agar bridges connected the buffer vessels with saturated calomel electrode vessels. Power was supplied by two Heathkit power units (Model PS5 from the Heathkit Company, Benton Harbon, Michigan) in series. In these experiments the potential difference was about 12 volts per cm. at 50 to 60 mamp. The duration of electrophoresis was 12 to 15 hr. in a cold room at 5°. The electrode vessels were exchanged between consecutive runs.

At the completion of the electrophoresis, the block was viewed by transmitted light and photographed as desired.

For the isolation of the hemoglobin fractions, the appropriate section of the block was cut out and mixed with a measured volume of ice-cold distilled water or chromatographic developer (saturated with carbon monoxide) on a sintered disc funnel. After the liquid had been sucked through the filter, the removal of the hemoglobin was completed with a few more ml. of water or developer.

All operations of the electrophoretic procedure from the time of application of the sample to the isolation of the fractions were carried out in a cold room at 5°.

Chromatographic Procedures.—The chromatographic procedures were essentially those of Allen, Schroeder and Balog[10] and Clegg and Schroeder[1] with some modifications. For most experiments, the columns were of the usual size, namely, 1 × 35 cm. Most chromatograms used Developer No. 5[1] although others[10] were sometimes advantageous as was a new developer devised by Jones.[13] This new developer, No. 6, has a *p*H of 6.70 ± 0.02 and is prepared by dissolving 18.37 g. of monosodium phosphate monohydrate, 4.74 g. of disodium phosphate and 2.60 g. of potassium cyanide in 4 l. of water. Developer No. 6 is especially designed to retard the movement of the more rapidly moving hemoglobin components in whole human hemoglobin.

When whole hemoglobin was chromatographed, the 50-mg. sample was dissolved in 2 ml. of developer but the samples for the minor components frequently were as dilute as 10 mg. in 4 or 5 ml. Far more regular zones can be obtained by a new technique[13] for placing the sample on the column. Instead of permitting the sample to drain into the column and then rinsing with developer, the hemoglobin sample was carefully layered over with one or two ml. of developer, and the whole was permitted to drain into the column before the main development was begun.

In all of these chromatograms, the hemoglobin was the carbonmonoxy instead of the oxy derivative.[1,10] Prior to chromatography the sample was dialyzed against carbon monoxide-saturated buffer. However, although the developer for the chromatogram was not saturated with carbon monoxide, the carbonmonoxyhemoglobin form seemed to be quite stable and to chromatograph satisfactorily.

Chromatograms with Developer No. 5 were run in a slightly different manner than described.[1] After the first 150 ml. of development, the initial flow rate of 6 ml. per hr. was increased to 12 ml. per hr. for the remainder of the chromatogram. The volume of individual fractions was as follows: to 80 ml. of effluent, 2 ml.; to 150 ml., 4 ml.; to 700 ml., 8 ml.; and the remainder, 4 ml. After 700 ml. of developer had passed through the column, the leading edge of the main component was within 3 to 4 cm. of the bottom of the column. In order to obtain the main component and following components in concentrated fractions, the temperature must be raised at this point. Clegg and Schroeder[1] changed to "room temperature" but it was found in the present experiments that a difference of a few degrees in room temperature had a profound effect on the chromatographic results. Consequently, a more exact procedure was initiated. After the effluent volume was 700 ml., the chromatogram was stopped and the temperature of the circulating water in the jacket around the chromatographic column was changed from 6 to 28°. After allowing 30 min. to reach temperature equilibrium, the flow was again started and adjusted to 12 ml. per hr. Under these conditions, all remaining components passed from the column in less than 300 ml. of developer. Before a column was used again, it was reëquilibrated with 1000 ml. of developer at 6° in the course of 4 days.

Larger scale preparative chromatograms were carried out on columns 2.5 or 3.5 × 35 cm. in dimension. When Developer No. 5 was used, 500 or 1000 mg., depending upon the size of the column, could be applied in 12 ml. of developer. Large scale chromatograms were carried out in a cold room at 5° so that the fractions could be collected at this temperature. Flow rates of developer were 40 ml. per hr. with the 2.5-cm. columns and 60 ml. per hr. with the 3.5-cm. columns.

For the preparation of relatively large amounts of hemoglobin A_2, a combination of chromatography and electrophoresis has been advantageous. Thus, 2 g. of whole hemoglobin were chromatographed on a 3.5 × 35-cm. column with Developer No. 2 until A_I had emerged from the column. If the chromatogram is now warmed to 28°, the next 200 ml. of effluent contain most of the A_{II} while the following 400 ml. contain about 20% A_{III}. This procedure avoids too long a time at 28° with attendant decomposition of A_{III}, yet when the fraction rich in A_{III} is then submitted to starch electrophoresis as described above, an excellent yield of A_2 is obtained. For example, 2 g. of whole hemoglobin gave 500 mg. of the mixture of A_{II} and A_{III} from which 50 mg. of pure A_2 were isolated after starch block electrophoresis.

Concentration of Dilute Solutions of Hemoglobin.—After chromatography or starch block electrophoresis, the hemoglobin fractions are in such dilution that concentration is required before further chromatography or electrophoresis. Concentration against solutions of polyvinylpyrrolidone as used by Clegg and Schroeder[1] led to considerable losses in the present work. Accordingly, recourse has been had to the method of concentration that was recently devised.[14] In this method, the dilute solution is centrifuged at 40,000 r.p.m. for 16 to 24 hr. at 0 to 5° in the No. 40 head of a Spinco Model L centrifuge. Under these conditions, the hemoglobin concentrates in a few tenths of a ml. at the bottom of each centrifuge tube and can be readily removed with a hypodermic syringe equipped with a long needle. Concentrations of 3 to 7% hemoglobin can be obtained in this way. Before or after concentration, the solution was dialyzed against the carbon monoxide-saturated buffer or developer appropriate to the next procedure that was planned.

Spectrophotometry.—The optical density of chromatographic fractions was determined at 280 or 415 mμ as described by Allen, *et al.*[10] For determining the concentration of solutions, the maximum at 541 mμ was used with the factor 1.14 × optical density of 1 cm. of solution equal to the concentration in mg. per ml.

Results

Electrophoretic Results.—When the larger starch block was used, a total of 5 to 6 ml. of a 10% solution of carbonmonoxyhemoglobin could be applied to 20 to 24 individual spots. Each spot gave a pattern very much like that presented by Kunkel and Wallenius[2]: a photograph of a typical pattern from one spot is shown in Fig. 1a. The usual three components designated as A_1, A_2 and A_3 were observed. The distance from the point of application to the center of the main component usually was 6 to 7 cm.

By combining the appropriate sections from the many spots of a single electrophoresis, sufficient

(13) R. T. Jones, private communication.

(14) J. R. Vinograd and W. D. Hutchinson, *Nature*, **187**, 216 (1960).

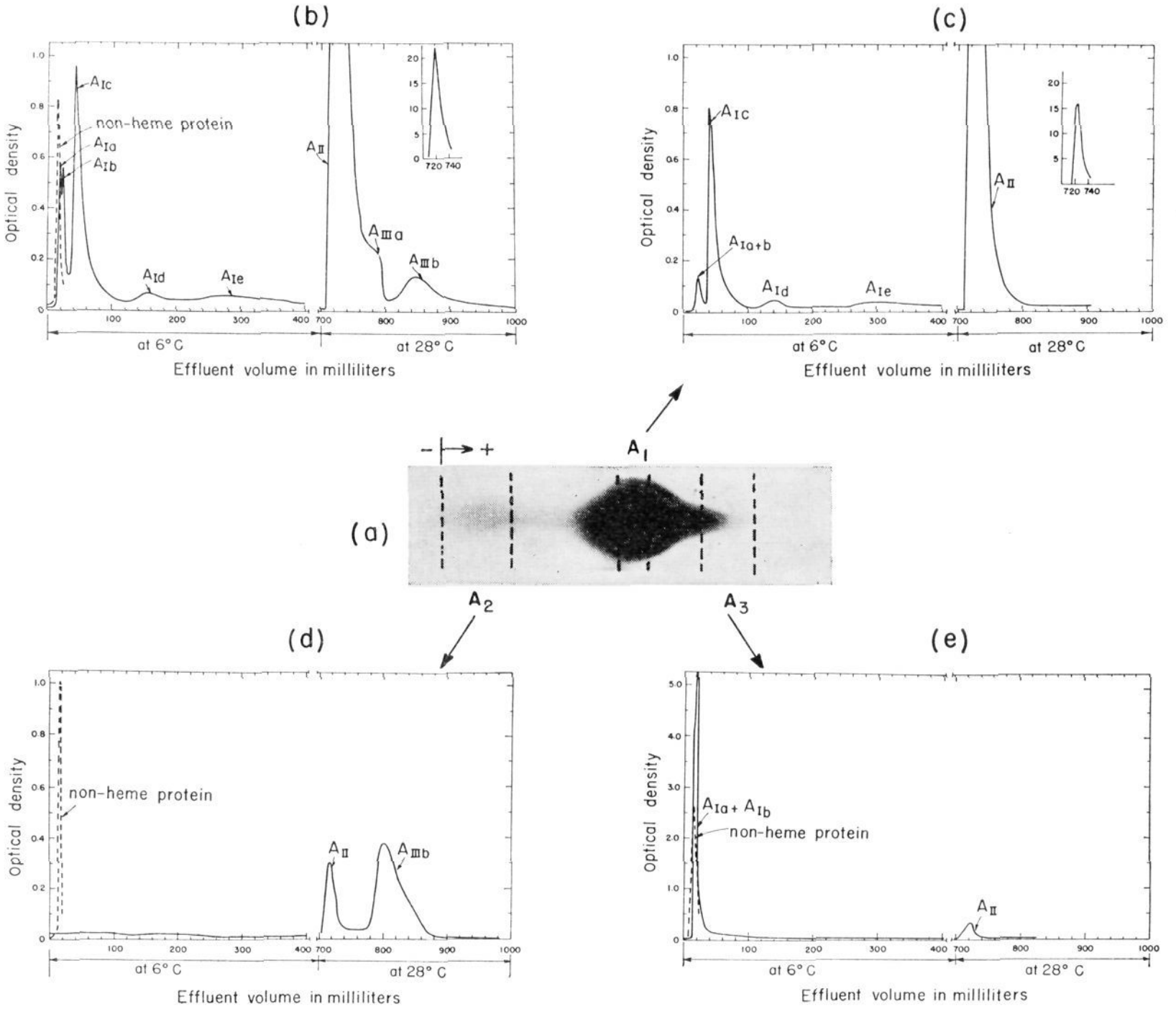

Fig. 1.—Summary of the chromatographic properties in Developer No. 5 of the three electrophoretic fractions of whole adult human hemoglobin: (a) starch block electrophoresis at *p*H 8.6 of whole hemoglobin; (b) chromatogram of whole hemoglobin; (c) chromatogram of A_1 (23 mg.); (d) chromatogram of A_2 (7 mg.); (e) chromatogram of A_3 (7 mg.).

material could be obtained to study each portion chromatographically. First, however, the electrophoretic behavior of each isolated fraction was compared with that of whole hemoglobin. Component A_2 contains only one heme protein after one electrophoresis but the poor yields (only 10 mg. from a gram of hemoglobin) required the development of a preparative procedure in order to isolate sufficient material for other more elaborate experiments. To obtain good A_3, the extreme part of the spot must be selected as shown in Fig. 1a, electrophoresed again and the extreme part again chosen. A_1 behaves in the same manner as the main component. The electrophoretic and isolative procedures thus do not alter the properties of the components and, hence, they were considered to be suitable for the study of their chromatographic behavior.

Chromatography of the Electrophoretically Isolated Components.—Fig. 1b shows the typical chromatographic pattern of whole hemoglobin. It differs from that published by Clegg and Schroeder[1] only in the presence of A_{IIIa}. On some chromatograms, A_{IIIa} was separated more completely. Table I presents the percentages of the various components in whole hemoglobin as calculated from five chromatograms. The agreement with the data of Clegg and Schroeder[1] is satisfactory.

TABLE I

PERCENTAGES OF THE COMPONENTS AS DETERMINED BY CHROMATOGRAPHY WITH DEVELOPER NO. 5

Chrom. no.	A_{Ia+b}	A_{Ic}	A_{Id}	A_{Ie}	A_{II}	A_{IIIa}	A_{IIIb}
286	2.1	5.7	1.6	5.5	79.3	3.4	2.3
288	2.7	5.4	2.5	4.1	81.0	1.4	2.8
294	2.7	5.2	1.6	4.1	81.2	2.1	3.0
316	2.1	5.6	1.4	4.5	80.9	3.0	2.6
324	2.4	5.9	1.9	6.9	77.1	2.0	3.9
Average	2.4	5.6	1.8	5.0	79.9	2.4	2.9

Component A_1, when chromatographed with Developer No. 5, showed the pattern of Fig. 1c. Thus, A_1 contains A_{Ic}, A_{Id}, A_{Ie} and A_{II} but little A_{Ia+b} and no A_{IIIa} and A_{IIIb}. The quantitative

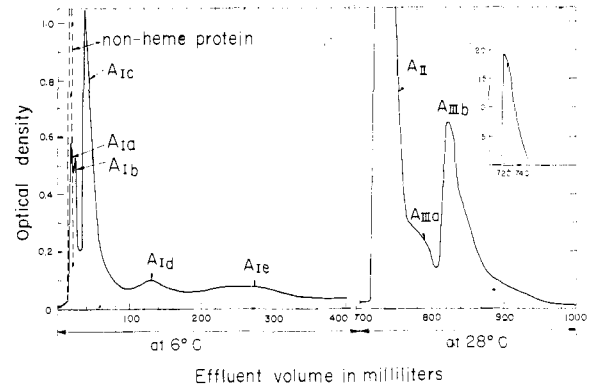

Fig. 2.—Chromatogram of a mixture of whole hemoglobin (47 mg.) and A_2 (6 mg.) with Developer No. 5.

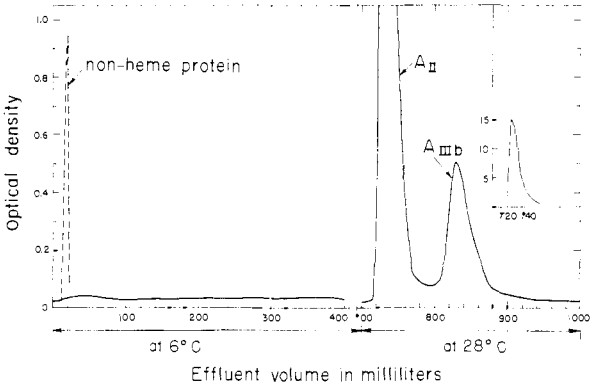

Fig. 3.—Chromatogram of a mixture of chromatographically isolated A_{II} (35 mg.) and electrophoretically isolated A_2 (9 mg.) with Developer No. 5.

relations of the several components from three experiments are given in Table II. The different proportions as compared to whole hemoglobin (Table I) suggest that the positions of the components in A_1 are not exactly coincident on the starch block and vary from experiment to experiment.

TABLE II

PERCENTAGES OF THE CHROMATOGRAPHIC COMPONENTS IN FRACTION A_1

Chrom. no.	Amount on column, mg.	A_{Ia+b}	A_{Ic}	A_{Id}	A_{Ic}	A_{II}	A_{IIIa}	A_{IIIb}
291	52	1.4	5.8	3.8	1.9	87.1	...	...
299	20	2.0	7.0	5.4	3.5	82.2	...	...
301	23	1.5	5.7	2.7	3.5	86.5	...	...
	Average	1.6	6.2	4.0	3.0	85.3		

Component A_2 is labile and is difficult to isolate solely by starch block electrophoresis in sufficient quantity to permit completely satisfactory study. However, preliminary experiments using electrophoretic isolation only showed the correspondence of A_2 with A_{IIIb} and also the presence of non-heme protein as presented in Fig. 1d. Component A_2 was also added to whole hemoglobin and the mixture was chromatographed with Developer No. 5. The result is shown in Fig. 2 where there is an obvious increase in A_{IIIb} and also non-heme protein as compared to whole hemoglobin alone (Fig. 1b). When component A_2 is isolated preparatively by chromatography and electrophoresis, the non-heme protein is absent. The quantitative results from the chromatograms of the mixtures of whole hemoglobin and A_2 as presented in Table III correspond to an increase in A_{IIIb} of 50 to 60% of the added A_2. There is no increase in the other minor components and the decrease in the *percentage* of A_{II} is roughly what one would calculate from the composition of the mixture. When a mixture of chromatographically isolated A_{II} and electrophoretically isolated A_2 was chromatographed with Developer No. 5, the result depicted in Fig. 3 further substantiates the identification of A_2 with A_{IIIb}. Kunkel and collaborators[5] report that the percentage of A_2 in normal whole hemoglobin is about 2.5% of the total hemoglobin: the percentage of A_{IIIb} in whole hemoglobin (Table I) was found to be 2.8%—indeed, it is 2.5% if the one widely different value is excluded. The correspondence in the quantities of A_2 and A_{IIIb} is added proof of their identity.

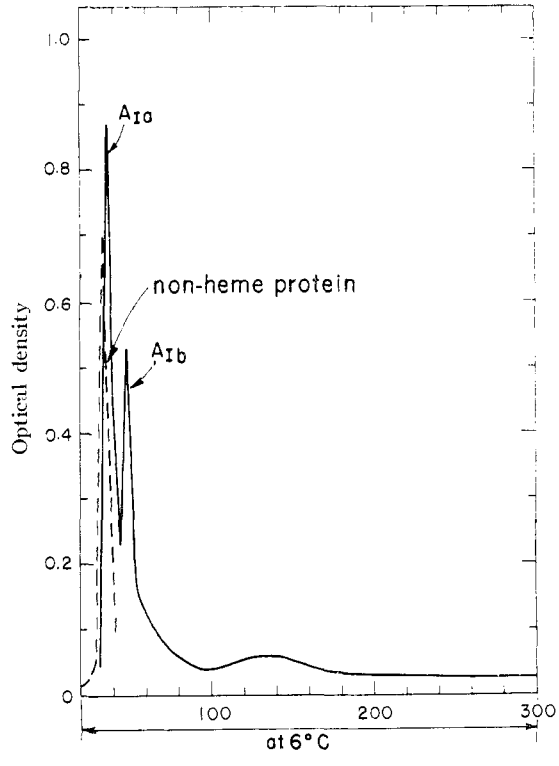

Fig. 4.—Chromatogram of A_3 with Developer No. 4.

Component A_3, upon chromatography with Developer No. 5, clearly corresponded to A_{Ia+b} (Fig. 1c) and also contained some non-heme protein. The definite presence of both A_{Ia} and A_{Ib} was proved by chromatographing with Developer No. 4 with the results depicted in Fig. 4. The quantitative data in Table IV show that A_{Ia} and A_{Ib} make up 70 to 80% of A_3.

Electrophoresis of the Chromatographically Isolated Components.—In order to have a sufficient amount of each of the chromatographically isolated minor components for electrophoretic studies, a gram of whole hemoglobin was chromatographed with Developer No. 5 on a large column (3.5 ×

TABLE III

PERCENTAGES OF THE CHROMATOGRAPHIC COMPONENTS AFTER ADDITION OF A_2 TO WHOLE HEMOGLOBIN A

Chrom. no.	Amount of Hb, mg.	A_{Ia+b}	A_{Ic}	A_{Id}	A_{Ie}	A_{II}	A_{IIIa}	A_{IIIb}
287	50 A + 6.3 A_2	2.7	6.8	1.5	5.6	72.3	2.9	8.2
296	47 A + 6.0 A_2	1.8	5.6	1.4	4.9	76.0	2.1	8.2
327	42 A + 7.3 A_2	2.2	4.4	1.3	5.5	73.4	2.0	11.1

TABLE IV

PERCENTAGES OF THE CHROMATOGRAPHIC COMPONENTS IN FRACTION A_3

Chrom. no.	Amount on column, mg.	A_{Ia}	A_{Ib}	$A_{Ic,d+e}$	A_{II}	Dev. no.
298	7	48.1	22.6	13.7	15.6	5
289	13	38.0	37.2	25.0	..	4
292	7	32.1	47.2	20.6	..	4
302	9	36.5	39.9	23.6	..	4

35 cm.). Fractions corresponding to A_{Ia+b}, A_{Ic}, A_{Id} and A_{II} were pooled and concentrated. A_{Ia} and A_{Ib} were then separated on a 1 × 35 cm. column with Developer No. 6. A mixture of A_{II} and A_{III} with a high concentration of A_{III} was prepared by the use of Developer No. 2 as described in the Experimental section. A portion from each component (concentration 25 mg. per ml. except A_{Id}, 5 mg. per ml.) was then spotted on a starch block with whole hemoglobin for comparison. A photograph of the results (Fig. 5) shows that the behavior of each chromatographically isolated component is as would be expected from the chromatographic behavior of the electrophoretically isolated components.

Discussion

The procedure of Masri, *et al.*,[3] for the large scale isolation of the electrophoretic components had been published shortly before this investigation was initiated. Despite many attempts to use the method, we were never able to achieve satisfactory separations without a second electrophoresis. In addition, the use of buffer of 0.1 ionic strength required electrophoresis for as long as 40 hr. in order to obtain even a fairly good separation. The procedure was not only time-consuming but the heating of the starch block, even in a cold room, probably led to some denaturation of the hemoglobin fractions. Kunkel's procedures were much more satisfactory and the time of electrophoresis was decreased to 12 to 16 hr. When a particular sample of starch had been used several times, increased electro-osmosis toward the cathode was observed as noted by Masri, *et al.*,[3] and was an effective aid in improving the separations. The component termed A_4 by Masri, *et al.*,[3] has not been observed.

The correlation between the chromatographic and electrophoretic minor components that was the object of this study has been achieved and the results are summarized in Table V.

TABLE V

SUMMARY OF CORRELATIONS

Electrophoresis	Chromatography
A_1	A_{Ic}, A_{Id}, A_{Ie}, and A_{II}
A_2	A_{IIIb} and non-heme protein(s)
A_3	A_{Ia}, A_{Ib} and non-heme protein(s)

No electrophoretic fraction seems to be entirely homogeneous by chromatography. Thus, A_1, in addition to the four components listed, contains a little A_{Ia} and A_{Ib}; A_2 also a little A_{II}; and A_3 also a little A_{Ic} and A_{II}. One cannot be certain whether the apparent contamination is present because of some overlapping of the electrophoretic spots or whether there has been some decomposition to produce materials that behave like the contaminants. Kunkel, *et al.*,[5] suggest that certain operations such as concentration, dialysis, etc., produce A_3 from A_1.

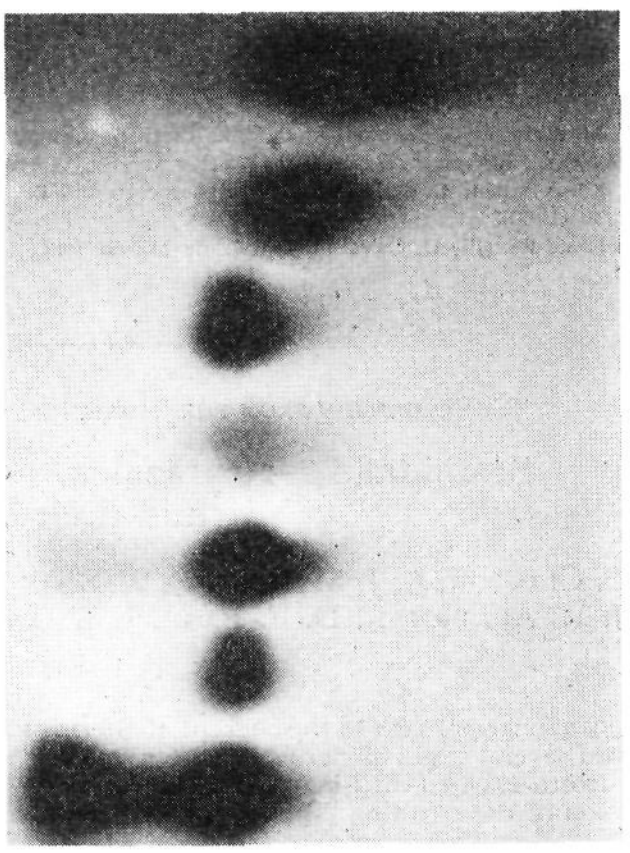

Fig. 5.—Starch block electrophoresis of chromatographically isolated components. The order from top to bottom is: A_{Ia}, A_{Ib}, A_{Ic}, A_{Id}, whole hemoglobin, A_{II}, and a mixture of A_{II} and A_{III}. The movement of the spots is from left to right.

Huisman and Meyering[9] have presented a partial correlation of the components that may be isolated by starch block electrophoresis with some of the components that may be isolated by chromatography on carboxymethylcellulose. Chromatograms of the electrophoretically isolated components do not show all of the components that are apparent by chromatography alone. Their procedure for chromatography on IRC-50 is so different from the procedures of the present investigation that there is no obvious relation between the components that they and we separate. They find three very well separated components. It is somewhat disconcerting, however, that each of these, when rechromatographed on carboxymethylcellulose, shows the presence of a component that they term A_1^c.

It is pertinent to consider the results in relation to some information in the literature. Thus Stein, Kunkel, Cole, Spackman and Moore[15] have determined the amino acid composition of A_1 as isolated by starch block electrophoresis. Although this sample may well have been the most homogeneous sample of hemoglobin A yet analyzed, it clearly must have contained at least four components.

Recently, Rossi-Fanelli, de Marco, Benerecetti and Guacci[16] have published an amino acid analysis of A_2 that had been isolated by starch block electrophoresis. According to their analysis, there is a very considerable difference in the amino acid composition of A_2 and hemoglobin A as analyzed by Rossi-Fanelli, *et al.*[17] The present investigation has shown that A_2 may be contaminated with non-heme protein as Kunkel, *et al.*,[5] have also pointed out. It seems highly probable, therefore, that this analysis of A_2 is in reality the analysis of a mixture of A_2 and non-heme protein. The rather extreme differences that they report in amino acid composition of A and A_2 seem unlikely.

(15) W. H. Stein, H. G. Kunkel, R. D. Cole, D. H. Spackman and S. Moore, *Biochim. Biophys. Acta*, **24**, 640 (1957).

(16) A. Rossi-Fanelli, C. de Marco, A. S. Benerecetti and L. Guacci, *ibid.*, **38**, 380 (1960).

(17) A. Rossi-Fanelli, D. Cavallini and C. de Marco, *ibid.*, **17**, 377 (1955).

A_2 contains two α chains like those in hemoglobin A[18] and apparently has only a few changes in amino acid sequence.[19] Clearly, any reports of the properties of the components that may be electrophoretically isolated cannot ignore their heterogeneity.

The question may naturally be raised as to the extent of the heterogeneity of the minor components that may be chromatographically isolated. It is hoped that investigation now in progress[20] on the characterization of these components will throw some light on this subject. The main component, A_{II}, especially, may still hide other minor components. Component A_{IIIa}, no doubt, was observed by Clegg and Schroeder[1] but continues to be elusive and the conditions under which it may consistently be well separated from A_{II} have not yet been determined.

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(18) A. G. Schnek, R. T. Jones and W. A. Schroeder, to be published.

(19) A. O. W. Stretton and V. M. Ingram, *Fed. Proc.*, **19**, 343 (1960).

(20) R. T. Jones, unpublished.

[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE, RENSSELAER, N. Y.]

Steroidal[3,2-c]pyrazoles. II.[1] Androstanes, 19-Norandrostanes and their Unsaturated Analogs

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Utilizing a new approach to the concept of altering the structure–activity relationship in anabolic steroids, there have been synthesized several types of steroidal[3,2-c]pyrazoles. These were prepared from 3-ketoandrostanes, 3-ketoandrost-4-enes, 3-ketoandrosta-4,6-dienes, 3-keto-19-norandrostanes and 3-keto-19-norandrost-4-enes by formylation at the 2-position and reaction of the resulting 2-hydroxymethylene-3-ketosteroids with hydrazine or a substituted hydrazine. The derived steroidal[3,2-c]pyrazoles frequently possessed enhanced or unusual endocrinological activities. These included greatly increased anabolic/androgenic ratios, or the unexpected development of estrogenic activity.

Since the initial discovery by Kochakian and Murlin[2] that the administration of testosterone can reverse nitrogen loss in the castrate dog, much effect has been expended on the synthesis of compounds with the high anabolic potency but without the undesirable masculinizing effects of the male hormone.[3] That there still exists a need for an anabolic agent with few or no androgenic or progestational side-effects is amply demonstrated by the abundance of very recent publications in this field.[4] Without exception, these approaches to the problem have continued previous efforts to vary endocrine activity patterns by means of alterations within, or substitutions on, the steriod nucleus.

Several years ago, as a result of investigations of the known relationships between structure and anabolic activity in steroids, we began a program directed toward the synthesis of anabolic agents with a high specificity of action. We assumed, as have others, that the target receptors (enzyme systems, for example) at the sites of anabolic and androgenic

(1) Preceding communication. R. O. Clinton, A. J. Manson, F. W. Stonner, A. L. Beyler, G. O. Potts and A. Arnold, *J. Am. Chem. Soc.*, **81**, 1513 (1959).

(2) C. D. Kochakian and J. R. Murlin, *J. Nutrition*, **10**, 437 (1935).

(3) An excellent summation and critical review (through early 1957) has been made by V. A. Drill and B. Riegel, "Recent Progress in Hormone Research," Vol. XIV, Academic Press, Inc., New York, N. Y., 1958, Chapter 2.

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