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Studies on dissociation and re-association of hapto-globins. Hybridisation between Hp^1Hp^1 and Hp^2Hp^2

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Genetica. — *Studies on dissociation and re-association of haptoglobins. Hybridisation between* Hp^1Hp^1 *and* Hp^2Hp^2 (*). Nota di LUIGI BERNINI, CARLA BORRI-VOLTATTORNI e MARCELLO SINISCALCO presentata (**) dal Socio G. MONTALENTI.

RIASSUNTO. — È stata ottenuta la riattivazione di aptoglobine umane dopo che, purificate, erano state sottoposte a clivaggio riduttivo in presenza di urea 8 M e di 2-mercaptoetanolo 0,5 M. Il recupero della proteina nativa è stato molto elevato (70-80 %).

È stato possibile ottenere delle proteine ibride partendo da miscele di aptoglobine purificate di tipo 1-1 e di tipo 2-2.

È stato inoltre dimostrato che lo scostamento dal rapporto 50 : 50 tra le due aptoglobine nella miscela originale dà origine alla formazione di proteine riattivate con un quadro elettroforetico su gel d'amido estremamente simile a quello del fenotipo *2-1-Modificato* se è in eccesso l'aptoglobina 1-1, oppure al fenotipo *Carlberg* se invece è in eccesso l'aptoglobina 2-2.

In the last few years considerable attention has been devoted to the mechanism of formation of tertiary and quaternary structure of proteins.

Several experiments have demonstrated that the tridimensional configuration of proteins is dependent upon the primary structure of the polypeptide chain itself. That is to say that as soon as the polypeptide chains are released from the polyribosomes into the cytoplasm, they are likely to assume the type of configuration which is the most stable from a thermodynamical point of view.

In this process—as is well known—non-covalent and covalent bonds (disulphide bridges) are involved. The latter, though strongly contributing to the stability of the molecules, are not an indispensable feature for the formation of a stable molecule. In fact several molecules are known which do not involve disulphide bridges, independently from the actual presence of SH-groups in the molecule itself.

One of the most fruitful approaches for understanding the intimate mechanism of folding and assembling of protein subunits is to obtain their dissociation and reassociation *in vitro* under different experimental conditions.

Several *in vitro* experiments have been performed thus far on the dissociation and reassociation of a large number of proteins consisting of one or more polypeptide chains and involving or not intra and/or inter-chain disulphide bonds [1]. Usually the splitting of the non-covalent bonds is obtained

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by exposing the purified protein to denaturing conditions such as extreme changes in pH and treatment with concentrated solutions of urea or guanidine hydrochloride. But whenever the tridimensional structure of the protein is dependent also on the presence of one or more disulphide bridges, the use of reducing agents such as mercaptoethanol, thyoglycolate or cysteine is necessary for a complete development.

When the denaturing agents are slowly removed, the protein regains its native configuration and functional activity. For those proteins involving disulphide bridges, the reactivation can be obtained only in the presence of oxygen which permits the reformation of the disulphide bonds.

Classical examples of these types of experiment are the dissociation of ribonuclease [2], insuline [3] and aldolase [4]. The first consisting of only one chain and four disulphide bridges, the second of two chains with one intra and two inter-chain disulphide bridges and the third consisting of three chains with no disulphide bridges at all.

Besides their intrinsic value for the understanding of the basic principles underlying the mechanism of formation of tri–dimensional structure in proteins, the experiments on the *in vitro* dissociation and reassociation of polypeptide chains can be a valuable tool for locating possible structural alterations in proteins made up of more than one subunit.

The hybridisation between abnormal haemoglobins after dissociation at acidic or alcaline pH is a clear-cut example of this type of approach and has become a widely used method to individuate the mutated chain usually bearing one aminoacid substitution [5].

Other well known examples of hybridisation experiments are those on the alkaline phosphatase of *E. Coli* [6] and those on 7S human gamma globulins [7].

The studies which are going to be reported here refer to some preliminary results obtained from experiments on the dissociation and reassociation of human haptoglobins and on the hybridisation of haptoglobin subunits.

The genetics of human haptoglobins has been very thoroughly studied in the last ten years, especially by Smithies and his co-workers [8] who showed that the common genetical polymorphism, as detectable by starch gel electrophoresis, involves only one of the two polypeptide chains (α and β) of which the haptoglobin molecule is formed.

Thus, so far, three common alleles have been identified: $Hp^{2\alpha}$, $Hp^{1\alpha F}$, $Hp^{1\alpha S}$, the first of which has most likely been derived through duplication events resulting from unequal crossing-over in individuals heterozygote for the last two genes.

Additional variation at the haptoglobin locus has been identified in recent years, involving rare patterns that have been grouped in two classes [9]:

(1) those with the appearance of new Hp-bands after ordinary or urea-starch gel electrophoresis,

(2) those with quantitative differences in the relative amounts of the single Hp-bands both on ordinary as well as on urea starch gel electrophoresis.

Among the first group the so-called Johnson type and the variants recently described by Robson et al. [10] are usually mentioned; whereas the second group includes the 2.1 modified [11], the 2.1 Trans [9], the 2.1 Haw [9], the Carlberg phenotype [12] and, most likely, the weak 2.2 patterns reported by Harris et al. [13] in the Ferrara family showing an aberrant segregation of haptoglobins.

The idea that the 2.1 modified and the Carlberg phenotypes could be instances of mutations affecting respectively the relative production of 1 α and 2 α polypeptide chains, has occurred to many workers in this field [14] during the last few years.

This suggestive hypothesis could in fact be efficiently tested with the kind of hybridisation experiments described above and starting from different amounts of haptoglobin 2.2 and haptoglobin 1.1 after complete dissociation.

This has been the main aim of the investigations reported below.

MATERIALS AND METHODS.

a) *Purification of Haptoglobins.*

Adequate amounts of Hp *1.1*, *2.1* and *2.2* were prepared from pooled plasma according to the procedure of Connell and Shaw [15].

The eluates from the DEAE columns were brought to 5 % saturation with ammonium sulphate at pH 7. The precipitate formed was dissolved in a small amount of distilled water, and dialysed against three changes of distilled water. Further purification was achieved by zone electrophoresis on Pevikon block in 0.05 M barbital buffer pH 8.6. Final preparations, checked by starch gel electrophoresis did not show impurities.

b) *Reduction and reoxidation of haptoglobins.*

Five to sixty mg of purified haptoglobins were reduced in a reaction mixture containing 8 M. cyanate free [16] urea, 0.5 M mercapto-ethanol (Light), 0.0134 M Na_2 EDTA and 0.5 M TRIS-HCl, pH 8.6 in a final volume of 6-18ml.

The mixture was kept under nitrogen for four hours at room temperature. At the end of this period the reduced proteins were separated from reagents by gel filtration on a column of Sephadex G. 25 equilibrated with a 0.05 M Tris-NaH_2PO_4 buffer pH 8.15 made 0.002 M in Na_2 EDTA. The reagent free protein eluted from the Sephadex column was pooled and reoxidation was allowed to take place in a large vessel at room temperature for 24 hours. Protein concentration in the eluate ranged from 0.3 to 0.6 mg/ml. The yield of protein recovered after gel filtration was close to 100% (97-100%). After

reoxidation the diluted protein solution was concentrated by ultrafiltration on collodion filters (Membran Filter Geselleschaft-Göttingen) at 4° C and stored at —20° until used.

c) *Starch gel and paper electrophoresis.*

Starch gel electrophoresis was carried out horizontally according to Poulik [17].

Reduced α and β chains from Hp 1.1 were separated by electrophoresis on 4 × 17 cm gelatinized cellulose acetate strips (« Cellogel » Chemetron Milan-Italy). The electrophoresis was performed in a Universal Shandon tank using a Na–formate buffer 1.4 M pH 1.9 and 6 mA/strip were applied for one hour.

After completion of the run the strips were stained in alcoholic Amido Black 10 B.

d) *Immunological techniques.*

Antisera against type 2.2 HpHb complex were obtained by immunizing albino rabbits with *Hp–Hb complex* in Freunds adjuvant. 4.8. mg of Hp were dissolved in 2 ml of complete Freunds adjuvants and 0.1 ml of this mixture was injected in each foot pad of 3 rabbits and 0.6 ml were given hypodermically.

After 1 week the treatment was repeated and after 13 days from the beginning of the immunization the rabbits were bled from the marginal ear-vein. Since the antisera contained antibodies against minor quantities of other serum components, they were absorbed with 100 mg/ml of a lyophilized ahaptoglobinemic serum.

The immuno-diffusions in agar gel were carried out according to the method described by Ouchterlony [18].

e) Total protein concentration was determined either with a microbiuret method [19] or, occasionally, by spectrophotometry at 280 mμ [20] in a Beckman DU.

Results and discussion.

Dissociation of haptoglobins in subunits.

Hp 1.1 consists of a single molecular species and behaves as a monomer both in starch gel electrophoresis and in the ultracentrifuge, whereas Hp 2.2 and Hp 2.1 consist of a series of stable polymers of increasing molecular weight [21]. In spite of this difference it is most likely that all haptoglobin types share the same fundamental unit composed of two α (M.W. = 8.850) and probably two β–chains (M. W. = 40.000) held together by disulphide bridges [22].

The stability of Hp polymers in presence of 8 M urea [8 *b*] and the possibility of reducing them to monomers following treatment with mercaptoethanol [23] suggests that also the polymerization is dependent on the existence of one or more disulphide bridges between monomers.

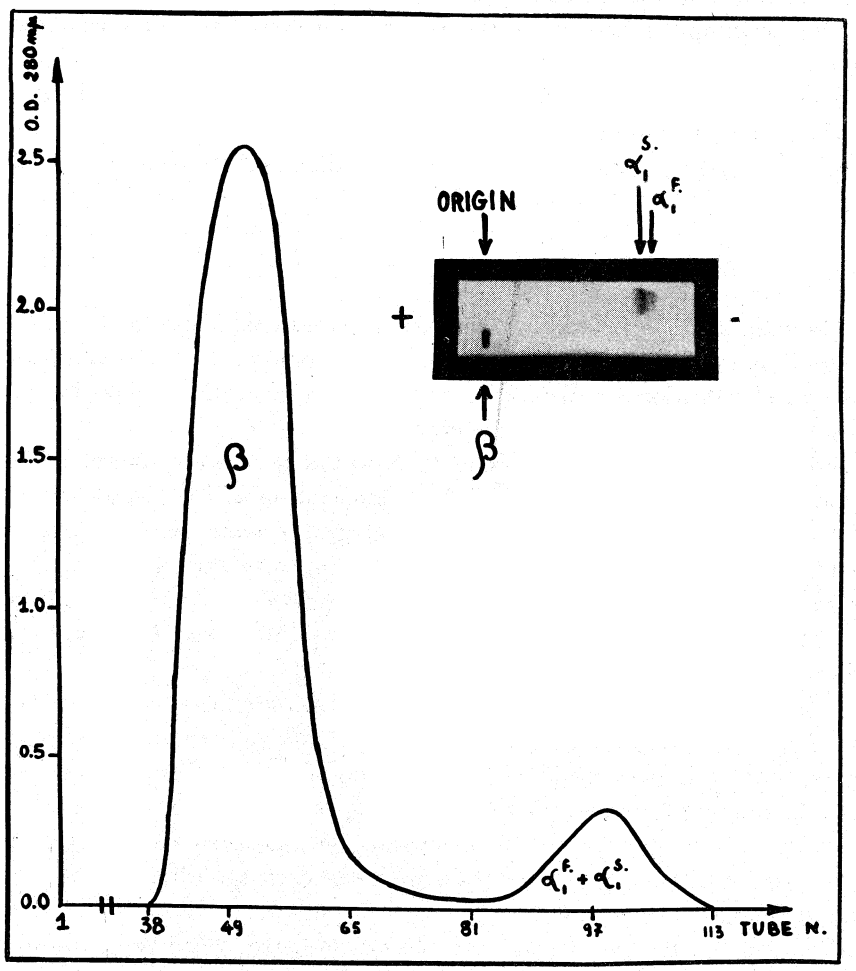

Fig. 1. – Cromatography of reduced Hp 1.1 on a column (120×1.5 cm) of Sephadex G 75 equilibrated with 5 % acetic acid. Flow rate = 8 ml/h.

The photograph shows the electrophoresis on Cellogel of the two concentrated peaks (see text for details).

Thus the first step of our study was to prove the complete dissociation of native Hp into α and β–chains, under the experimental conditions described above.

For this purpose 58 mgr of Hp 1.1 were reduced (see methods) and submitted to gel filtration on column of Sephadex G 75 equilibrated with 5 % acetic acid. In these conditions the reduced haptoglobin was resolved in two well distinct peaks (see graph in fig. 1), the faster (β–chain) being about 80 %

of the total protein put on the column. The central portions of each peak were concentrated by rotary-evaporation and submitted to electrophoresis on cellogel for a better discrimination of the Hp–chains and for controlling their purity (see photo in fig. 1): it may be noted that the β–chain has remained at the origin while the α–chain is splitted in two bands (the 1 F α and the 1 S α) for the simple reason that the purified protein had been prepared from a random pool of Hp 1.1 sera.

From these results, which are similar to those reported by Bearn [22], it is clear that a complete cleavage of Hp 1.1 molecule into its constituent polypeptide chains, has been obtained. Similar results were obtained with Hp 2.1.

Reduction-oxidation experiments.

The next step consisted in attempting to reassociate unfolded and reduced haptoglobins of the three common types after removal of the denaturing reagents by gel filtration on Sephadex G 25 and re-oxidation according to techniques described in Material and Methods.

Since the preliminary experiments reported here were meant to investigate only the qualitative aspects of the reassociation of the haptoglobin molecule, we cannot thoroughly define at this stage the exact experimental conditions necessary for an optimal reactivation, nor specify with accuracy the recovery of the native protein. It is possible, however, to anticipate that:

(1) the recovery of the active protein in the described experimental conditions is certainly not below 70–80%;

(2) the highest recovery of functional haptoglobin and the best reintegration of the starch gel electrophoretis patterns can be obtained when the re-oxidation is performed in the presence of 3×10^{-3}M mercaptoethanol and when the concentration of the protein in the medium is increased.

In fig. 2, which reports the electrophoretic pattern of the re-activated proteins 1.1, 2.1, 2.2 run side by side with the corresponding native samples, it can be clearly seen that:

(1) the recovered proteins are always showing a full restoration of the haemoglobin binding capacity;

(2) the original electrophoretic pattern of the 1.1 type is fully re-integrated, but those of the 2.1 and 2.2 types show a greater amount of light polymers;

(3) in all types of reactivated proteins, but especially in the 2.2, and the 2.1, there is a certain amount of high molecular weight material with slow mobility and an appreciable haemoglobin binding capacity;

(4) an additional fast migrating and haemoglobin binding component, whose nature is under investigation, is also present in the reactivated samples of all types. The mobility of this component is faster than that of free haemoglobin on starch gel and its elution volume on a Sephadex colum G 200 is intermediate between those of free haemoglobin and Hb–Hp 1.1 complex.

Besides the electrophoretic behaviour and the haemoglobin binding capacity, the criteria of nativeness included a double diffusion test on agar gel to compare the reactivity of the re-activated proteins with that of the native haptoglobins against a specific anti-haptoglobin 2.2 serum prepared as described in Material and Methods.

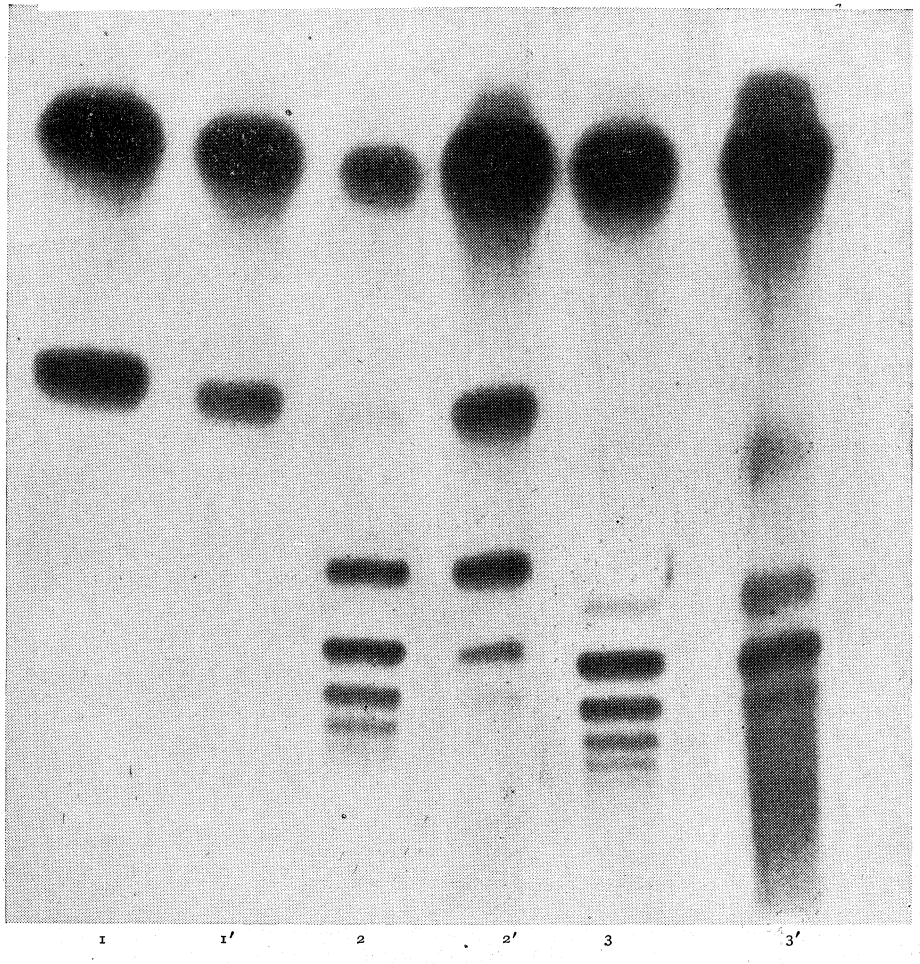

Fig. 2. – Starch gel electrophoresis of native and reactivated Hp. Before the run haptoglobins were saturated with suitable amounts of pure HbA.

1=Hp 1.1 native 1'—Hp 1.1 after reduction and reactivation
2=Hp 2.1 native 2'=Hp 2.1 » » » »
3=Hp 2.2 native 3'=Hp 2.2 » » » »

With this test it was possible to establish not only that the reactivated proteins gave the expected precipitation lines (fig. 3), but also that the fast migrated component does not react with the anti-Hp serum (partially degraded Hp?).

Hybridization experiments.

Having established the possibility of reactivating the native haptoglobins from the reduced material, we tried to obtain the haptoglobin 2.1 by *in vitro* hybridization of Hp 1.1 and Hp 2.2 sera.

Thus mixtures of purified 1.1 and 2.2 native haptoglobins were reduced and re-oxydized with the already described procedures, and the reactivated protein obtained from each experiment was concentrated by ultra filtration and run on starch gel electrophoresis side by side with the original mixture of native haptoglobins.

These experiments were performed in series on a total of 10 mgs of haptoglobins per each experiment, but using each time different amounts of 1.1 or 2.2 haptoglobins.

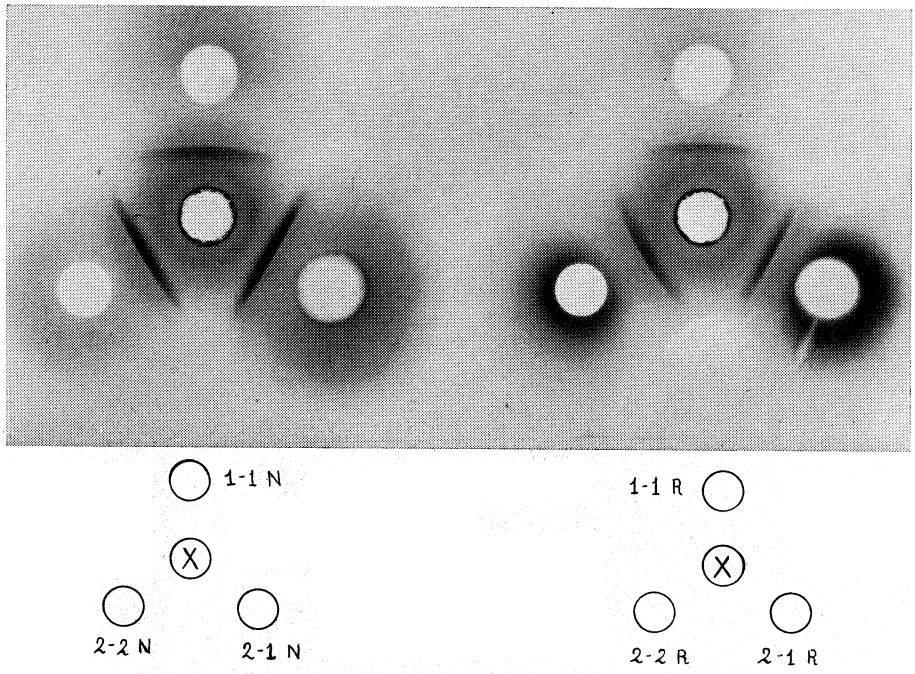

Fig. 3. – Reaction of native and reactivated Hp with specific anti–Hp 2.2 antiserum. See scheme reported.

N=native Hp.
R=reduced and reoxidized Hp.
X=anti–Hp 2.2 antiserum.

Fig. 4 summarizes the critical data demonstrating beyond reasonable doubt the occurence of hybridization and shows also the striking differences in the electrophoretic pattern of the reactivated material when the starting mixture departs significantly from a 50 : 50 ratio.

It is important to emphasize at this point that the electrophoretic pattern of the hybrid obtained from a mixture with equal amounts of Hp 2.2 and

Hp 1.1, though not entirely corresponding to that of the native 2.1 phenotype, is however identical to the pattern observed after dissociation and reassociation of the native Hp 2.1 (see fig. 2). This suggests that in normal conditions the haptoglobin polymers observed in a 2.1 individual must consist of approximately equal amounts of Hp 2 and Hp 1 monomers, as on the other hand it

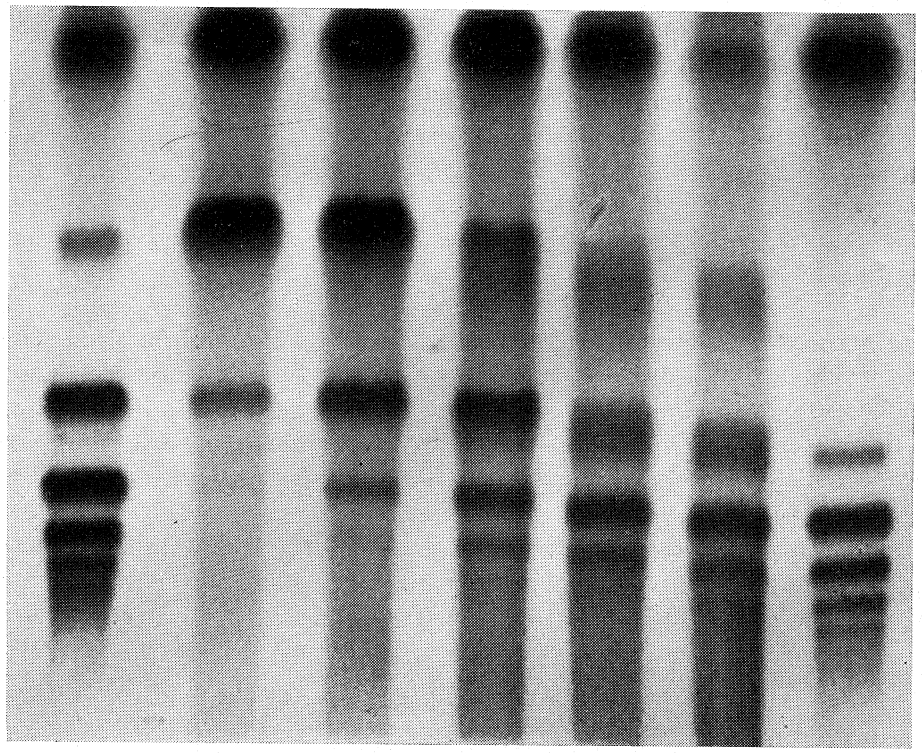

Fig. 4. – Benzidine stained starch gel electrophoresis of mixtures of Hp 1.1 and 2.2 after Hybridization.

1 = Native Hp 2.1
2 = 90 % Hp 1.1 + 10 % 2.2 after reduction and reactivation
3 = 75 % Hp 1.1 + 25 % 2.2 » » » »
4 = 50 % Hp 1.1 + 50 % 2.2 » » » »
5 = 25 % Hp 1.1 + 75 % 2.2 » » » »
6 = 10 % Hp 1.1 + 90 % 2.2 » » » »
7 = Native Hp 2.2

can be argued from the fact that the subtyping of the 2.1 haptoglobins yields 1 α and 2 α chains in approximately equal amounts.

But one of the most interesting aspects of the experiments summarized in fig. 4 is that when the original mixture included a greater amount of Hp 1.1, the pattern of the resulting reactivated protein resembled the so-called 2.1 modified phenotypes. In the course of our experiments we were able to reproduce very clearly at least two of them, merely the 2.1 mod. (*d*) and the 2.1 mod. (*c*) of the series reported by Sutton [11 *c*).

On the contrary a gradual increase of Hp 2.2 in the original mixture produced a reactivated protein with an electrophoretic pattern very similar to that of native Hp 2.2 but with the unquestionable presence of a least one band of the 2.1 phenotype (fig. 4). Such a pattern is similar to that described for the so-called Carlberg phenotype [9], though in the present instance the fastest band is not exactly located in the 1.1 position as happens for the Carlberg phenotype. Further investigations are in progress to elucidate this point.

Even at the present stage of the investigation we feel, however, confident to conclude that the hybridization experiments reported above permit us to support the hypothesis that all the so-called 2.1 modified mutations (probably including Hp 2.1 Trans and Hp 2.1 Haw) as well as the Carlberg phenotypes may be interpreted in a unitary manner as mutations affecting in some way the rate of synthesis of Hp^2 and Hp^1 genes.

Thus a relative excess of Hp^1 monomers, in a 2.1 heterozygote, would lead to the formation of a 2.1 modified phenotype whereas the opposite situation would lead to a Carlberg phenotype. In the extreme case of a heterozygote individual with a very low production or complete suppression of one of the two monomers, the end-result would probably be a weak Hp 2.2 pattern, if the Hp^1 gene is suppressed, or a nearly normal 1.1 pattern if the Hp^2 gene is suppressed.

Probably one of these two alternatives may be the explanation of the aberrant segregation of haptoglobins observed in the pedigree, described by Harris et al. [13].

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