

IV DISCUSSION

4.1 SEPHADEX-ACRYLAMIDE GEL ELECTROPHORESIS

A number of techniques for the high resolution separation of complex protein mixtures have already been developed. These include isoelectric focusing for pI determinations, and electrophoresis on various types of gel media, such as polyacrylamide, agarose and starch gel. All of these techniques and separation media are discussed comprehensively by Gordon (1975). Surprisingly, little consideration seems to have been given to the modification of the resolving power of such techniques by the incorporation of other materials into the original medium, which may then change the physical basis of the separating process and thereby result in a better separation of the proteins. As was pointed out before (see Section 1.2), electrophoresis in acrylamide gels containing agarose has been described (Uriel, 1966), but the resolving power was not significantly different from that of the conventional acrylamide gels. The addition of Ampholyte to acrylamide has been described and used by Kotbol (1970) and others with great success. Sephadex has also been used as a medium for electrophoresis by Whitehead *et al* (1971) and Johansson and Rymo (1964). Radola (1973) performed isoelectric focusing in layers of granulated gels using Sephadex and Biogel.

In the newly developed sephadex-acrylamide gel described in this study, Sephadex G-25 (Superfine) is used primarily as a mechanical stabilizer for low concentration polyacrylamide gels. Because of the low concentration of acrylamide in the sephadex-acrylamide gel, separation of the proteins (at pH 8.6) is achieved primarily on the basis of molecular charge, which is to be expected in low concentration polyacrylamide gels (Chrambach & Rodbard, 1971; Johansson, 1972). A pure polyacrylamide gel at such a low concentration is, however, practically impossible to handle. By determining sectional nitrogen and monosacharide concentrations of the sephadex-

acrylamide gel along the length of the gel, the existence of opposing acrylamide and sephadex gradients in the gel was established. The sephadex-acrylamide gels were frozen, sliced into 2 mm sections and weighed (Hattingh *et al*, 1978). The nitrogen and monosaccharide contents of each slice were then determined. The sephadex-acrylamide gels showed a marked decrease in nitrogen content corresponding to the area of the gel where the Sephadex beads are first found, followed by a non-linear two phase increase (Fig. 40). The monosaccharide content of the mixed gels showed the reverse of this pattern.

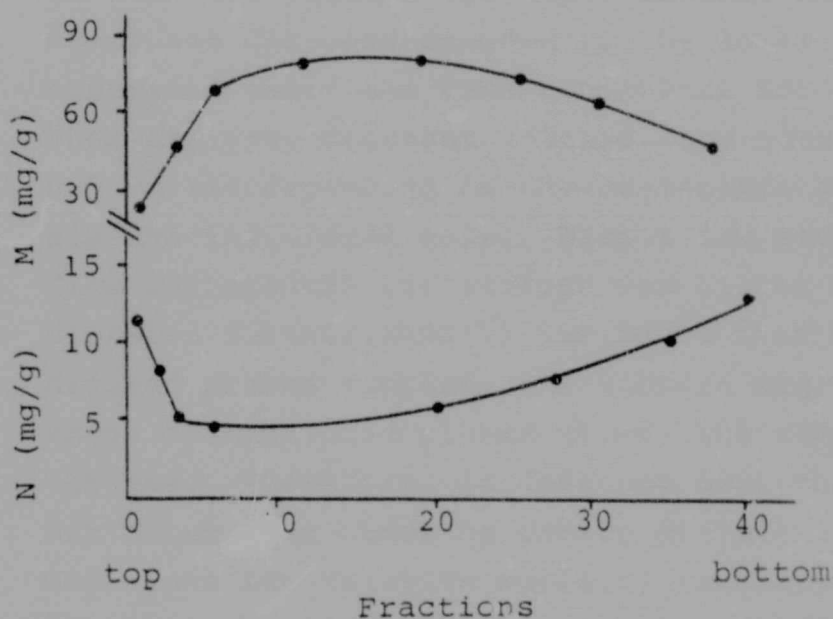


Figure 40

Nitrogen (N) and monosaccharide (M) gradients in sephadex-acrylamide gels (mg per gram of gel). After Hattingh *et al* (1978).

Most of the present results were obtained at pH 8,6. Changes in buffer pH from 7,0 to 9,0 did not yield results similar to those obtained at pH 8,6. Separation of albumin into two subfractions could not be achieved at pH 8,0, whereas the positions of the albumin subfractions as separated at pH 8,6 were found to be reversed at pH 7,0 (see section 3.1).

The reasons for these results are still not clear since the sephadex-acrylamide gel apparently separated proteins according to pI at pH 8,6 (Hattingh *et al*, 1978).

It would thus seem that the lower albumin has a lower pI than the upper albumin fraction, the pI

of the latter being 4,7 for human albumin (White et al, 1968). Further studies are required to elucidate the factors responsible for this condition in the present results, since they appear to indicate that other factors besides pI may also contribute to the electrophoretic mobilities of proteins in sephadex-acrylamide gels.

When using body fluids such as aqueous humor and CSF, which have a very low protein concentration, a constant current was applied during electrophoresis. This was done because at constant voltage the upper albumin bands of aqueous humor and CSF corresponded to the lower plasma albumin separated under the same conditions for the same animal. When applying constant voltage, the current through each gel varied depending on the resistance of the protein sample and individual gels. With a low protein concentration (low resistance) the current was higher and the proteins migrated further. Thus it was found that when electrophoresing diluted plasma samples, the albumin migrated further than those of undiluted plasma under the same separating conditions. Therefore, it does not mean that the upper albumin of aqueous humor or CSF is different from that of plasma, since its relative mobility is the same as that of lower plasma albumin. It was also found that the aqueous humor or CSF albumin band stained with BCG, indicating that it is the upper albumin. When applying constant current, the upper albumin bands of aqueous humor, CSF and plasma migrated to the same position under the same conditions.

When using sephadex-acrylamide gel for the separation of body fluid proteins of various animals, the results showed that a higher degree of protein resolution could be obtained than when conventional means of acrylamide gel electrophoresis is applied. Another advantage of this gel is that it is not time consuming for the estimation of pI of proteins (at pH 8,6) and, as with the polyacrylamide gel, only small volumes of proteins are required, a factor which may be important when valuable or expensive specimens are used.

The greatest disadvantage of the gel is that it is very soft and easily stretched, which causes difficulty in handling. Because it is opaque, conventional scanning is impossible whilst this characteristic also renders accurate photography difficult. In addition, the visual resolution of the proteins is poor in comparison with that of acrylamide gels.

However, by doing CIE, it could be seen that all the proteins were present in the sephadex-acrylamide gel and also that they can be quantitated by this method, since the area enclosed by the precipitate is proportional to the antigen concentration. This of course, only applies when the "rockets" are run to completion (Clausen, 1971). Several experiments were performed with varying ratios of antigen to antibody during the 2,5 hour CIE. The optimum ratio was used where the "rocket" peaks were fully grown during the 2,5 hours. At higher ratios the albumin "rocket" had migrated off the agarose plate while at lower antigen/antibody ratios the "rocket" peaks were too small for meaningful comparison.

4.2 HETEROGENEITY OF ALBUMIN

Albumin is one of the most comprehensively studied proteins mainly due to its ready availability in relatively large quantities. Reports indicate that human serum albumin, apart from its well known heterogeneity in the low pH range (below pH 4) which results in alteration from the normal form to a faster migrating form (N-F transition; Foster, 1960 ; Foster et al, 1965), may also be heterogeneous electrophoretically (Graber et al, 1960; Reinek et al, 1963), chromatographically (Janatova et al, 1968), isoelectrically (Reis & Wetter, 1969; Spencer & King, 1971) as a result of aging (Rosenoer et al, 1977) and also due to various physical manipulations such as dye binding, drugs, decay of fluorescence

and others (Peters, 1980). This, however, is not the classical condition of bisalbuminemia, but may be a transient phenomenon. A question that has often arisen, is whether human serum albumin is heterogenous in vivo or whether the observed heterogeneity results from preparative techniques. Electrophoretic and immunoelectrophoretic studies on normal human serum (Laurell & Niléhn, 1966), would seem to suggest that it might contain more than one species of albumin. The only reports of two distinct albumin fractions under normal conditions are those by Merler et al (1962) and Rotbol (1970), both of which studied urinary albumin. A multiplicity of albumin bands using a mixture of ampholytes having varying isoelectric points, have also been demonstrated by Kaplan and Foster (1971), Reis and Wetter (1969) and Wallevik (1973). Valmet (as quoted by Rosenoer et al, 1977) attributed this resolution mainly to fatty acid contaminants in the albumin, finding a distinct difference in pI between fatty acid-containing and fatty acid-free albumin components, but others have not observed such significant effects.

The two distinct albumin fractions obtained with SAGE of the body fluids studied here seem to be two species of molecules with the same molecular masses differing from one another only in pI, the lower albumin having a lower pI than the upper albumin. This was previously also proposed by Rotbol (1970), reporting a second urinary albumin with a lower pI as determined on acrylamide gel containing Ampholytes at pH 3 to 10.

From results obtained with the isolated lower albumin (human) as compared to BSA, the hypothesis is put forward that the lower albumin may be an unbound albumin, i.e. one which is a carrier molecule but is not loaded at the time of investigation (see section 3.1.4.1). The physiological importance of these observations lies in the possibility that the circulating albumin concentration may be underestimated by dye-binding assays, as can be seen from the results obtained with

BcG (see Table 2).

It has been shown that interstitial fluid has a relative higher concentration of the lower albumin as compared to the upper albumin than has plasma (see section 3.2.2.3). It would seem reasonable to assume that interstitial fluid albumin contains relatively more unbound albumin than does plasma, due to the nutrient requirements of the tissues, and that the lower albumin therefore represents an unbound molecule or one which is bound to metabolites. These suggestions are, however, not conclusive and further studies are necessary in order to ascertain the functions and characteristics of the lower albumin.

The ability of the lower albumin to combine with dyes such as Amido Black, seems to be much more variable than that of the upper albumin (final staining intensity). After staining with Amido Black it would appear that the lower albumin is less concentrated than the upper albumin. However, in doing CIE, the upper and lower albumin can be shown to be of equal concentration when the rockets are run to completion, proving that Amido Black does not stain both types of albumin to the same degree. It was also found that the lower albumin does not bind at all to dyes such as BcG, BpR and BpB. The effect of BpB added to plasma before SAGE seems to be dependant on the final BpB concentration (Section 3.1.3). Presumably, at high concentrations of BpB all of the upper albumins are bound to BpB,

which thus increase their mobility, and therefore it seems as if the upper and lower albumins are not different. By decreasing the BpB concentration, only a certain percentage of the upper albumin is bound, thus resulting in two different mobilities of the upper albumin. This would then lead to the result of three visually distinguishable albumin bands (one lower and two upper) after staining with Amido Black (section 3.1.3).

From the results obtained with BpB during PAGE, it can be seen clearly that adding of BpB to the plasma caused a definite artefactual increase in the densitometric estimation of albumin concentrations (Fig. 1). This effect has also been pointed out by Schultze and Heremans (1966). Erroneously high values for albumin when using BcG has also been reported by Webster *et al* (1974). When using BpB or BcG as albumin prestainer during PAGE, it was possible to determine that the dye-bound albumin represents a faster migrating portion of the albumin band as shown by scanning of the acrylamide gels at the specific wave-lengths for the dye involved. This faster migrating section was also found to correspond to the upper albumin during SAGE. Since BpB is a negatively charged molecule, it will bind to positive groups (Lind *et al*, 1974) of the albumin and result in a more negatively charged albumin which will, at an alkaline pH, have a higher relative mobility and move faster than the unbound albumin. The resultant electrophoretic heterogeneity of the albumin will, however, seldom reach such proportions as to mimic bisalbuminemia (Schultze & Heremans, 1966).

Bromo-phenol Blue is an organic compound (Conn, 1977) and therefore dissolves in plasma. The densitometric increase of albumin concentration with increasing concentration of BpB (see Table 1) was found to be significant ($P < 0,001$). Due to this erroneous increase in estimated albumin, it was decided not to use any dyes during electrophoretic studies of body fluids reported here.

Transferrin and α_2 -macroglobulin were found to be two closely associated prominent protein fractions in the central regions of the sephadex-acrylamide gels (Figs. 2 and 9). By CIE the upper of the two fractions was identified as transferrin, and the lower, faster migrating one, α_2 -macroglobulin. In only a few human plasma samples did the two fractions migrate as one (Fig. 8). By doing CIE it could be seen in such cases, that both proteins were present, the one within the other. As with albumin, the different positions of transferrin could be due to binding of ions. Transferrin labeled with radioactive iron migrated slightly further on the sephadex-acrylamide gel. A prominent "shoulder" towards the anodal side of the gel appeared on the transferrin rocket after CIE (Fig. 8). The slight cathodal "shoulder" (Fig. 8) also seems to be more noticeable after iron labelling. These results could indicate that not all the transferrin transports iron to the same extent and that the molecular charge of transferrin might be altered when transferrin binds to iron (Fig. 8).

In summary, the sephadex-acrylamide gel can be described as a low concentration acrylamide gradient gel, mechanically supported by Sephadex beads. This provides a high resolution gel medium, separating certain proteins into more components than is possible with conventional polyacrylamide gels. This gel was found to be of value for studying changes in the albumin molecule during stress conditions (see Section 3.2.2). The results obtained with this gel medium indicate the possible existence of protein fractions or sub-fractions in plasma, which have not yet been identified and may on identification lead to a better understanding of their characteristics and functions (see also Section 4.5).

4.3 BODY FLUID PARAMETERS

During this study, plasma samples were stored frozen for long periods of time. It has been reported that repeated

thawing of frozen plasma has an adverse effect on the physical and biochemical properties of plasma proteins (Pensinger *et al*, 1959; Schultze & Heremans, 1966). For this reason, the plasma samples used in this study were divided into a number of aliquots of small volume before freezing. When needed for experimental purposes, an aliquot of plasma was used and then discarded. In this way the same plasma sample could be used repeatedly without repeated thawing and freezing thereof. It was established that plasma or serum samples kept frozen were not adversely affected, regarding protein concentration or electrophoretic separation pattern, for periods of up to 18 months.

The results obtained with plasma, sampled from various animals, all revealed a double albumin fraction after SAGE. In all cases the albumin was represented by a single band on PAGE. An exception was horse plasma where at least two major albumin fractions could be observed, as well as a few minor fractions with greater mobilities on the acrylamide gel (Fig. 19). However, during SAGE only the characteristic double albumin fraction could be demonstrated. An explanation of these results at present can be, at best, speculative and by no means conclusive. It may possibly be that the different protein fractions observed in horse plasma on acrylamide gel in the albumin region, are indeed all representative of the albumin component. The continuous nature of the albumin rocket peaks enclosing the more mobile protein fractions in the albumin region (see Fig. 20) proves that these fractions are albumin. Polymorphism of horse plasma albumin has been demonstrated by several workers (e.g. Osterhoff *et al*, 1974), but the reason why polymorphism of the horse would be indicated during PAGE and not for sheep (polymorphism of sheep albumin as indicated by Tucker, 1975), or cattle (Osterhoff, 1968), cannot be explained. In the context of the results obtained with SAGE where the albumin component consisted of two major fractions only, it would appear that the lesser fractions observed on the polyacrylamide gel are species of albumin molecules similar to the major albumin fractions in pI, but differing in molecular mass.

This heterogeneity of albumin was also verified by crossed-immunoelectrophoretic studies. From these results it became clear that the phenomenon of albumin variants was not species-specific nor was it restricted in its distribution amongst body fluids within a species.

Body fluid protein concentrations determined in this study were found to compare favourably with those reported in the literature, with few exceptions. The protein concentration of $79,7 \pm 0,76$ g/l for bovine plasma reported here is in agreement with the $75,0 \pm 5,8$ g/l reported by Kawamura *et al* (1974). Conversely, a much lower protein concentration of 41,0 g/l was reported by Pappenheimer and Soto-Rivera (1948) for bovine plasma. Plasma protein concentration of the pig ($69,3 \pm 5,5$ g/l) was found to be similar to the average 67 g/l reported by Cartwright *et al* (1948). Similarly, a plasma protein concentration of $75,6 \pm 2,75$ g/l for the horse strongly supports a previous report of $75,3 \pm 1,6$ g/l (Kirk *et al*, 1975).

Gibson and Segal (1975) reported a plasma protein concentration for the dog of $72,8 \pm 2,6$ g/l which is substantially higher than the $50,6 \pm 0,84$ g/l reported for the same animal in this study. However, plasma protein concentrations for the dog of $49,0 \pm 6,0$ g/l (Vreim & Staub, 1976) have also been reported and compare favourably with the results of this study. The plasma protein concentration of the cat reported here ($63,0 \pm 5,3$ g/l) was also found to be in agreement with the $63,5 \pm 4,8$ g/l reported by Schultze *et al* (1972). Similarly, the rabbit plasma protein concentration reported in this study is well within the range of values reported in the literature for the same animal (see Table 14). A protein concentration of $2,6 \pm 1,64$ g/l for rabbit CSF was found in this study and is in agreement with the 2,9 g/l reported by Cserr *et al* (1972). These results indicate that the protein concentration of 0,3 g/l for rabbit CSF reported by Davson (1967) is not representative for the species.

Rabbit lymph protein concentration determined in this study ($41,7 \pm 6,7$ g/l) was found to be substantially higher than the 24,7 to 30,0 g/l previously reported for the same animal species (Benson *et al*, 1955; Lewis, 1969; Bach & Lewis, 1973; Rutili & Arfors, (1977) and Tibes *et al* (1977)). Although most results reported in the literature were obtained on muscle lymph, whilst this study was based on lymph collected from the femoral lymphatic duct, it is unlikely that the differences could be attributed to this factor alone. Since the lymph in the femoral lymphatic duct originates from both the muscle and the cutaneous area, and since lymph from the cutaneous area is reported to have a protein concentration less than that of muscle lymph (Bach & Lewis, 1973), femoral lymph could be expected to have a lower protein concentration than muscle lymph. The differences are more likely due to differences in period of time during which lymph was collected. In the present study, lymph was collected directly after cannulation of the lymph duct for periods not exceeding 15 min, whereas the results reported in the literature are based on lymph samples collected over periods of time exceeding 60 min. It is reasonable to assume that, during the period of cannula insertion, lymph accumulated in the lymph ducts distal to the operative area as a result of the incomplete return of lymph due to inactivity of the anaesthetized animals, could account for the differences reported. During passive movements of the legs of anaesthetized animals by manipulation, a lymph protein concentration of 31,0 g/l was recorded, which compares favourably with the 30,0 g/l reported by Tibes *et al* (1977).

4.4 PHYSICAL EXERCISE, HEAT STRESS AND FEVER CONDITIONS

4.4.1 Physical Exercise

4.4.1.1 Controlled laboratory conditions

Reports on comparative haematological data of physically trained and untrained subjects during periods of stress are relatively scarce. In this study, no significant difference between the haematocrits of physically trained and of untrained human subjects could be demonstrated. It was also found that no significant change in haematocrit of either group occurred during physical stress. These results are contradictory to those of Tibes *et al* (1974), reporting a significant increase in haematocrit of physically trained and untrained human subjects during periods of physical exercise. The reasons for the difference in results obtained in this study and those reported by Tibes *et al* (1974) are, at present, not clear. Although excessive water loss during physical exercise may explain increased haematocrit due to haemoconcentration, excessive water intake during exercise may cause haemodilution, resulting in a decreased haematocrit.

From the results in Table 16 it can be seen that the A/G ratios of the untrained subjects and that of the same subjects after the fitness course, is not significant. During physical exercise, a significant increase in A/G ratio of the physically trained as well as the untrained subjects occurred ($P < 0,05$). This could indicate that their circulating albumin increased relative to the globulins.

De Lanne et al (1958) also reported an increase in the albumin concentration during muscular activity, while Poortmans (1971) reported an increase in both albumin and globulin concentration.

It was reported by McAlpine et al (1971) that changes in the electrophoretic pattern of plasma proteins from physically untrained humans occurred in the β -globulin region following physical exercise. In this study, however, similar results could not be obtained with the polyacrylamide gel medium. No difference in electrophoretic banding patterns of the plasma proteins before and after exercise of either physically trained or untrained subjects could be demonstrated on this gel medium. However, by determining the % composition of each protein, more conclusive results would be available.

The results obtained with SAGE, appear to be of more interest. A marked increase in the lower albumin of the physically untrained subjects after exercise was found to occur. A similar significant change in lower albumin during exercise could not be demonstrated for physically trained subjects. Seen in the context of the characteristics of the lower albumin reported elsewhere in this study, it would appear that loading of the lower albumin (unloaded) was more complete in the physically trained subjects, thus explaining the increased lower albumin (unloaded) in the untrained subjects after physical exercise (see also Section 4.5).

4.4.1.2 Field conditions

Results obtained on the blood parameters of athletes participating in strenuous physical exercise under field conditions,

were in accordance with those obtained during a training programme (Section 4.4.1.1). A significant decrease in Hct ($P < 0,001$; $P < 0,005$) for athletes running a distance of 42 km was found in this study. This could, however, not be demonstrated in subjects participating over longer (96 km) or shorter (32 and 16 km) distances. An increase in Hct after a race was reported by most workers in the field (Astrand & Saltin, 1964; Poortmans, 1971; Maron *et al*, 1975; Davis *et al*, 1976; Noakes & Carter, 1976). At present a satisfactory explanation for the fact that the present results differ from those reported in the literature, cannot be given. Nor is it clear why only the 42 km race yielded significant results. From these results it may be concluded that studies on blood parameters during physical exercise, would have to take into account the water loss and water intake of the subjects during the period of physical exercise. If no water, or an amount less than that lost through perspiration, was taken *ad libitum* during the race, haemoconcentration and an increased Hct can easily be explained.

Since the blood flow and capillary hydrostatic pressure increased during exercise (Kjellmer, 1964), this results in a movement of fluid from the capillary blood into interstitial fluid and then into lymph (White *et al*, 1933). The latter authors also reported an increased lymph flow from the area of activity as a result of muscle movement. Thus the protein present within muscle interstitial space is transferred back to the vascular circulation resulting in an increased protein concentration and Hct. The idea of protein transfer was presented by Senay (1972). Röcher *et al* (1976) similarly reported that the change in plasma protein concentration is also due to a protein redistribution between the intravascular and extravascular pool, and not only due to transcapillary fluid shifts.

An increase in plasma protein concentration after muscular activity has been reported by Ebert and Stead (1941), Bojanovski and Filip (1956), De Lanne et al (1958), Tiblin et al (1966), Haralambie (1969), Poortmans (1971), Tibes et al (1974), Maron et al (1975), Senay and Fortney (1975) and Noakes and Carter (1976). This increase in protein concentration could be due to a filtration of water out of the vascular bed (Barcroft & Kato, 1915; Landis, 1931; Kaltreider & Meneely, 1940; Ebert & Stead, 1941; Nylin, 1947; Adolf et al, 1947, Holmgren, 1956; De Lanne et al, 1958; Saltin, 1964; Kozlowski & Saltin, 1964; Ekelund, 1967a). Tissue breakdown has also been hypothesized to be the cause of an increased plasma protein concentration (Poortmans, 1971). Thus the interstitial fluid volume increases and this will lead to an increased lymph flow (Rusznayak et al, 1967) containing extravascular protein that has been shown to exist in considerable amounts in the cutaneous tissue spaces (Schultze & Heremans, 1966). Consequently the delivery of protein to the vascular volume by way of the lymph ducts would increase (McCarrell, 1940). In this study, however, no significant changes in plasma protein concentrations could be demonstrated. More extensive studies are necessary in order to satisfactorily explain the anomalous results.

As was the case with physically trained subjects under laboratory conditions, no definite change in electrophoretic banding pattern of athletes during physical exercise could be observed on either acrylamide or sephadex-acrylamide gel.

The plasma albumin component of athletes was represented mainly by an upper albumin (during SAGE), with a less prominent lower albumin which was either unchanged or slightly decreased after the race. In one case (subject

no. 30) a lower albumin fraction present in the resting plasma could not be detected after the race (Fig. 31). These results agree with those obtained with human subjects after a physical training programme and lend support to the idea that the upper and lower albumins respectively, represent loaded and unloaded albumin molecules. In the case of athletes, being adapted to regular physical exercise, loading of the albumin molecules with nutrients could be expected to be efficiently accomplished even during exercise, and therefore no significant increase in lower albumin occurs during physical exercise.

4.4.2 Fever Conditions

The lack of published information on the effects of fever on body fluids of animals, appears to indicate that they have not been extensively studied. In this study, no significant changes in Hct or plasma A/G ratios of rabbits were found to occur during a period of endotoxin-induced fever. Haematocrits and plasma A/G ratios both before and during fever were also found to be well within the respective ranges reported in the literature (see Section 3.2.1.9). A decrease in A/G ratio has been reported by Rosencr et al (1977, p 244) which is due to an immediate outpouring of globulins, fibrinogen and haptoglobin, and a decrease in albumin synthesis.

Although electrophoretic studies (on acrylamide and sephadex-acrylamide gel) as well as CIE, failed to demonstrate any significant changes in plasma proteins during fever, electrophoresis of plasma labeled with radioactive tryptophan indicated an increase in the lower albumin component during fever. In view of the contradictory results obtained, the latter should be regarded with caution.

Bovine serum albumin in itself was found to cause fever in rabbits. Despite a report by Bito (1977) to the effect that BSA may be contaminated with bacterial endotoxin, it was shown that BSA purified from bacterial endotoxin by column chromatography, was still pyrogenic in rabbits (Hattingh *et al*, 1979). It is quite possible that the lower albumin component may be involved as a carrier for pyrogen in the mechanism of fever.

4.4.3 Heat Stress

During this study an average plasma protein concentration for the rat prior to heat exposure was found to be $73,8 \pm 14,8$ g/l. Values reported in the literature are slightly lower e.g. $61,2 \pm 1,9$ g/l (Aukland & Fadness, 1973) $56 \pm 4,4$ g/l (Aukland & Johnson, 1974) and 57,0 g/l (Lieberman *et al*, 1972). These differences could be related to different rat species, sex, seasonal variations (Bass & Herschel, 1956; Bazett *et al*, 1940) etc.

For interstitial fluid a protein concentration of $29,5 \pm 8,8$ g/l was measured. Interstitial fluid protein concentrations of $34,4 \pm 3,0$ g/l, $28,8 \pm 4,2$ g/l and 34,0 g/l (Aukland & Fadness, 1973; Aukland & Johnsen, 1974; Lieberman *et al*, 1972 respectively) have been reported which is slightly higher than the concentration reported in this study. The results have also shown a significant decrease ($P < 0,001$) in Hct of rats during a period of heat stress. These results indicate haemodilution in the rats during a period of heat stress, probably due to excessive water intake. According to Senay (1975) this haemodilution will persist if the water balance is maintained, and if it is not maintained, the subjects are allowed to dehydrate and haemoconcentration will occur (Kozlowski & Saltin, 1964). Although Walshe and Gilles (1962) and Whittow (1965) could not find a significant decrease in Hct during prolonged heat exposure, reports of

haemodilution and decreased Hct during heat exposure are manifold (Bazett et al, 1940; Glickman et al, 1941; Stein et al, 1948; Best & Taylor, 1955; Bass & Henschel, 1956; Senay & Christensen, 1965; Senay, 1973; Senay & Fortney, 1975; Noble et al, 1976). A possible explanation for this could be that during heat exposure vasodilation occurs (Bass & Henschel, 1956), and as a result of an inadequate blood volume to this change, the hydrostatic pressure drops causing protein-free (Senay, 1970) interstitial fluid to move into the vascular circulation.

Following the first week of heat exposure, it was found in this study that plasma and interstitial fluid protein concentrations decreased concurrently. A similar decreased plasma protein concentration has also been reported (Senay, 1972). The decrease in plasma protein concentration (although not significant) could be as a result of haemodilution due to excess water intake, although water intake was not measured. During the successive weeks, plasma protein concentration did not change significantly, although interstitial fluid protein concentration increased significantly ($P < 0.05$).

Both plasma and interstitial fluid A/G ratios appeared to increase during the first week of heat exposure, followed by a subsequent decrease. These results were significant in the case of interstitial fluid ($P < 0.001$), whereas no significant changes throughout the entire period of heat exposure occurred in plasma A/G ratio. This significant decrease in A/G ratio for interstitial proteins seems to be due to an increase in globulin concentration (see Fig. 38). From these results it may be concluded that a redistribution of body fluid proteins occurs during heat exposure. In this regard it has to be considered that decreased capillary permeability to albumin as a result of heat exposure has

been reported (Borut et al., 1972; Horowitz & Borut, 1973) and an increase to albumin permeability (Senay and Christensen, 1965, 1968).

By means of CIE following electrophoretic separation on sephadex-acrylamide gel, it was shown that the lower albumin concentration of interstitial fluid was relatively higher than that of plasma. During heat exposure, the lower albumin of plasma could be shown to increase, whereas a similar change could not be shown for interstitial fluid. Possibly the lower albumin attached to the surface of the erythrocytes (Williams, 1973; as discussed in section 4.5) are released as a result of the higher body temperature, causing a higher plasma lower albumin concentration. These results, however, certainly merit more extensive investigation.

Sephadex-acrylamide gel electrophoresis of rat plasma revealed the presence of an additional protein band in the β -globulin region after heat exposure. This additional protein could not be shown to appear in the interstitial fluid after heat exposure. As the protein(s) involved was not identified, meaningful discussion of the possible/probable significance of this result is not possible here, and will have to be shelved until such time as more extensive studies have been done.

4.5 SUMMARY AND CONCLUSION

Several interesting results have been obtained during the course of this study. At the outset the aim was to study the proteins in the body fluids of several animal species on a comparative basis. One of the most popular methods used for the separation of proteins is the polyacrylamide gel electrophoresis (PAGE) method (Davis, 1964). This separation method is based on differences in molecular masses and isoelectric points (pI) of the proteins (Feinstein, 1966). Acrylamide polymers consist of long chain molecules with a varying proportion of cross linkages resulting from the addition of bisacrylamide before polymerization. With increasing proportions of bisacrylamide the extra cross links lead to an increase of gel concentration (Gordon, 1975). The pore diameter in the gels is greatly dependant on total acrylamide concentration (Gordon, 1975). Polymerization of acrylamide occurs rapidly at room temperature if ammonium-persulphate is present. In the absence of excess O_2 the reaction continues until gelation is complete (Gordon, 1975).

As this study is based mainly on electrophoretic results, an attempt was made to develop a separating medium which extended the versatility, yet retained the simplicity, of the polyacrylamide gel. Much of the work reported here was devoted to the development of such a separating medium and the resultant combined sephadex-acrylamide gel was found to allow rapid separation of proteins. It was found that the sephadex-acrylamide gel provided better resolution than existing media, especially with regard to albumin, which can be separated into two subfractions. Unfortunately, the new gel medium is not without disadvantages, most notably the difficulty in handling and the fact that evaluation of the results by conventional densitometric methods is impossible due to its opaque nature. Hopefully, future refinements of the medium could minimize or eliminate the problems encountered in this regard.

Probably the most significant result of this study, is the separation of albumin into two distinctly separate subfractions. On the basis of the results obtained here, attempts were made to determine a few of the characteristics and functions of the more mobile albumin subfractions. The results of several experimental approaches indicated that the two so-called albumin subfractions are indeed characteristic albumins, although they differ in some respects.

By means of CIE it could be shown that the two albumin subfractions are present in plasma at approximately equal concentrations. It was also shown that the two albumin subfractions were immunologically related, producing a uniform continuous "rocket" with two distinct peaks in CIE. Dye-binding studies have also revealed that, in spite of their similarities, the lower albumin does not bind certain dyes (e.g. BcG and BpR), as does the upper albumin. These results indicate that albumin concentration determinations based on dye-binding studies, should be approached cautiously, as the presence of a lower albumin component would cause underestimation of the total albumin concentration by this method of assay. The reasons for the lower albumin not binding to certain dyes can be no more than speculative at this stage. If the lower albumin is an unbound form of the upper albumin, it is conceivable that binding to fatty acids, hormones etc. caused conformational changes in the protein molecule, thus exposing specific binding sites for the dyes involved.

Evidence was also obtained that the lower albumin subfraction was associated with the erythrocytes, probably with the outer surface of the cell membrane (Section 3.1.4.1). It has previously been shown that crystalline bovine albumin, as well as human albumin, binds to the cell membrane of fresh red blood cells (Williams, 1973). The precise nature and extent of the interaction between any given concentration of either human or bovine serum albumin in the intact erythrocyte membrane depends upon the chronological age of the cell concerned (Williams, 1973). Thus from these results it would

appear that the lower albumin released from the liver, attaches to the erythrocyte membrane. Splitting of the lower albumin from the erythrocyte membrane then probably occurs in the blood, releasing a functional albumin (lower albumin) with binding sites available for the binding (loading) of certain molecules to be transported. Loading of the lower albumin with the latter (e.g. hormones, fatty acids etc.) then transforms the lower albumin into a functional upper albumin.

Studies on the lower albumin of lymph and interstitial fluid support the postulations above. It was shown that a relatively higher concentration of lower albumin occurs in these fluids than in plasma. This result is to be expected if it is considered that the nutrients required by the tissue have already been removed from the fluids in question, including molecules transported by albumin. This would result in a transformation of some upper albumin to lower albumin due to unloading. It has been established that loading and unloading of albumin is a reversible process which probably occurs at a faster rate in tissue with high metabolic rates such as muscle (Hawkins, 1961; McFarlane, 1963).

Results obtained on the albumin fractions during periods of stress, also support the view that the lower and upper albumin subfractions respectively, are unbound and bound albumin molecules. Physical exercise of subjects not participating regularly in physical exercise, was shown to result in increased relative concentration of lower albumin in plasma as compared to physically trained subjects. During physical exercise, increased metabolic rate of muscle tissue espe-

cially, would be expected to result in an increased rate of unloading of the upper albumin. In addition, the muscle movements associated with physical exercise would also ensure more complete return of interstitial fluid and lymph, containing relatively large amounts of the lower albumin, to the blood.

In subjects regularly participating in physical exercise, the relative concentration of lower albumin in resting plasma was found to be less than that of physically untrained subjects. Furthermore, no significant changes in the relative concentrations of the upper or lower albumins could be found to occur during physical exercise of the physically trained subjects. From these results it would appear that the rate of albumin loading increases during a training period of regular physical exercise and therefore results in a physiological adaptation which enables physically trained subjects to withstand physical stress more efficiently than their untrained counterparts. In this regard it should also be considered that the entire transformational process of albumin, from the pro-albumin stage through to the loading of the lower albumin to produce a functionally loaded upper albumin, may be involved in the physiological adaptation to physical stress.

In summary, it can be said that the results of this study have cast new light on the characteristics and functions of plasma albumin. Being the most abundant and certainly one of the most important plasma proteins, plasma albumin has been extensively studied, but the exact role of this plasma protein is still not entirely understood. It is towards this end that this study hoped to contribute, especially with the new line of approach made possible by the development of a new electrophoretic medium.

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