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Binding of S^{35}-Labeled Sulfate in Serum of Rats as Studied by Filter-Paper Electrophoresis.* (22033)

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Filter paper electrophoresis of blood serum or plasma has been used to study the protein binding of radioactive substances(1-5). Dziewiatkowski(6) recently reported some observations on the association of sulfate sulfur with serum proteins. The present study was undertaken to extend our knowledge of sulfate binding by serum constituents. The results indicate that about 40% of the S^{35}-sulfate administered to Sprague-Dawley rats becomes bound to a substance or substances which migrate to the α_1-globulin region during electrophoresis on filter paper.

Materials and methods. Male Sprague-Dawley rats weighing 300-400 g were injected intravenously with 1200 μc of carrier-free $Na_2S^{35}O_4$. Blood was withdrawn from the heart 24 or 48 hours postinjection with a syringe containing 0.2 ml of mineral oil. The clot was spun down and the serum drawn off with a syringe. The S^{35} activity of the whole serum was determined by counting 100-μl samples in a gas flow proportional counter. Duplicate samples were spread on flat metal planchets and air dried for counting. The serum to be used for electrophoresis was stored at −30°C. Electrophoresis of 25 μl of blood serum placed on 3 by 29 cm strips of Whatman No. 1 chromatography filter paper was carried out in an apparatus similar to that described by Durrum(7). The samples were subjected to electrophoresis for 10 hours in a barbiturate buffer of ionic strength 0.05 and pH 8.6. Best results were obtained when the strips were immersed in the buffer and thoroughly blotted before the sample was applied. The current was maintained at 1.0 ma/strip, and the initial and final potentials were 300 and 150 volts, respectively. Following electrophoresis, the strips were dried at 60°C and developed in tetrabromophenolsulfonphthalein according to the procedure of Durrum(7). The dried and stained strips were scanned with a white light used in conjunction with a Photovolt photometer, and the optical density was recorded at 3-mm intervals. A standard serum electrophoresis pattern was obtained by plotting the optical densities against the distances from the origin. As an indication of the presence of carbohydrates, some of the strips were restained by the periodic acid-Schiff (PAS) routine according to the method of Köiw and Grönwall(8). The radioactivity associated with the serum proteins was determined by cutting the strips into 6 to 24 mm segments. Long or short segments were used where the activity was low or high, respectively. The segments were taped to planchets, and the S^{35} activity was determined in a gas flow proportional counter.

In view of the low S^{35} activity present on the segments of filter paper, it was necessary to

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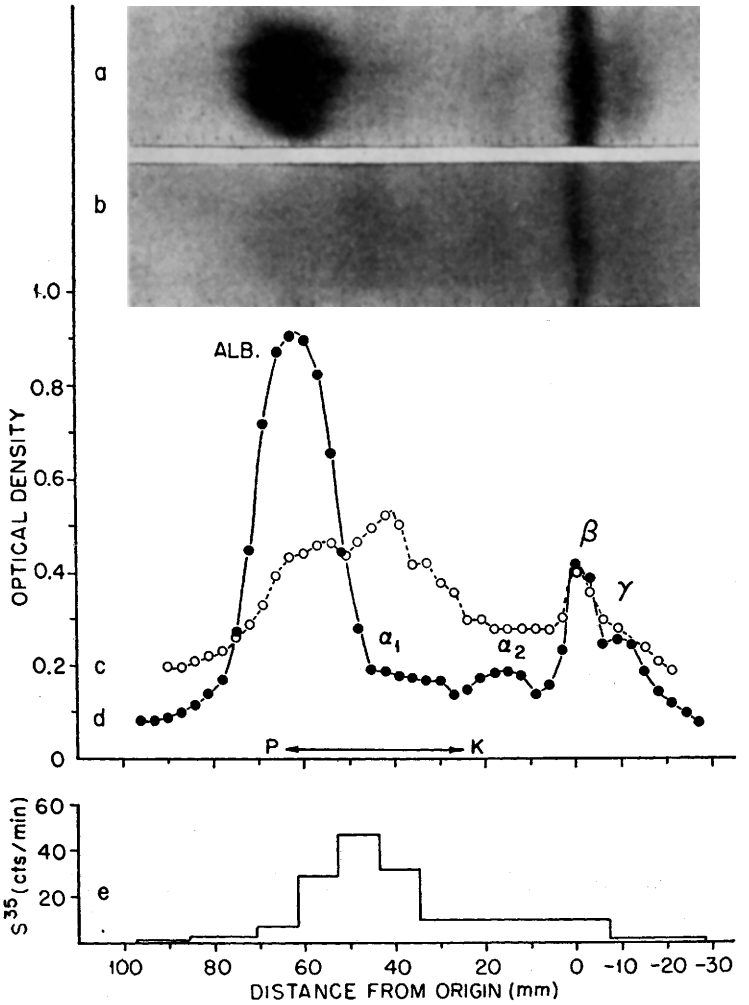

FIG. 1. Electrophoretic pattern of serum proteins as revealed by staining with tetrabromophenolsulfonphthalein (a) and periodic acid-Schiff routine (b). Graph (c) represents the optical densities of (b), and graph (d) depicts the optical densities of (a). Corresponding S^{35}-sulfate activities on the strip are shown in graph (e). S^{35} activity included between points P and K is referred to in the text as the activity peak.

calculate a counting time of sufficient length to insure the significance of the sample counts. A background count was established by averaging the background counts for 31 days. The daily counting periods ranged from 5-20 min., and a single day's value often represented an average of 2 or more such counts. The average value for each day always fell within one estimated standard deviation ($\sqrt{\bar{x}}$) of the mean background for the 31-day period. Since the background did not vary appreciably from day to day, it was considered to be a constant in calculating the sample counting time. It was decided to count each sample long enough so that the gross counts per minute minus 5 times the standard deviation of the gross counts per minute was greater than the background counts per minute. At the maximum sample counting time used, a value of 8 net cts/min. or greater was at least 5 standard deviations from the mean background count per minute. Thus any sample count greater than 8 cts/min. above background was considered to be significant.

TABLE I. Distribution of S^{35} Activity after Electrophoresis on Filter Paper of Serum from Normal Rats Injected Intravenously with $Na_2S^{35}O_4$.

Animal No.	Strip No.	Time post-inj., hr	Whole serum, cts/min./25 μl	Cts/min. in whole strip†	Cts/min. in peak†	% total counts recovered		% of recovered counts found in peak
						On strip	In peak	
640	91	24	3987	2134	1382	53.5	34.7	64.8
	106	24	3987	2370	1599	59.4	40.1	67.5
	161	24	3987	2270	1510	56.9	37.9	66.5
	159*	24	3987	2426	1596	60.8	40.0	65.8
641	92	24	4468	2384	1671	53.4	37.4	70.1
	100	24	4468	2925	2111	65.5	47.2	72.2
654	143	48	2581	1451	1006	56.2	39.0	69.3
655	144	48	2110	1345	914	63.7	43.3	68.0
645	118	48	1910	1359	844	71.2	44.2	62.1
					Mean:	60.0 ± 1.8	40.5 ± 1.3	67.6 ± 1.0

* This sample was dialyzed for 24 hr in cold against 3 changes of barbiturate buffer prior to electrophoresis.

† Corrected for self-absorption.

To determine what percentage of the whole serum activity remained on all or parts of the strip following electrophoresis, it was necessary to calculate a self-absorption factor. This factor was determined by comparing the activity of aqueous $Na_2S^{35}O_4$ dried on a planchet with the activity of the same solution dried on a disc of Whatman No. 1 filter paper attached to a planchet. The average of several determinations gave a self-absorption factor of 0.359. This factor was used to calculate the aforementioned percentages, but the activity values used to construct graph e in Fig. 1 are the original counts per minute uncorrected for self-absorption.

Results. A representative set of values obtained from the electrophoretic and S^{35} analysis of one serum sample is depicted in Fig. 1. The major part of the S^{35} activity coincided with a region on the strip which contained the α_1-globulin fraction. Occasionally it was observed that this maximum was shifted slightly into the ascending limb of the albumin region, but in no case did this maximum coincide with the albumin peak. The major part of the S^{35}-sulfate also coincided with a region on the strip which stained intensely with the PAS routine.

Calculations from the data for the S^{35} distribution on 9 strips are shown in Table I. It is seen that 60% of the whole serum activity was recovered on the strip, 40% of it being concentrated in a peak,† with the remainder distributed over the rest of the area occupied by the serum proteins (Fig. 1).

In one experiment (strip 159, Table I), the serum was dialyzed in the cold (2°C) against 3 portions of the barbiturate buffer during the 24 hours prior to electrophoresis. This procedure did not alter the pattern of distribution or the magnitude of sulfate activity on the strip.

To eliminate the possibility that the observed S^{35}-sulfate distribution on the paper strips resulted from independent migration of free sulfate ions, a serum sample to which $Na_2S^{35}O_4$ had been added *in vitro* was subjected to electrophoresis. The activity in this mixture was twice the amount found in the serum of rats injected with 1200 μc of $Na_2S^{35}O_4$, yet activity analysis of the stained strip showed no S^{35} present on any of the segments.

Discussion. In a paper on the effects of age on sulfate metabolism in the rat, Dziewiatkowski(6) reported that *in vivo* sulfate sulfur "becomes associated with serum proteins, mainly with albumin, and to a lesser extent with α- and β-globulins." The results of the present investigation showed that a major

† The S^{35} peak includes the activity present between the maximum point of the albumin curve and the low point anodal to the α_2-globulin region, *i.e.*, between points P and K in Fig. 1.

fraction of the serum sulfate is combined with a substance having the electrophoretic mobility of an α_1-globulin.

Sulfur is known to be present in 3 forms in the serum: one fraction is present as the sulfur in amino acids; another fraction is in the form of inorganic sulfate ion; and a third fraction is represented by several sulfuric acid esters, *e.g.*, heparin and indoxyl sulfuric acid. It has been shown by Tarver and Schmidt(9), and Boström and Jorpes(10) that administered S^{35}-sulfate does not become incorporated into the sulfur-containing amino acids of the rat, or becomes incorporated in negligible amounts (11). Our results, therefore, cannot be explained in terms of -S-S- or -SH groups.

The absence of activity on the paper strip following electrophoresis of serum mixed with S^{35}-sulfate *in vitro* confirms the recent report of Dziewiatkowski(6) that binding does not occur under *in vitro* conditions. In addition it indicates that the S^{35} in the α_1-globulin region did not result from the presence of free sulfate ions. Furthermore, the removal of free sulfate ions by dialysis of S^{35}-sulfate-labeled serum prior to electrophoresis resulted in no change in the amount of activity remaining on the strip after electrophoresis. These findings do not eliminate the possibility of a salt linkage between the sulfate ion and a protein, but the following preliminary experiments in this laboratory do not support such an hypothesis. After dialysis of S^{35}-sulfate-labeled plasma against a buffer of pH 12.0, 38% of the whole activity remained in the plasma, essentially the same amount (40%) as was found in the α_1-globulin region following electrophoresis of undialysed plasma at pH 8.6. At pH 12.0, few, if any, positively charged groups of a protein would be available for anion binding through a salt linkage. Even at pH 8.6, it is expected that the number of available groups of a protein capable of binding $SO_4^=$ would be small. In addition, anion binding would have to be quite strong to overcome the attraction of the sulfate ion to the anode during electrophoresis at pH 8.6. Although anion binding to a protein through a salt linkage might explain the present results, it seems more likely that the S^{35}-sulfate activity peak reflects the presence of sulfuric acid esters.

Proteins combined with sulfomucopolysaccharides such as chondroitin sulfate occur in many tissues. Protein-carbohydrate complexes such as the mucoprotein described by Winzler *et al.*(12) and an α_1-glycoprotein crystallized by Schmid(13) have been found in plasma. These 2 substances, isolated from human plasma and apparently identical, are acid although the composition of the acid groups of the carbohydrate moiety is unresolved(14). Schmid reported that his α_1-glycoprotein did not contain a sulfuric acid ester. Winzler *et al.*(12), and Weimer *et al.*(15) found, however, that after mild hydrolysis of their mucoprotein preparations a precipitate was formed by the addition of barium chloride to the hydrolyzate, suggesting that labile sulfuric acid groups had been removed from the mucoprotein. Winzler's mucoprotein has the electrophoretic mobility of an α_1-globulin at pH 8.4 (16), and contains approximately 30% carbohydrate(15). In the present studies it was observed that the S^{35}-sulfate activity peak corresponded to an area on the strip which contained PAS-positive constituents. Since the S^{35}-sulfate activity peak and the PAS-positive region appear to coincide electrophoretically with the mucoprotein described by Mehl *et al.*(16), it is possible that the carbohydrate moiety of this mucoprotein is esterified with sulfuric acid. A similar interpretation was previously suggested by Mehl *et al.* (16) to account for the observation of Winzler and Burk(17) that sulfur in excess of that required by cystine and methionine was present in a mucoprotein preparation from rat blood. With the use of human blood, Weimer *et al.* (15) made the same observation.

Summary. The binding of S^{35}-labeled sulfate in rat serum has been investigated by filter paper electrophoresis. About 40% of the whole serum S^{35}-sulfate activity was found to be combined with a serum constituent having the electrophoretic mobility of an α_1-globulin. Although the chemical identity of the labeled substance has not yet been established, the results of these experiments suggest that it may be a protein with a sulfonated carbohydrate prosthetic group.

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Effect of Some Centrally Acting Drugs on Compulsive Circling. (22034)

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The group of signs and symptoms observed, following a unilateral intracarotid injection of di-isopropyl fluorophosphate (DFP) into rabbits, has been called the adversive syndrome (1) and the latter has been attributed to the anticholinesterase action of DFP(1-5). As a result of DFP administration, an asymmetric distribution of cholinesterase (ChE) activity is produced in the frontal cortex and caudate nucleus. The enzymatic activity is profoundly depressed on the injected side of the brain whereas the opposite side is relatively unaffected. This is especially marked in the right caudate nucleus which retains only 4% of its normal ChE activity. This inhibition permits asymmetric accumulation of acetylcholine (ACh) in the brain(6) which in turn may be directly responsible for the observed effects. It has been established in cats and monkeys that the adversive syndrome is the result of a biochemical lesion of the central nervous system(7). Although it is possible that peripheral receptors are more important in rabbits than in cats(7), many factors also point to a central mechanism for the production of the adversive syndrome in the former animal. First, the direct application of DFP to the peripheral vestibular receptors elicits no behavioral change in rabbits(8). Secondly, central stimulants can modify the forced circling behavior(5). Thirdly, a characteristic asymmetric brain cholinesterase pattern is found in all animals exhibiting the adversive syndrome, and this pattern is absent when compulsive movements are not observed (3,9,10). Finally a temporary or permanent reversal in the direction of the forced movements usually follows a minor convulsive seizure(2). The factors mentioned above appear to implicate the central nervous system as the primary site for the production of compulsive circling in rabbits. Previous work(5) showed that the adversive syndrome can be corrected by intravenous injections of small amounts of atropine sulfate after which the animal exhibits its normal mode of progression. When large doses (i.v.) of atropine are given the forced circling is reestablished, however, the animal turns in the opposite direction, toward the injected side.

In the present study a further analysis of

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