Dealkylation and Loss of Capacity for Reactivation of Cholinesterase Inhibited by Sarin

Abstract. *Inhibition of rat brain acetylcholinesterase by ^{32}P-sarin in vivo results initially in ^{32}P-isopropylmethylphosphonylated enzyme. The percentage of inhibited enzyme that could not be reactivated by pyridinium aldoxime methochloride (aged enzyme) approximated the amount of radioactivity identified as ^{32}P-methylphosphonate. The ^{32}P-isopropyl methylphosphonate not released from the inhibited enzyme by the oxime accounted for 51 ± 10 percent (standard deviation) of the radioactivity fixed to brain tissue. It showed no correlation with aging and was probably bound to sites other than acetylcholinesterase.*

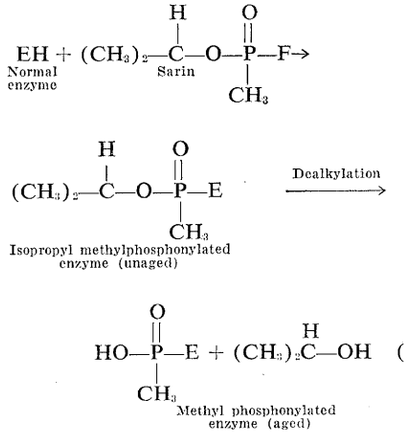
The normal transmission of nerve impulses at cholinergic sites by acetylcholine is regulated by the hydrolytic action of acetylcholinesterase (AChE) (*1*). Poisoning by anticholinesterases containing organophosphorus interferes with this hydrolysis in that AChE is phosphorylated, resulting in inhibition of enzyme activity (*2*) and accumulation of ACh (*3*).

One of the most potent compounds acting in this manner is isopropyl methylphosphonofluoridate (sarin) (*4*). Effective chemical treatment of cholinergic poisoning, due to administration of sarin to animals, requires the injection of atropine and an oxime such as monoisonitrosoacetone (*5*), pyridine-2-aldoxime methiodide (2-PAM) (*6*), or 1, 1′-trimethylene-bis(4-formylpyridinium bromide) dioxime (*7*). The antidotal action of these oximes in animals poisoned with sarin involves a dephosphorylation of the inhibited enzyme (*8*), and, as a result, the AChE activity is restored (*9*). However, the fraction of activity that can be restored to the phosphorylated enzyme decreases exponentially in the absence of prompt addition of an effective oxime (*2*, *10*). This effect has been called "aging." Since the development of resistance of phosphorylated AChE to reactivation may have important implications for treatment of individuals poisoned by organophosphorus insecticides, the molecular basis for the aging phenomenon has been under study in a number of laboratories.

When butyrocholinesterase, which is present in horse serum, is inactivated with the anticholinesterase diisopropylphosphorofluoridate in vitro, the molecular mechanism associated with the aging is the loss of one isopropyl group from the initially diisopropylphosphorylated enzyme (*11*). The loss of the alkoxy substituent promotes the acquisition of a negative charge on the phosphorus bound to the enzyme and results in resistance to nucleophilic attack by oximes whose active species is oximate anion (*12*).

Fleisher and Harris (*13*) have shown that the decrease in reactivatibility and in dealkylation—by loss of a pinacoloxy group from AChE previously inhibited with ^{32}P-labeled pinacolyl methylphosphonofluoridate (soman)—occurred at approximately equal rates in vitro with a half-time of about 2 minutes. Evidence for dealkylation of the enzyme in rats poisoned with ^{32}P-soman in vivo was also presented (*13*). However, the rapidity of the aging process prevented accurate measurement of the relation between the rate of dealkylation and the rate of aging of the phosphonylated enzyme in vivo.

We have taken advantage of the slower aging rate of AChE inhibited by sarin (*8*) to study the applicability of the dealkylation mechanism in the case of poisoning by this compound both in vitro and in vivo. If the postulated mechanism shown in Eq. 1 is correct, then the rate of dealkylation



could be measured by (i) the decrease in isopropyl methylphosphonylated enzyme, (ii) the increase in methylphosphonylated enzyme, or (iii) by the production of isopropanol. Utilization of ^{32}P-sarin (*14*) permitted the dealkylation process to be studied by the aforesaid procedures (i) and (ii). Treatment with 2-PAM (*15*) releases isopropyl methylphosphonic acid (IMPA) from the unaged inhibited enzyme with concomitant reactivation of the enzyme. Methylphosphonic acid (MPA) is not released from the aged enzyme by 2-PAM treatment; however, it can be displaced by digestion in alkali. Determination of IMPA and MPA in a mixture of the two could be made by partition in immiscible solvents.

^{32}P-Sarin was purified according to the method used for purification of ^{32}P-soman (*16*); ^{32}P-labeled IMPA and MPA were then synthesized as reference compounds by hydrolysis of the labeled sarin in excess NaOH and 12*N* HCl, respectively. The corresponding unlabeled phosphonic acids were made in a similar manner from unlabeled sarin, and were characterized as pure compounds by elemental analysis and by paper chromatography (*17*) in the solvent system containing isopropanol, H_2O, and concentrated NH_4OH (75:1:24 by volume) (*18*). The labeled phosphonic acids were identical to the unlabeled reference compounds in the above system. Both ^{32}P-IMPA and ^{32}P-MPA were further characterized by partition in an equal volume of 1 : 1 isobutyl alcohol and chloroform with aqueous medium at pH = 0.5. The distribution coefficients, solvent to aqueous phase, were determined from six trials of each acid; the distribution coefficient (D_A) for IMPA was 2.05 ± 0.10; and for MPA it was (D_B) = 0.062 ± 0.004 (P = .05). By use of the previously derived formula (*13*), the mole fraction of ^{32}P-IMPA (F_A) in a mixture of ^{32}P-MPA and ^{32}P-IMPA can be calculated from Eq. 2,

$$F_A = \left(\frac{D_A + 1}{D_A - D_B}\right)\left(\frac{R - D_B}{R + 1}\right) \qquad (2)$$

where R is the ratio of total radioactive counts in the organic phase to those in the aqueous phase. Substituting the values of D_A and D_B into Eq. 2, then

$$F_A = [1.53\ (R - 0.062)]/(R + 1) \qquad (3)$$

For study in vivo of aging and dealkylation, ^{32}P-sarin (60 μg/kg; approximately 1.5 LD_{50}) was injected intravenously into rats that had been treated with atropine (10 mg/kg), and separated groups of ten survivors each were sacrificed at ½, 3, 6, 9, 14, and 24 hours after the injection. At each interval a pair of unpoisoned animals were treated identically to ascertain the normal brain AChE activity. The brains were excised, blotted, and pooled in

pairs; the tissue (10 g/100 ml) was homogenized in ice cold 0.9 percent NaCl in 0.01*M* borate buffer at *p*H 8.8. The homogenates were divided into two equal fractions and centrifuged at 105,000*g* for 15 minutes at 2°C, and the supernatants were discarded. The residues were washed twice with the cold saline borate solution and centrifuged, the supernatant being discarded, as before. The residue from one of the fractions of the original homogenate was suspended in 2 ml of $10^{-1}M$ PAM in 0.1*M* phosphate buffer, *p*H 7.6, while the other (control) fraction was suspended in the same volume of phosphate buffer alone. Each was incubated, with gentle agitation, for 90 minutes at 25°C, a period sufficient for PAM to completely dephosphorylate the IMP from the unaged cholinesterase enzyme. The suspensions were centrifuged, as above, and the supernatants were retained. Each residue was washed twice with successive 1-ml portions of cold 0.9 percent NaCl and centrifuged; the washings were pooled with the supernatant. The residues were retained in ice for further study. The supernatants were treated with sufficient 50 percent trichloroacetic acid (TCA) to give a final concentration of 5 percent, and with one drop of 10*N* HCl to approximate a *p*H of 0.5. The samples were centrifuged at 2000 rev/min for 10 minutes. The supernatant was removed, the radioactivity was determined on a portion, and the remaining supernatant was shaken with an equal volume of a mixture of isobutyl alcohol and chloroform (1:1) to characterize the ^{32}P displaced by PAM as IMPA, and to establish that no MPA had been released by this treatment. The phases were then separated and clarified by centrifugation, and a portion was transferred to planchets, dried, and counted with a Geiger-Mueller end-window counter. The radioactivity due to IMPA liberated to the supernatant by PAM incubation was calculated (after correcting for any radioactivity in the supernatant of the phosphate controls) by the formula for the mole fraction of IMPA given above.

The residues that had been retained were now washed twice more with 10 ml of ice-cold saline and centrifuged at 105,000*g* to remove traces of PAM which could interfere with the assay of cholinesterase activity, because of its own anticholinesterase properties (*19*). The washed residues were now made to a 2 percent suspension with cold 0.3*M* KCl. The AChE activity was estimated colorimetrically by incubating 1 ml of the brain suspension for 30 minutes at 25°C with 1 ml of 0.006*M* acetyl-β-methylcholine (Mecholyl) in 0.3*M* NaCl buffered to *p*H 7.6 with 0.05*M* phosphate buffer. The remainder of the assay has been described (*20*). The percentage of ChE activity reactivated was calculated from the formula suggested by Hobbiger (*21*):

$$\text{Percentage of ChE reactivated} = 100 \times \left[\frac{(\text{AChE})_T - (\text{AChE})_I}{(\text{AChE})_C - (\text{AChE})_I}\right] \quad (4)$$

where $(AChE)_T$ is the enzyme activity of oxime treated phosphonylated tissue $(AChE)_I$ is the enzyme activity of sarin inhibited tissue $(AChE)_C$ is the enzyme activity of oxime treated tissue prepared from unpoisoned controls. The reactivability of the AChE after poisoning with sarin is given in Table 1.

Table 1. Distribution of ^{32}P from rat brain poisoned with ^{32}P-sarin after incubation with PAM in relation to aging in vivo. Six pairs of brains were used for each time interval studied.

Time of aging* (hr)	Enzyme activity reactivated (% ± *P* = .05)	^{32}P – IMPA released by PAM (% ± *P* = .05)	^{32}P – MPA remaining (% ± *P* = .05)
½	91.6 ± 13.0	91.0 ± 6.7	9.1 ± 7.2
3	73.9 ± 9.5	72.0 ± 9.5	20.8 ± 11.1
6	45.5 ± 6.7	55.0 ± 3.2	45.0 ± 5.6
9	31.5 ± 5.5	36.3 ± 8.3	70.0 ± 6.2
14	17.2 ± 5.0	23.3 ± 3.7	76.7 ± 3.3
24	7.8 ± 3.0	8.6 ± 5.6	91.4 ± 5.8

* Time of aging represents the time in hours between poisoning with sarin and sacrifice.

The remainder of the 2 percent homogenates from the PAM treatment were centrifuged at 105,000*g*, the supernatants were discarded, and the residues were dispersed in 2 ml of 0.1*N* NaOH, after digestion at 100°C for 2 hours to cleave any phosphonate bound to the brain tissue. The *p*H of the digest was adjusted to approximately 1.0 with 10*N* HCl, and TCA was added to 5-percent concentration. The mixture was centrifuged at 2000 rev/min, and the supernatant was collected. The residue was washed twice with 5 percent TCA adjusted to *p*H 0.5 with HCl, and then centrifuged. The washings were added to the supernatant already collected. The solution was then shaken with a mixture of isobutyl alcohol and chloroform (1:1); the phases were separated and centrifuged; samples were put on planchets and the radioactivity was counted as before. If we now assume that any ^{32}P-IMPA found in the residue was not bound to ChE [the evidence for this has been discussed (*13*)], then the ^{32}P-MPA counts are obtained from

$$M = Z - I$$

where M is the number of ^{32}P-MPA counts in the residue, Z is the total counts in residue, and I is the number of ^{32}P-IMPA counts, which is also equal to $F_A Z$. Substitution for the value of F_A, gives

$$M = Z[1 - 1.53(R - 0.062)/(R + 1)] \quad (6)$$

Furthermore, the mole percentage of ^{32}P-IMPA dephosphorylated by PAM is given equal to 100 times the ratio of the ^{32}P-IMPA released by PAM to the sum of the ^{32}P-IMPA released by PAM and the ^{32}P-MPA in the residue.

Correspondence between the percentage of the radioactivity released by PAM exclusively as IMPA and the fraction of the AChE enzyme that could still be reversed by incubation of the sarin-poisoned brain tissue with PAM is close (Table 1). By subtracting the percentage of AChE reactivated from 100 percent, one may obtain that fraction of the enzyme which is not reactivated. There is quantitative parallelism between the percentage of AChE not reactivated, that is, the percentage of aged enzyme, and the percentage of the radioactivity bound to AChE as ^{32}P-MPA (Fig. 1).

The rate of aging of rat brain AChE phosphorylated by sarin in vitro was compared with that obtained in vivo.

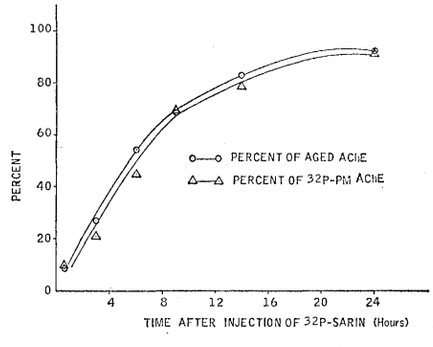


Fig. 1. Relation between "aging" and ^{32}P bound to AChE as ^{32}P-MPA in rat brain poisoned with ^{32}P-sarin.

Table 2. Decrease [percentage ± (P = .05)] in activity that was reactivated in rat brain AChE after inhibition with sarin.

Time of aging (hr)	Decrease (%)	
	In vivo	In vitro
½	87.0 ± 6.3	88.5 ± 4.4
2	78.0 ± 12.0	77.0 ± 5.7
6	44.2 ± 5.4	45.6 ± 10.2
14	17.4 ± 6.5	18.4 ± 3.8

For this purpose, rat brain (11 g/100 ml of solvent) was homogenized in ice cold 0.9 percent NaCl solution containing 0.01M borate buffer at pH 8.8. The homogenate was incubated with 2 × $10^{-7}M$ sarin for 20 minutes at 0°C. Almost complete inhibition with minimum aging occurred under these conditions. The preparation was immediately transferred to a bath maintained at 37°C by a thermostat and allowed to equilibrate to this temperature. Samples were taken before and at ½, 3, 6, and 14 hours after adjusting the pH to 7.4 with phosphate buffer (final concentration, 0.05M) to initiate aging. For reactivation, 1-ml portions were transferred into 1 ml of 2 × $10^{-2}M$ monoisonitrosoacetone in 0.05M phosphate buffer at pH 7.8. Thirty minutes at 25°C was allowed for reactivation. At the end of this time the samples were diluted with 3 ml of 0.3M KCl, to yield a 2 percent brain homogenate. The AChE activity was determined with Mecholyl. Controls on uninhibited brain homogenate without, and in the presence of, reactivator and also on inhibited homogenate were run concurrently. The final concentration of

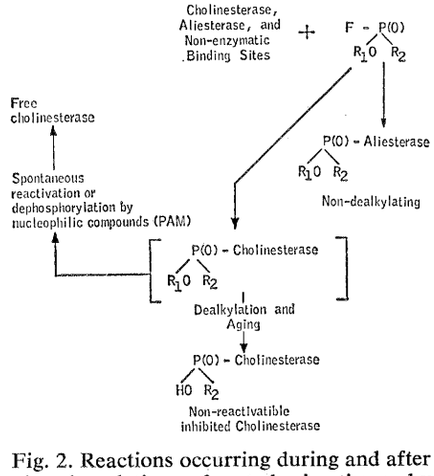


Fig. 2. Reactions occurring during and after phosphonylation of rat brain tissue by sarin or soman. For soman, R_1 = pinacoloxy, R_2 = CH_3; for sarin, R_1 = isopropoxy, R_2 = CH_3.

2 × $10^{-3}M$ monoisonitrosoacetone produced little or no inhibition of rat brain AChE.

In the study in vivo unlabeled sarin (60 μg/kg) was injected intravenously into rats treated with atropine (10 mg/kg). Separate groups of 8 to 10 survivors were killed at the same time intervals as in the in vitro study above. The brains were homogenized in 0.9 percent NaCl buffered to pH 8.8 with 0.01M borate buffer. Portions were promptly incubated with an equal volume of 2 × $10^{-2}M$ monoisonitrosoacetone or with buffer alone. The logarithm of the mean values for AChE reactivated (Table 2) obeyed first-order kinetics when plotted as a function of time. The times for loss of 50 percent of the initial AChE reactivatability approximated 5.8 hours both in vitro and in vivo.

A fraction identified as IMPA which was not dephosphorylated by PAM or converted to ^{32}P-MPA, retained 51 ± 10 percent (S.D.) of the total radioactivity bound to rat-brain tissue for each of the time intervals after poisoning with ^{32}P-sarin. Approximately the same percentage of ^{32}P derived from labeled soman was found in the alkylated fraction and also showed no direct correlation with the aging process (*13*). Probably this residual alkylated ^{32}P-phosphonate is bound to brain aliesterase (*22*), an enzyme that does not undergo dealkylation (*23*), or to other nonspecific sites exhibiting the same property.

The distribution of ^{32}P compounds bound to rat brain tissue after poisoning with soman (*13*) or with sarin in vivo in relation to inhibition, dealkylation, and the effect of oximes is shown in Fig. 2. The close similarity between the rates of aging in vitro and in vivo (Table 2) had been previously reported for chicken brain ChE inhibited with either dimethyl-2,2-dichlorovinyl phosphate or Malathion (*24*) and for sheep erythrocytes inhibited with sarin, TEPP, DFP, and dimethoxy-*p*-nitrophenoxyphosphine oxide (*25*). Our results and those cited suggest that in a given species estimation of the rate of aging of ChE in vitro after inhibition with an organophosphate may be helpful as a guide to the time during which oxime administration would be effective in vivo in the case of intoxication by the same anticholinesterase. To insure maximum applicability to situations in vivo, the aging process should be followed in vitro under conditions simulating those present physiologically with respect to pH, temperature, and ionic strength of the medium. Studies in vitro with organophosphates producing phosphorylated ChE which dealkylate and age rapidly are facilitated by inhibition at 0°C and at a pH approximating 8.5. Aging and dealkylation are thus minimized (*8*, *13*) during the initial inhibitory process. Ideally, the rate of reactivation should also be very much greater than the rate of aging during incubation of the phosphorylated ChE with the oxime. This is promoted by the high concentrations of PAM or of monoisonitrosoacetone used in this study. The anticholinesterase properties of the oximes which could interfere with subsequent enzyme assay were practically eliminated by washing out, and by dilution, for PAM and monoisonitrosoacetone respectively.

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Inherited Variations of Human Serum α_1-Antitrypsin

Abstract. *The normal serum α_1-antitrypsin migrates as a three banded pattern when separated electrophoretically in starch gel with a sodium acetate–ethylenediaminetetraacetic acid buffer of pH 4.95. The results obtained when certain inherited variants of the serum α_1-antitrypsin are separated electrophoretically suggest that the previously described variations in the region preceding the albumin band represent inherited variations of the serum α_1-antitrypsin.*

The α_1-antitrypsin (α_1-at), one of the four known protease inhibitors in human serum, is of interest because of the possible deleterious consequences of an inherited deficiency of this protein (*1*). The genetically determined deficiency, as well as a recently discovered structural mutation, might serve as additional genetic markers in man. A large pedigree in which both mutations occur has been reported (*2*).

The method commonly used to detect a deficiency of the α_1-at is to measure the capacity of serum to inhibit the hydrolysis of the synthetic substrate N,α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) by trypsin (*3*). Electrophoretic variants have been identified by the antigen-antibody crossed electrophoresis as developed by Laurell (*4*). This method requires electrophoretic separation of serum in two dimensions, consumes relatively large amounts of specific antiserum, and is therefore not ideally suited for screening populations for genetic variants.

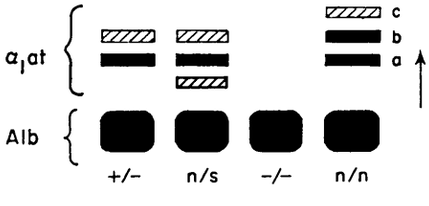

Fig. 1. Diagram of starch-gel electrophoresis of serums obtained from four individuals with different α_1-antitrypsin phenotypes. Buffers: Cathodal vessel contained 0.125*M* sodium acetate, 0.011*M* ethylenediaminetetraacetic acid (EDTA). Anodal vessel contained the same buffer used at half concentration. The gel buffer was 0.031*M* sodium acetate, 0.004*M* EDTA; *p*H of all buffers, 4.95. Electrophoresis for 9.5 hours at 250 volts. Stain: Amido black. *n/n*, normal phenotype; *n/s* heterozygous for slow variant; −/−, homozygous for deficiency gene; +/−, heterozygous for deficiency gene; $\alpha_1$at, α_1-antitrypsin; *Alb*, albumin; *a*, *b*, and *c* are the three major α_1-antitrypsin bands.

Between *p*H 8.0 and 9.0 the α_1-at migrates electrophoretically more slowly than albumin and forms a large part of the α_1-band in both paper electrophoresis and agarose-gel electrophoresis. Although under these conditions the α_1-at migrates as a single band, the antigen-antibody crossed-electrophoresis technique reveals that there is a second minor component migrating at the cathodal edge of the main band (*5*). When a buffer of a lower *p*H is employed in the vertical starch-gel system (*6*), the normal α_1-at migrates as three distinct bands preceding the albumin band. Two of the bands have approximately equal staining intensity, whereas a third band, migrating more anodally, is much weaker and in some serums is barely visible (Fig. 1). An antigen-antibody crossed electrophoresis, performed with a specific antiserum, indicates that the observed bands are indeed α_1-at (Fig. 2A). Moreover, it is apparent that, at least with the antiserum used in our experiments, there is no major antigenic difference between the three components, since the precipitation lines fuse completely. Additional evidence which supports the view that these bands represent α_1-at is that the serum from an individual known to be homozygous for the deficiency gene shows, with the exception of a very faint band, a virtual absence of bands in the region anodal to the albumin (Fig. 1, −/−). Whether this faint band represents small quantities of α_1-at or a trace protein unrelated to the α-at which migrates in a similar position is unknown. The evidence from the antigen-antibody crossed-electrophoresis experiments suggests that small quantities of α_1-at are present in the region preceding the albumin band of this serum. Trypsin inhibition by normal serum can be demonstrated with the fibrin-agar technique (*7*). A broad zone of inhibition corresponding to the position of the α_1-at banding pattern was observed.

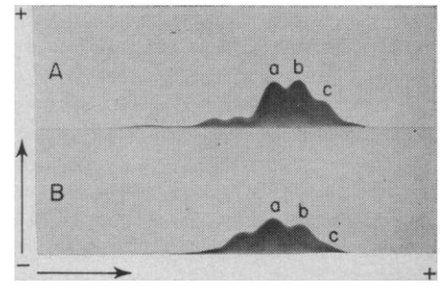

Fig. 2. Antigen-antibody crossed electrophoresis of serums. (A) Normal individual (*n/n*). (B) Heterozygous for slow variant (*n/s*). The α_1-antitrypsin peaks *a* and *b* from normal individual are of similar height; in *n/s* peak *a* is higher than peak *b*. After starch gel electrophoresis a longitudinal strip of starch was removed and placed on 1 percent agarose gel (containing 4 percent specific antiserum to α_1-antitrypsin) on a glass plate. Electrophoresis, in calcium lactate containing 0.051*M* barbital buffer, *p*H 8.65, in horizontal direction (1.5 hours, 10 volt/cm). After the second electrophoresis the starch strip was removed, and the plate was kept in 0.15*M* NaCl for 12 hours, then dried and stained.

Using the conditions described in the legend to Fig. 1, we examined a number of variations of the α_1-at. We discovered a family, some of whose members were heterozygous for a slow α_1-at variant, from an examination of the serum by antigen-antibody crossed electrophoresis with an alkaline *p*H for both electrophoretic separations. Under these conditions the results (Fig. 3) were very similar to those of Axelsson *et al.* (*2*). The two mutations are probably indistinguishable, or even identical. The electrophoretic pattern in starch gel at *p*H 4.95 of serums from those heterozygous individuals is illustrated in Fig. 1 (*n/s*). In addition to

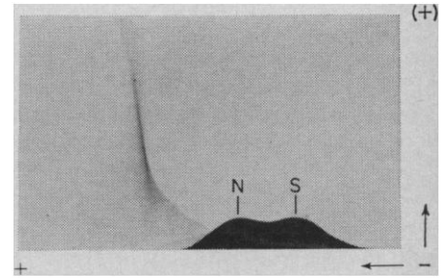

Fig. 3. Antigen-antibody crossed electrophoresis of serum from an individual heterozygous for the slow α_1-antitrypsin variant. The supporting medium was agarose. Calcium lactate containing barbital buffer, *p*H 8.65, for both electrophoretic separations. Double-peaked precipitate is due to α_1-antitrypsin. Normal antitrypsin (*N*), slow variant (*S*). The faint immune precipitate anodal of the normal α_1-antitrypsin is due to serum albumin.