EFFECTS OF ACIDIC AZOPROTEINS ON PLASMA TRIGLYCERIDE LEVELS AND ON THE HEPARIN-ACTIVATED CLEARING SYSTEM*

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Heparin injected into animals of several species activates a powerful lipid clearing system in the blood plasma, as was first shown by Hahn (1). The factor responsible for clearing is recognized to be a lipase (2) but despite considerable work, recently reviewed by Robinson and French (3, 4), its precise role in the physiological processes of fat transport remains uncertain.

The principal evidence suggesting the functional importance of the clearing system is the finding that certain substances which raise the level of blood triglyceride *in vivo* also inhibit the clearing system *in vitro*. Brown (5) and Bragdon and Havel (6) showed that protamine and toluidine blue, long recognized as antagonists of the anticoagulant action of heparin, produce raised blood triglyceride levels after injection into rats. Similarly, 2 non-ionic surface active agents, triton A 20 and tween 80 whose hyperlipemic effect was described by Kellner *et al.* (7), have also been shown to inhibit this system (8). Recently Rudman and Seidman showed that pituitary extracts when injected into rabbits caused delayed hyperlipemia (9). Kellner *et al.* showed that the extracts alone produced no effect on the heparin-activated clearing system *in vitro*, but plasma from animals made hyperlipemic by them was strongly inhibitory (10).

The purpose of the present publication is to add to the short list of agents producing hyperlipemia members of a distinctly different group of chemical substances, the acidic azoproteins. The effects produced depend entirely on the particular acidic group present in the azoprotein. For while the 4-arsonophenyl causes a marked and prolonged hyperlipemia, 2 other groups, the 4-sulfonophenyl and the 4-carboxyphenyl, produce more complex results consisting of an initial raising and later lowering of the levels of blood triglyceride. It is further intended to show that these end results can be related to the effects of the particular azoprotein on the lipase clearing system, thus providing further evidence that this system has a significant role in regulating blood triglyceride levels.

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Although azoproteins have been considerably used for antigenic studies since their first introduction by Landsteiner more than 40 years ago (11), the possibility that they might have other biological effects has been largely overlooked. Recently however, Kidd found that 4-arsonophenylazoproteins are strongly inhibitory to proliferating cells of a number of transplantable mouse lymphomas (12). Investigations of the general biological properties of 4-arsonophenylazoproteins undertaken to elucidate the cause of this inhibition showed that they possessed marked anticoagulant activity, which was to a large extent due to complex formation with fibrinogen, producing a substrate resistant to the action of thrombin (13). Other acidic azoproteins possessed similar anticoagulant properties. In the course of these investigations the further observation was made that the plasma of animals injected with 4-arsonophenylazoproteins became within a few hours markedly lactescent.

Materials and Methods

Preparation of Azoproteins.—Diazotates were prepared using standard methods described by Eagle and Vickers (14) and Kidd (12), from the following substances: arsanilic acid (Eastman Organic Chemicals, Rochester), *p*-aminophenylarsene oxide (prepared by reduction of arsanilic acid with SO_2 according to Ehrlich and Bertheim, reference 15), sulfanilic acid, *p*-aminobenzoic acid, and aniline (reagent grades). Bovine albumin (Armour Pharmaceutical Company, Kankakee, Illinois, Cohn's fraction V) and human albumin (Merck and Company Inc., Rahway, Cohn's fraction V) were used for conjugation. By varying the proportions of diazotate and protein in the reaction mixture, azoproteins with different degrees of conjugation were obtained, as was shown by analysis for their content of arsenic and of sulfur in sulfonic groups (performed by Schwartzkopf Microanalytical Laboratories Inc., Woodside, New York). Conjugation was performed usually at pH 9.0 and 0° to 4°C. After 2 or more hours the pH of the reaction mixture was adjusted to 6.5 and the azoprotein was then freed from other reaction products by dialysis against water for 48 hours. The final products were lyophilized.

The degree of conjugation in unsubstituted phenylazoprotein and in 4-carboxyphenylazoprotein could not be determined by direct analysis. However, the materials used in the present experiments were prepared using quantities of diazotate and proteins equivalent to those which consistently produced 4-arsonophenylazoproteins of 3 to 4 per cent arsenic content, that is, using 1 mmole of diazotate per gm of protein and allowing the reaction to proceed to completion under the conditions already described. The optical densities at 380 mμ of 0.01 gm per cent solutions of these azoproteins in 0.1 M phosphate buffer at pH 7.4 were: 4-arsonophenylazoalbumin (As 3.0 per cent) 0.244, 4-carboxyphenylazoalbumin 0.386, phenylazoalbumin 0.243.

Animals.—Market bought albino rabbits of both sexes were used, weighing 2.3 to 3.8 kg. Azoproteins and other substances were administered to these animals by intravenous injection. Rats were of the Nelson strain obtained from Carworth Farms Inc., New City, New York, and mice were ZBC animals obtained from the late Dr. J. J. Bittner of the University of Minnesota, Minneapolis.

Blood Samples.—Samples usually of 1 to 2 ml, but of up to 4.5 ml where analyses of both total and triglyceride lipids were to be performed, were obtained from rabbits by warming the ear and cutting a small marginal vessel. They were anticoagulated by mixing 9 parts with 1 of isotonic sodium citrate solution. Plasma was separated by centrifugation at 3000 RPM for 20 minutes.

Postheparin plasma (PHP) was obtained by bleeding rabbits 15 minutes after the intra-

venous injection of sodium heparin (Organon Inc., West Orange, New Jersey) in a dosage of 7.5 mg/kg.

Lipid Analysis.—Estimations of total plasma lipids were performed according to procedure 2 or Sperry and Brand (16).

Estimations of plasma triglycerides were performed according to Van Handel and Zilversmit (17) using triolein (Mann Research Laboratories Inc., New York) as a standard.

Plasma Lipid Clearing Activity.—This was measured in the manner described by Kellner *et al.* (10). Mixtures were prepared containing 0.7 ml PHP or other test plasma, 0.2 ml saline, 1.0 ml imidazole buffer (0.2 M, pH 7.4) and 0.1 ml of the stabilized coconut oil suspension ediol (Schenlab Pharmaceuticals Inc., New York) diluted $\frac{1}{50}$ in 0.15 M NaCl. The ediol was added last and clearing of the mixture at 37°C was followed from periodic measurements of optical density at 700 mμ in a Coleman junior spectrophotometer. In testing their effects on the clearing system, solutions of azoproteins and other substances (0.02 ml) were added immediately before the ediol. In experiments directly measuring the rate of hydrolysis of coconut oil, ediol diluted $\frac{1}{25}$ was used in the incubation mixture. 0.5 ml samples were removed initially and after 60 or 90 minutes for determination of their triglyceride contents.

TABLE I

Hyperlipemia Induced by 4-Arsonophenylazoprotein in Mice and Rats

Experimental animal	Dosage		Plasma triglyceride conc.	
	mg azo-protein/kg	mg As/kg	Average	Range
			mg per cent	*mg. per cent*
Mice 1–3	0	0	37.5	31.1–44.1
4–6	615	40.0	1893.5	1606.1–2192.8
Rats 1–3	0	0	69.8	58.7–79.5
4–6	308	20.0	2096.2	2045.7–2151.4

Experimental animals (ZBC mice 25 to 30 gm and Nelson strain rats 450 to 500 gm) were fasted during the course of the experiment and for the previous 10 hours. Injections of 4-arsonophenylazoprotein (preparation M9) were made intraperitoneally. Mice were killed by inhalation of CO_2 immediately before bleeding from the posterior vena cava, and rats were lightly etherized before cardiac puncture.

RESULTS

The Hyperlipemia Produced by 4-Arsonophenylazoprotein.— When rats and mice were injected with quantities of 4-arsonophenylazoprotein normally used for therapeutic experiments in the treatment of transplanted lymphomas, and which caused no external evidence of toxicity, a marked plasma lactescence was produced within 2 to 3 hours. As anticipated, this was found to be associated with a considerable elevation of the plasma triglyceride level (Table I). 6 hours after the injection of azoprotein the triglyceride content of the plasma of 3 mice gave an average value of 1894 mg per cent, and of 3 rats 2096 mg per cent. To

investigate the phenomenon further it was necessary to use animals of a larger species in which repeated blood samples could be easily obtained. For this purpose the rabbit proved particularly suitable. Lipemia was readily and consistently produced by quantities of azoprotein which produced no other apparent toxic effect.

Table II shows the effect of injections of a number of different 4-arsonophenyl-

TABLE II

The Effect of 4-Arsonophenylazoproteins on the Plasma Triglyceride Concentrations of Rabbits

Rabbit No.	Treatment			Dosage		Plasma triglyceride conc.	
	4-Arsonophenylazoprotein used	Preparation	As	mg azoprotein/kg	mg As/kg	Before injection	6 hrs. after injection
			per cent			*mg per cent*	*mg per cent*
1	None, Isotonic NaCl	—	—	—	—	28.3	10.4
2	None, Isotonic NaCl	—	—	—	—	69.4	32.7
3	Azoalbumin (human)	M9	6.5	7.7	0.5	38.3	59.0
4	Azoalbumin (human)	M9	6.5	15.4	1.0	57.4	249.1
5	Azoalbumin (human)	M9	6.5	38.5	2.5	37.1	334.5
6	Azoalbumin (human)	M9	6.5	77.0	5.0	40.6	982.8
7	Azoalbumin (bovine)	K9	1.4	357.2	5.0	16.0	280.4
8	Azoalbumin (human)	K15	2.2	227.3	5.0	4.4	322.7
9	Azoalbumin (bovine)	K(4x)	5.2	96.4	5.0	5.8	785.1
10	Azoalbumin (human)	M8	17.0	29.4	5.0	12.9	275.3

Albino rabbits (3.0 to 3.7 kg) injected intravenously with solutions of azoproteins in 0.9 per cent sodium chloride in a volume of 5 to 6 ml. Rabbits 1 and 2 received 0.9 per cent sodium chloride solution only. All animals were fasted during the course of the experiment and for the preceding 12 hours.

azoproteins on the plasma triglyceride levels of fasting rabbits. The azoprotein preparation M9, which contained 6.5 per cent As, produced in 6 hours a marked rise in triglyceride level when given in a dosage of 15 mg/kg, which was equivalent to 1.0 mg As/kg. Increasing doses produced progressively more powerful effects. By contrast, controls injected with physiological saline showed a slightly declining plasma triglyceride level during the experiments.

It will also be seen from Table II that the effectiveness of azoproteins in producing hyperlipemia varied with their arsenic content. Even though an equal amount of arsenic (5.0 mg/kg) was given to each of the animals in this experiment, conjugates containing 1.4 and 2.2 per cent As were considerably less

effective in influencing blood triglyceride levels than those containing 5.2 and 6.5 per cent As. But above this, increasing the arsenic content to 17 per cent produced materials which in relation to their arsenic content were again considerably less effective.

Since the 4-arsonophenylazoprotein preparation M9 was the most potent of

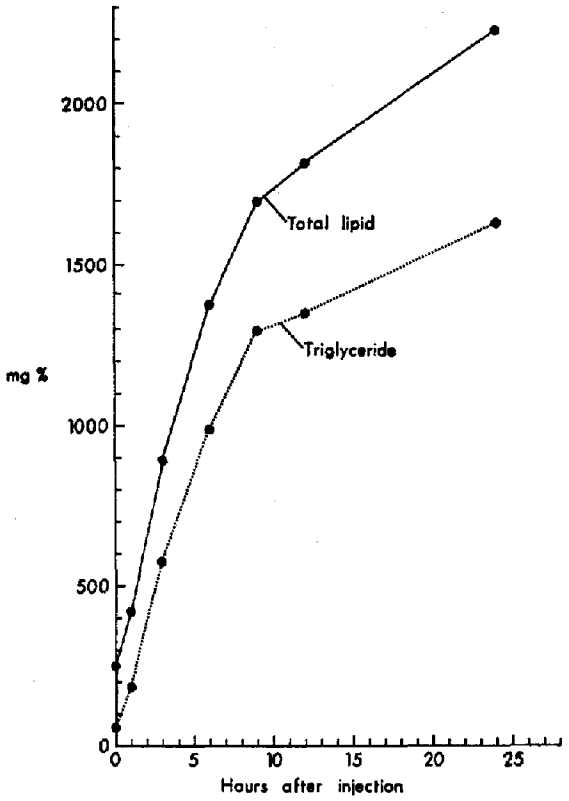

FIG. 1. Changes in the plasma triglyceride and total lipid concentrations of a rabbit injected with 4-arsonophenylazoprotein (preparation M9, 6.5 per cent As, dosage 5 mg As/kg).

those tested, it was used in most of the subsequent experiments. In all 42 rabbits were injected with this azoprotein, in doses containing from 1 to 5 mg As/kg, and each showed a marked hyperlipemia.

Other experiments, following the early observations which have been described, revealed further features of the hyperlipemic effect.

The Rapid Onset of Hyperlipemia.—In 6 rabbits tested, the total fat content of the plasma began to rise within 1 hour of the injection of azoprotein. Fig. 1 shows the results from an animal in which changes in the total fat content

were examined at intervals for 24 hours. In the 1st hour the fat content rose from an initial value of 250 to 420 mg per cent and after this continued to rise steeply for the following 12 hours. Indeed, it was still increasing when the experiment was ended after 24 hours. At this time, although the animal had been fasting since 12 hours before the beginning of the experiment, its plasma was extremely lactescent and contained 2225 mg per cent lipid. Examination of the plasma samples showed that almost all the increase was due to triglyceride fat. A moderate increase of non-triglyceride lipids occurred, which from a preinjection level of 205 mg per cent rose to 592 mg per cent at 24 hours, but this was a

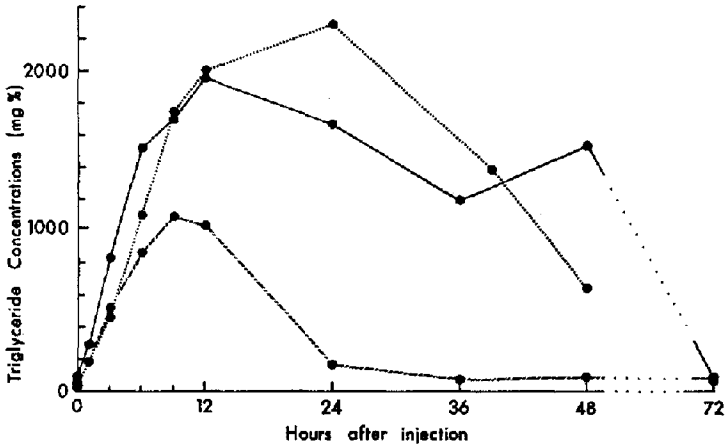

FIG. 2. Changes in the plasma triglyceride concentrations of 3 rabbits injected with 4-arsonophenylazoprotein (preparation M9, dosage 5 mg As/kg).

small change when compared with the massive accumulation of triglyceride in the same period.

Further experiments showed more of the persistence of the hyperlipemic effect.

The Prolonged Duration of the Hyperlipemia Produced by 4-Arsonophenylazoproteins.— Fig. 2 shows the result of injecting 3 rabbits with 4-arsonophenylazoprotein in the dose usually used, that is, 5 mg As/kg. In 2 the hyperlipemic effect persisted for 48 hours, while in the 3rd, a less responsive animal, although the peak was reached at 9 hours, the level at 24 hours was 175 mg per cent, at least twice that found in untreated fasting animals. These results were brought about only by the arsenic-containing azoproteins, as further experiments showed.

The Failure of Arsenic-Containing Substances Other Than 4-Arsonophenylazoproteins to Produce Hyperlipemia.— Following the experiments with 4-arsonophenylazoproteins just described, the effects of 4 other arsenic-containing

substances on the blood fat level were examined. First, in an experiment parallel to that shown in Fig. 1, a mixture of arsanilic acid and human albumin both in quantities equivalent to those given in the earlier experiment as azoprotein, failed to produce any significant change in either total or triglyceride fat levels in the plasma throughout a similar 24 hour period. This may be seen in Fig. 3. As the hyperlipemia produced by 4-arsonophenylazoprotein became

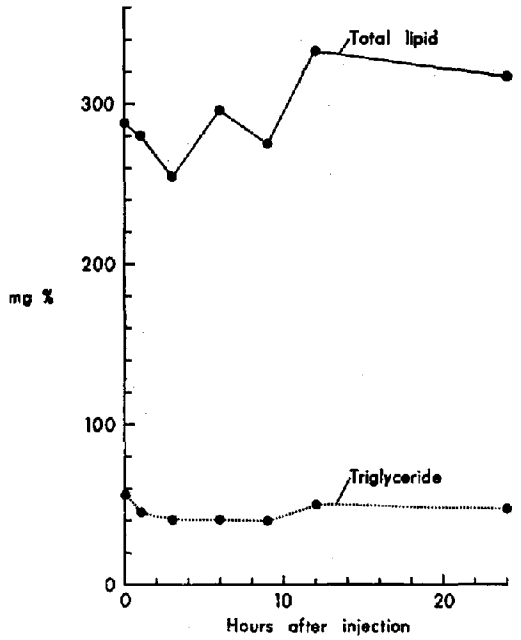

FIG. 3. The failure of mixtures of arsanilic acid and human albumin to influence plasma triglyceride concentrations in the rabbit. A rabbit (weight 3.1 kg) fasting from 12 hours before the experiment, was injected with human albumin (72 mg/kg) and arsanilic acid (5 mg As/kg), dissolved in 6 ml physiological saline and pH adjusted to 7.3.

marked soon after its administration the effect of 3 other arsenicals was examined in detail for 24 hours following injection. As shown in Table III, inorganic arsenic in trivalent or pentavalent form (as sodium arsenite or sodium arsenate) failed to influence the blood triglyceride level. Organic arsenic in the trivalent form (as mapharsen) similarly was without effect.

Although 4-arsonophenylazoprotein in a dose of 5 mg As/kg produced no external evidence of toxicity, other arsenic-containing substances were far more toxic and at the doses given (2 to 3 mg As/kg) 3 out of 6 animals died within 30 hours. It appears clear, therefore, that the hyperlipemia is not simply a feature of arsenic intoxication, but depends on specific structural features of the

arsenic-containing azoproteins. This point will be discussed more fully later, in relationship to the activity of 4-arsonophenylazoproteins on the heparin-activated lipase.

The Effect of 4-Arsonophenylazoprotein in Vivo on the Activity of the Heparin-Activated Lipid Clearing System.—When heparin was given to rabbits which had previously received injections of 4-arsonophenylazoprotein, plasma samples subsequently obtained (PHP) failed to show any lipid clearing activity. An

TABLE III

The Failure of Arsenic in a Number of Chemical Combinations to Influence the Level of Plasma Triglyceride in Rabbits

Rabbit No.	Arsenical injected	Dose, mg As/kg	Plasma triglyceride concentrations at intervals after the injection of arsenicals						
			0 hrs.	1 hr.	3 hrs.	6 hrs.	9 hrs.	12 hrs.	24 hrs.
			mg per cent	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*
1	Sodium arsenite	2	7.7	7.1	3.3	2.5	—	—	—
2	" "	2	23.5	15.0	15.9	14.8	13.1	28.2	—
3	Sodium arsenate	2	16.7	13.3	14.9	15.2	—	11.3	44.3
4	" "	2	19.4	14.7	7.6	7.3	6.1	15.6	26.3
5	Mapharsen	3	48.4	34.4	21.9	26.2	—	19.8	25.9
6	"	3	8.7	5.4	8.8	6.4	—	7.5	12.6

Plasma samples were obtained from market bought albino rabbits of either sex, weighing 2.3 to 2.9 kg, from which food was withheld during the period of the experiment and for the preceding 12 hours. Sodium arsenite and sodium arsenate were dissolved in physiological saline and the pH adjusted to 7.2 with 0.15 N NaOH, each animal receiving an injection of 5 ml. Mapharsen (2-amino-4-arsenophenol hydrochloride, Parke Davis and Company, Detroit) was injected in 5 ml distilled water. The quantities of arsenic used proved toxic to the rabbits. Rabbit 1 died 9 hours after injection, rabbits 4 and 5 at 24 and 30 hours.

experiment demonstrating this is shown in Fig. 4. Here PHP was obtained from animals which 6 hours previously had been injected with equivalent quantities of either 4-arsonophenylazoprotein, or a mixture of arsanilic acid and human albumin, or with physiological saline. It will be seen that the usual strong clearing action was produced by plasma from the controls which first received saline. So too, animals first given a mixture of arsanilic acid with human albumin produced a strong plasma clearing activity. But plasma from animals first treated with 4-arsonophenylazoprotein and which was by this time considerably lactescent, produced no clearing effect whatsoever. This effect might be explained in one or both of two ways: azoproteins could prevent heparin from activating the clearing factor or inhibit the formed clearing factor directly. The second of these possibilities was readily tested.

The Effect of 4-Arsonophenylazoprotein Added in Vitro to the Heparin-Activated Clearing System.— When 4-arsonophenylazoprotein was added to PHP, a strong inhibition of the lipid clearing system occurred, as shown in Fig. 5. An effect was demonstrable at a concentration in the final mixture as low as 10 μg of 4-arsonophenylazoprotein/ml which represented only 0.65 μg As/ml. This

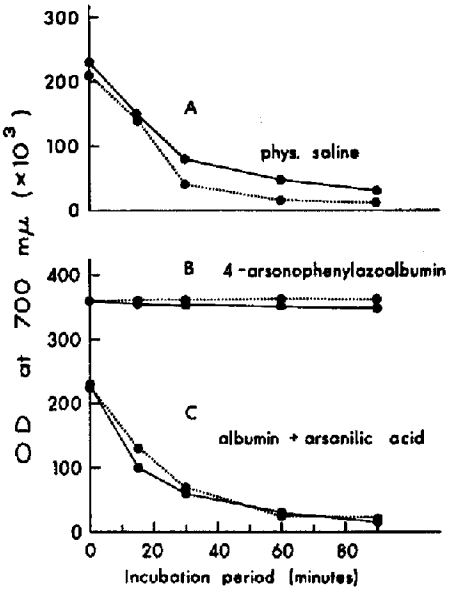

FIG. 4. The failure of heparin to bring about activation of the plasma clearing system in rabbits previously treated with 4-arsonophenylazoprotein.

A. Rabbits injected with 5 ml physiological (phys.) saline 6 hours before injection of heparin (7.5 mg/kg). The graph shows the clearing activity of plasma samples obtained 15 minutes later, tested on emulsions of coconut oil. *B*. As in *A*, but rabbits injected with 4-arsonophenylazoprotein (M9, 5 mg As/kg) 6 hours before the injection of heparin. *C*. As in *B*, but instead of 4-arsonophenylazoprotein, a mixture of arsanilic acid and human albumin (brought to pH 7.0) was injected. This provided amounts of arsenic and albumin equal to those given as azoprotein.

was a true inhibition of neutral fat hydrolysis, for when 4-arsonophenylazoprotein at a concentration of 0.05 mg/ml was added to 2 clearing systems each containing a different sample of PHP, less than 1 per cent of hydrolysis (from the initial triglyceride concentrations of 229.6 and 250.4 mg per cent) was produced during an incubation period of 60 minutes at 37°C. By contrast, in 2 controls in which 4-arsonophenylazoprotein was replaced by bovine albumin, 55.2 and 43.1 per cent respectively of the triglyceride underwent hydrolysis.

Other arsenic-containing substances failed to produce any notable effect on the clearing system. Figs. 6 and 7 show the effects of sodium arsanilate, sodium

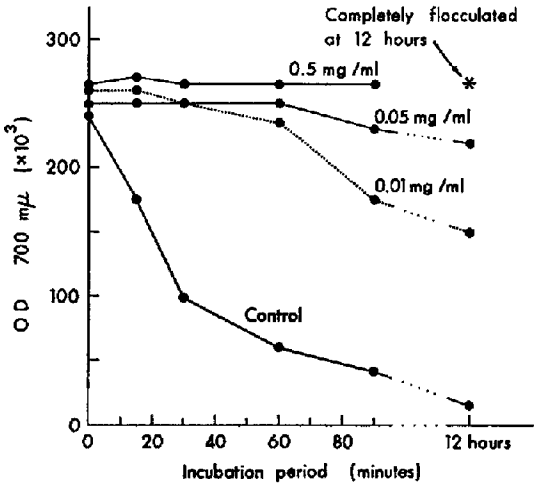

FIG. 5. The inhibition by 4-arsonophenylazoprotein of the heparin-activated clearing system *in vitro*. Concentrations shown are those of the azoprotein M9 in the clearing system.

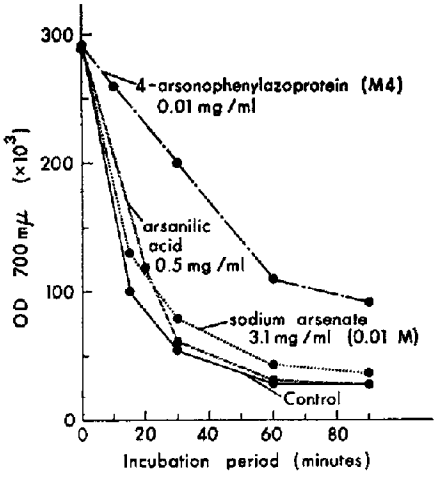

FIG. 6

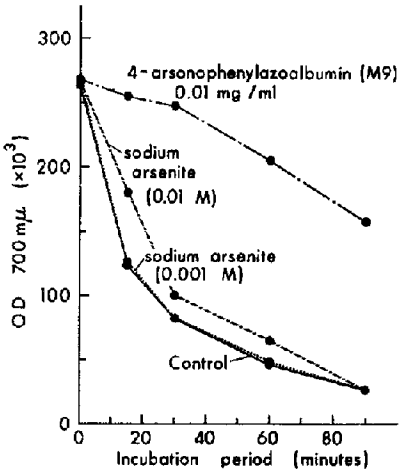

FIG. 7

FIG. 6. The effect of sodium arsenate and arsanilic acid on the lipid clearing action of PHP compared with that of 4-arsonophenylazoprotein. The pH of solutions of each test substance was adjusted to 7.3 with 0.15 N sodium hydroxide before addition to the clearing systems. The azoalbumin M4 contained 1.4 per cent As. Concentrations shown in this and succeeding figures are of test substances in the total clearing system.

FIG. 7. The effect of sodium arsenite on the lipid clearing action of PHP compared with that of 4-arsonophenylazoalbumin.

arsenate, and sodium arsenite. Even in concentrations of arsenic many times greater than that present as azoprotein, they produced only a minimal inhibition.

Overbeek and van der Vies (18) were able to produce a partial inhibition of lipid clearing systems by using concentrations of sodium arsanilate of 0.4 to 3 mg/ml and prolonged incubation with PHP before testing its clearing activity. But even under these conditions, a concentration of arsanilate of 0.5 mg/ml (which was that used in the present experiments) was only slightly inhibitory. It should be noted that the concentration of arsenic in this system was 250 times greater than that in others containing 4-arsonophenylazoprotein which were far more powerfully inhibited (see Fig. 5).

Since trivalent arsenic is a particularly powerful inhibitor of a number of sulfhydryl enzymes, several experiments were performed to examine the possibility that the presence of a contaminant of arsenic with this valency (perhaps formed by reduction during diazotisation) was responsible for inhibition of the lipid clearing system. However, an azoalbumin prepared from *p*-aminophenylarsene oxide (the analogue of *p*-aminophenylarsonic acid containing trivalent arsenic), while also inhibiting the clearing factor in PHP, possessed only 10 per cent of the activity of the pentavalent arsenic azoprotein (preparation M9) when the two were compared by arsenic content. Also, dimercaprol, L-cysteine, and glutathione when incubated with solutions of azoprotein at 50 times the molar concentration of arsenic present in these, failed to influence the inhibitory action of the 4-arsonophenylazoprotein solutions when they were subsequently added to lipid clearing systems. These results indicate that the strong inhibitory effect is in fact associated with the arsonic group, that is, with arsenic in the pentavalent form.

Since the results described showed that the 4-arsonophenylazoproteins have the capacity to inhibit the heparin-activated clearing system *in vitro*, experiments were performed to determine whether inhibition of this system could account for the different features of the hyperlipemia which were observed *in vivo*.

The Correlation of the Hyperlipemia Induced by 4-Arsonophenylazoprotein with Inhibition of the Heparin-Activated Clearing System.— A direct comparison of the degree of hyperlipemia in serial plasma samples from 4-arsonophenylazoprotein–treated animals with their inhibitory action in heparin-activated clearing systems was examined in 3 rabbits. Fig. 8 shows the typical result. Plasma samples following azoprotein treatment produced a considerable inhibition of the clearing action of PHP, even initially at a dilution of 1/5. At this dilution the inhibition became very slight in samples obtained after 12 hours, but undiluted plasma produced a marked inhibition for as long as 36 hours after its administration and even at 48 hours some inhibitory effect was still apparent. It will also be seen from the figure that these results corresponded

well with the degree of hyperlipemia. Thus, the triglyceride level reached its maximum at 24 hours and thereafter its declining values corresponded closely to the decreasing inhibitory activities of the plasma.

Further experiments were undertaken to determine whether the concentration of 4-arsonophenylazoprotein in the plasma of treated animals could be related to the degree of hyperlipemia. Measurements of the rate of clearance of

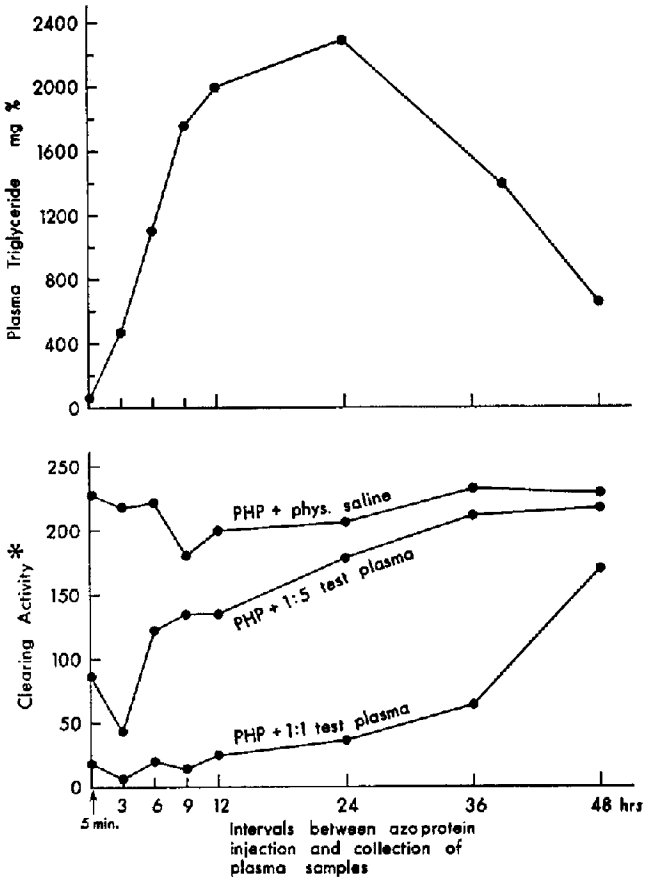

FIG. 8. The effect of adding plasma samples from a rabbit treated with 4-arsonophenylazo-albumin to heparin-activated clearing systems. Plasma samples were obtained at intervals from a rabbit injected with 4-arsonophenylazoprotein (M9, 5 mg As/kg). 0.1 ml of each of these plasma samples (either undiluted or diluted 1:5 in physiological saline) was added to mixtures of 0.7 ml PHP, 1.0 ml imidazole buffer, and 0.2 ml coconut oil emulsion, as in other clearing systems. Clearing activities were compared with those of controls in which physiological saline replaced the azoprotein-containing plasma.

*Clearing activity was measured by the decrease in optical density of the test systems ($\times 10^3$) during an incubation period of 30 minutes at 37°C.

4-arsonophenylazoprotein by 2 rabbits (Fig. 9) showed that no azoprotein could be detected in the plasma of either animal 24 hours after its administration. This finding was confirmed in 4 further animals which at 12 hours all contained demonstrable amounts of azoprotein in the plasma, but none by 24 hours. In 4 of these 6 animals the level of plasma triglyceride was approaching normality (90 to 170 mg per cent) at 24 hours and there was no obvious lactescence of the

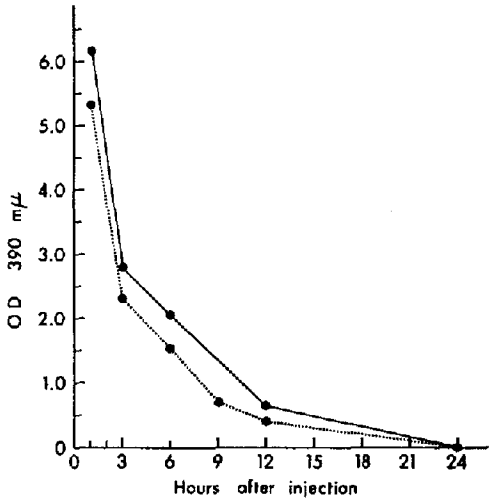

FIG. 9. Clearance of 4-arsonophenylazoalbumin from the plasma of injected rabbits. 2 rabbits, weighing 2.9 and 3.1 kg, fasted for 12 hours before the experiment, were injected intravenously with 4-arsonophenylazoprotein (M9, dosage 77 mg/kg). 2-ml blood samples were obtained before injection and at the intervals shown following it. To measure azoprotein concentrations, fat was first extracted by shaking the plasma samples with twice their volume of chloroform, and the optical density of the aqueous phase was measured at 390 mμ after suitable dilution in 0.1 M phosphate buffer pH 7.4. Preinjection plasma samples similarly treated served as blanks. Although other proteins were precipitated by chloroform, control experiments showed the concentration of azoprotein to be not significantly altered by this treatment.

plasma, but in the other two a high hyperlipemia persisted for 48 hours (triglyceride concentrations were then 2050 and 1783 mg per cent respectively). It appears, therefore, that although circulating azoprotein could be responsible for the hyperlipemia during the first 24 hours following injection, other mechanisms must be involved in some animals under the experimental conditions used which prolong the hyperlipemic effect for a considerable period following the clearance of azoprotein.

Whether this late hyperlipemia is an artifact caused by repeated bleeding to obtain samples for analysis (19) or is due to an effect of the azoprotein itself, remains to be determined.

In summary, 4-arsonophenylazoproteins produced a marked elevation in the blood levels of triglyceride fat when injected into 3 mammalian species. In the rabbit this was found to be associated with an inhibitory action of the azoprotein on the heparin-activated plasma lipase. But in the experimental systems used secondary effects in some animals were found to prolong the hyperlipemia after clearance of azoprotein from the plasma. The results differ strikingly from those produced by the injection of other acidic azoproteins, as will be described.

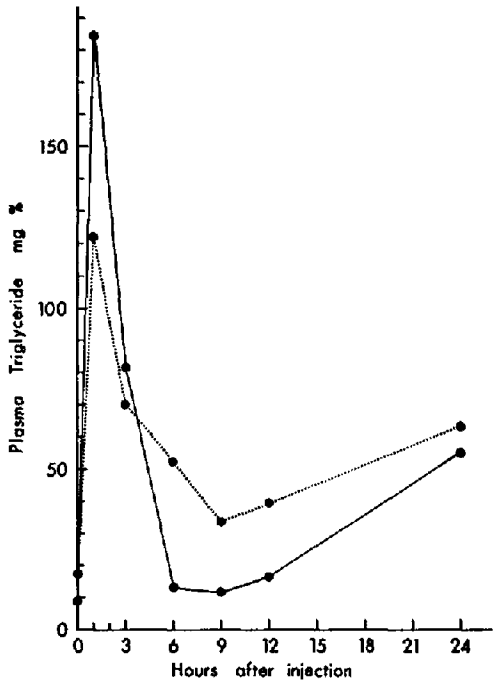

FIG. 10. Changes in plasma triglyceride concentrations following injection of 4-sulfonophenylazoprotein in rabbits. In both animals a dosage of 77 mg azoprotein/kg was used.

The Effects of 4-Sulfono- and 4-Carboxyphenylazoproteins on Plasma Triglyceride Concentrations.— A characteristic sequence of changes in blood triglyceride levels occurred when 4-sulfono- and 4-carboxyphenylazoproteins were injected into rabbits. Figs. 10 and 11 show the close similarity in the results following the injection of both azoproteins. 1 hour following injection, a notable rise in triglyceride content occurred, in one case, following injection of 4-sulfonophenylazoprotein a rise from 9.2 to 185.0 mg per cent was found. But after the 1st hour a sharp decline was observed, so that in each case at 6 hours the triglyceride concentration was in the normal fasting range and remained at this level until a slight final rise occurred at 24 hours. With the preparations used

the sulfono derivative produced a rather stronger effect than the carboxy derivative. However, neither sulfanilic acid nor *p*-aminobenzoic acid, when administered in greater or equivalent amounts as a simple mixture with protein, produced any change in the level of plasma triglyceride. So too, an azoprotein which lacked acidic groups (phenylazoalbumin) failed to produce any

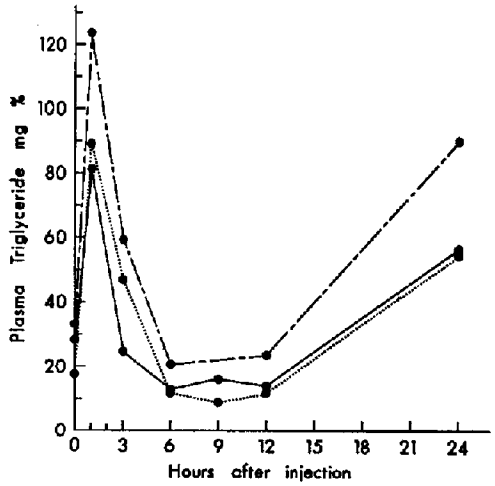

FIG. 11. Changes in plasma triglyceride concentrations following injection of 4-carboxyphenylazoprotein in rabbits. Dosages given were: rabbit 1, 125 mg azoprotein/kg (----); rabbit 2, 100 mg azoprotein/kg (····); rabbit 3, 77 mg azoprotein/kg (——).

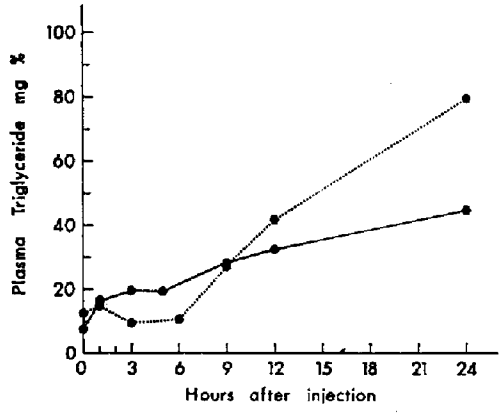

FIG. 12. Plasma triglyceride concentrations in rabbits following injection of phenylazoprotein. In both experiments shown, a dosage of 200 mg azoprotein/kg was used.

significant change (Fig. 12) in the early period, although as with the acidic azoproteins some rise in the concentration of triglyceride occurred after 12 hours.

The biphasic nature of the changes produced by 4-sulfono- and 4-carboxyphenylazoprotein suggested that they might cause several conflicting effects on the heparin-activated clearing system. Direct experiments confirmed this.

The Effects of 4-Sulfono- and 4-Carboxyphenylazoproteins on the Heparin-Activated Clearing System.— When 4-sulfono- and 4-carboxyphenylazoproteins

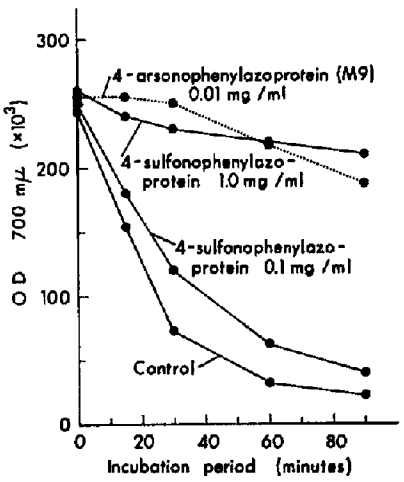

FIG. 13

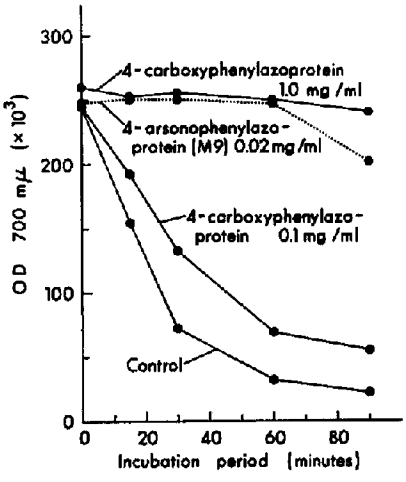

FIG. 14

FIG. 13. A comparison of the inhibitory activities of 4-arsonophenyl- and 4-sulfonophenylazoproteins on the heparin-activated clearing system *in vitro*.

FIG. 14. A comparison of the inhibitory activities of 4-arsonophenyl- and 4-carboxyphenylazoproteins on the heparin-activated clearing system *in vitro*.

were added to the heparin-activated clearing system *in vitro*, an inhibitory effect was produced. However, as may be seen in Figs. 13 and 14 neither of these substances acted so powerfully as the 4-arsonophenylazoprotein, for although the preparations of 4-arsonophenylazoprotein and 4-sulfonophenylazoprotein possessed approximately equivalent numbers of azo groups per molecule,[1] the preparation of 4-sulfonophenylazoprotein possessed only 1/100 of the inhibitory activity of the 4-arsono derivative. As with arsanilic acid no inhibitory activity was shown by simple mixtures of sulfanilic acid or *p*-aminobenzoic acid with serum albumin (Fig. 15).

A correlation between the inhibition of the clearing system and the initial hyperlipemia was shown on adding plasma samples obtained 15 minutes after

[1]Gm atomic weights of arsenic and sulfonic group sulfur were 0.088 and 0.107 respectively/100 gm azoprotein.

injection of azoprotein to heparin-activated clearing systems. As anticipated, these samples produced an inhibition of the clearing action of PHP, but samples obtained at 1 hour and later had no such effect, although, as shown by their color, a considerable quantity of azoprotein was still present. In complete contrast, these samples themselves produced, like PHP, a strong clearing action on fatty emulsions.

The Activation of the Plasma Lipid Clearing System by 4-Sulfono- and 4-Carboxyphenylazoproteins.—Figs. 16 and 17 show that plasma samples obtained at intervals following the injection of 4-sulfono- and 4-carboxyphenylazoprotein began to show clearing activity 1 or more hours after administration

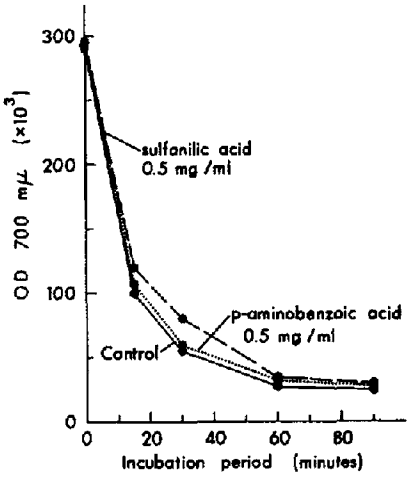

FIG. 15. The failure of *p*-aminobenzoic acid and sulfanilic acid to inhibit the heparin-activated clearing system *in vitro*. Sulfanilic and *p*-aminobenzoic acid solutions (pH adjusted to 7.0) were made up in 8.0 gm per cent bovine serum albumin.

of azoprotein, which reached its maximum at 3 to 6 hours and afterwards declined. No clearing action was produced by animals given the non-acidic phenylazoprotein.

As with PHP the clearing activity produced by 4-sulfono- and 4-carboxyphenylazoprotein was associated with the activation of lipase, as may be seen in Table IV. The injection of bovine albumin produced no activation of lipase, but plasmas from animals treated with 4-sulfono- and 4-carboxyphenylazoproteins were very actively lipolytic, and indeed in some experiments almost as strongly as preparations of PHP.

It is thus seen that under these experimental conditions the changes in plasma triglyceride level, both its initial rise and later fall, are closely correlated with changes in the activity of the plasma lipid clearing system.

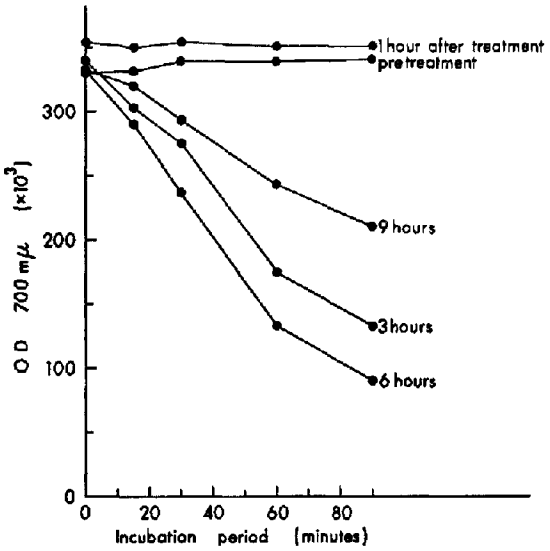

FIG. 16. The activation of clearing factor by 4-sulfonophenylazoprotein. A rabbit was injected with 77 mg/kg of 4-sulfonophenylazoprotein, and plasma samples were obtained at the intervals shown. In clearing systems these samples replaced the PHP used in earlier experiments.

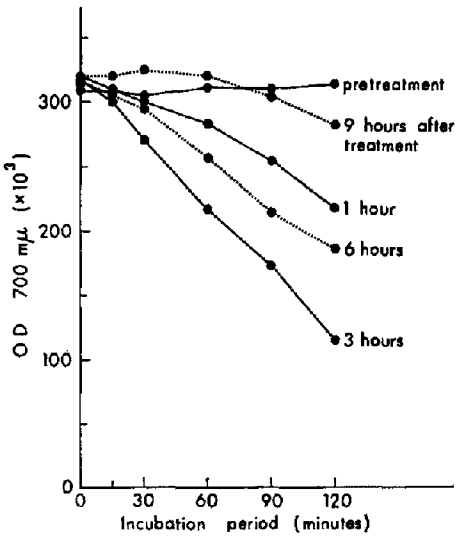

FIG. 17. The activation of clearing factor by 4-carboxyphenylazoprotein. A rabbit was injected with 100 mg/kg of 4-carboxyphenylazoprotein the experiment was otherwise performed as described in Fig. 16.

DISCUSSION

The experiments described show that 3 azoproteins produce marked effects on blood triglyceride levels. 4-Arsonophenylazoprotein produces a high and prolonged hyperlipemia, 4-sulfono- and 4-carboxyphenlazoproteins produce an initial raising and later lowering of blood fat levels. The hyperlipemia consists almost entirely of triglyceride; non-triglyceride lipids are increased relatively little by any of the azoproteins, in this resembling the changes induced in animals treated by Kellner *et al.* with tween 80 and tritons (7).

To account for the accumulation of fat in the blood of animals treated with azoprotein, one must postulate the occurrence of one or both of two processes:

TABLE IV

The Activation of Plasma Lipase in the Rabbit by 4-Sulfono- and 4-Carboxyphenylazoproteins

Rabbit No.	Substance injected	Dosage	Lipase activity (triglyceride fully hydrolyzed in 90 min./1 ml plasma)		Time after injection
			Before injection	After injection	
		mg/kg	*mg*	*mg*	*hrs.*
1	Bovine albumin	77.0	0.25	0.06	3
2	Heparin	7.5	0.17	4.76	¼
3	4-Sulfonophenylazoalbumin	77.0	0.21	3.48	6
4	"	77.0	0.26	3.71	6
5	"	77.0	0.17	2.90	6
6	4-Carboxylphenylazoalbumin	77.0	0.00	1.02	6
7	"	100.0	0.00	2.19	3
8	"	100.0	0.00	2.45	6

first, that there is an excessive mobilization of fat from the depots; or, secondly, that there is impairment of the removal of fat from the blood, since fat continuously enters it from the tissues (4). The present experiments provide no evidence on whether the first of these mechanisms is involved, but the very close correlation between changes in triglyceride fat levels and the activity of azoproteins on the lipid clearing system does provide evidence for a mechanism by which azoproteins could act through the second process and so cause the hyperlipemia observed. Recent evidence (Robinson and French, reference 4) suggests that the clearing system may, under physiological conditions be located in the capillary walls; the effects produced by azoproteins would be fully consistent with their acting on such a site.

On a structural basis, the action of 4-arsono-, 4-sulfono-, and 4-carboxyphenylazoproteins on blood fat levels is probably due to their being, like heparin, macromolecules carrying polar groups with strong negative charge; un-

substituted phenylazoprotein lacking the negative charge is without effect.

Korn, using a lipase extracted from tissues which has apparently identical properties with that induced by heparin in the plasma, has provided strong evidence that heparin forms an integral part of the enzyme molecule (20, 21). *In vivo* a number of sulfated polysaccharides can substitute for heparin in activating plasma lipase (Constantinides *et al.*, reference 22), and so too can macromolecules with other acidic groups, as shown by Havel and Bragdon (23). But although heparin in low concentration is a potent activator of clearing factor, in higher concentration it is in fact strongly inhibitory (24). A possible explanation for the effects of 4-sulfono- and 4-carboxyphenylazoproteins on the clearing system may be found by comparison with these effects of heparin. Initially, following the injections of these 2 azoproteins, when their concentration in the blood is high, inhibition of the clearing system occurs. But as the azoproteins are removed from the blood, their lower concentrations then become such as to cause activation of the clearing system possibly by acting as substitutes for heparin in the lipase molecule. The observed initial rise and later fall of blood triglyceride levels could be explained on this basis.

4-Arsonophenylazoproteins are exceptional in causing only inhibition of the lipase clearing system and in whatever dose given, no activation of the enzyme. The experiments which have now been described do not, however, show the precise means of this inhibition, which, indeed, is unclear for other inhibitors too. It is possible that the protein-combining activity of 4-arsonophenylazoprotein is involved (13), either causing complex formation with lipase or inactivation of its lipoprotein cofactor (20). However, features of structural similarity between 4-arsonophenylazoprotein and heparin (both are macromolecules with a high negative charge) suggest that inhibition could be caused by displacement of heparin from its combination with lipase. There is evidence that pyrophosphate and a number of other phosphate compounds inhibit the enzyme activity in this way (20). The similarity in charge and other properties between phosphate and arsenate groups is shown by their interchangeability in a number of biochemical reactions (see, for instance Rothstein, reference 25).

The results presented thus add 3 further substances, each an acidic azoprotein, to the list of agents known to produce changes in plasma triglyceride levels. It has been possible to correlate the effects of azoproteins on the lipid clearing system with the alterations produced in plasma triglyceride concentrations, providing further evidence for the physiological importance of this system. Further work may disclose whether the lipid clearing system is the sole site of action of the acidic azoproteins on the physiological mechanisms controlling blood triglyceride levels or whether actions on other as yet unrecognized processes are also involved.

SUMMARY

When injected into the rabbit 3 acidic azoproteins produced marked changes in the blood triglyceride levels. 4-Arsonophenylazoproteins, which were found to act similarly in 2 other mammalian species, produced a gross and prolonged hyperlipemia. 4-sulfono- and 4-carboxyphenylazoproteins in the rabbit produced an initial elevation and later lowering in blood triglyceride levels. The correlation between these changes and the action of the azoproteins on clearing factor lipase provides further evidence for the importance of this enzyme in fat transport mechanisms. 4-Arsonophenylazoprotein is powerfully inhibitory to the lipase. 4-Carboxy- and 4-sulfonophenylazoprotein *in vivo* initially inhibit the enzyme and later produce a heparin-like activation.

BIBLIOGRAPHY

1. Hahn, P. F., Abolishment of alimentary lipemia following injection of heparin, *Science*, 1943, **98**, 19.
2. Shore, B., Nichols, A. V., and Freeman, N. K., Evidence of lipolytic action by human plasma obtained after intravenous administration of heparin, *Proc. Soc. Exp. Biol. and Med.*, 1953, **83**, 216.
3. Robinson, D. S., and French, J. E., The heparin clearing reaction and fat transport, *Quart. J. Exp. Physiol.*, 1957, **42**, 151.
4. Robinson, D. S., and French, J. E., Heparin, the clearing factor lipase, and fat transport, *Pharmacol. Rev.*, 1960, **12**, 241.
5. Brown, W. D., Reversible effects of anticoagulants and protamine on alimentary lipaemia, *Quart. J. Exp. Physiol.*, 1952, **37**, 75.
6. Bragdon, J. H., and Havel, R. J., *In vivo* effect of anti-heparin agents on serum lipids and lipoproteins, *Am. J. Physiol.*, 1954, **177**, 128.
7. Kellner, A., Cornell, J. W., and Ladd, A. T., Sustained hyperlipemia induced in rabbits by means of intravenously injected surface-active agents, *J. Exp. Med.*, 1951, **93**, 373.
8. Robinson, D. S., Jeffries, G. H., and French, J. E., Studies on the interaction of chyle and plasma in the rat, *Quart. J. Exp. Physiol.*, 1954, **39**, 165.
9. Rudman, D., and Seidman, F., Lipemia in the rabbit following injection of pituitary extract, *Proc. Soc. Exp. Biol. and Med.*, 1958, **99**, 146.
10. Kellner, A., Hirsch, R. L., and Freeman, E. B., Inhibition of lipoprotein lipase activity following injection of pituitary extracts into rabbits, *J. Exp. Med.*, 1960, **112**, 1.
11. Landsteiner, K., The Specificity of Serological Reactions, Cambridge, Massachusetts., Harvard University Press, 1945.
12. Kidd, J. G., Effects of arsenic-azoproteins on mouse lymphoma cells *in vivo*. With observations on the effects of other "anti-lymphoma" agents, and on the susceptibility to these effects of lymphoma cells of various types, *J. Exp. Med.*, 1958, **108**, 665.
13. Broome, J. D., and Kidd, J. G., Some effects of arsenic azoproteins on lymphoma

cells, fibrinogen and the polymerisation of fibrin, Proceedings, 3rd International Congress of the International Academy of Pathology, London, June 13–18, 1960.

14. Eagle, H., and Vickers, P., On the nature of the reaction between diazotized sulfanilic acid and proteins, *J. Biol. Chem.* 1936, **114**, 193.
15. Ehrlich, P., and Bertheim, A., Reduktions produkte der arsanilsaure und ihrer derivate erste mitteilung: über *p*-aminophenyl-arsenoxyd, *Ber. Chem. Ges.*, 1910, **43**, 917.
16. Sperry, W. M., and Brand, F. C., The determination of total lipides in blood serum, *J. Biol. Chem.*, 1955, **213**, 69.
17. Van Handel, E., and Zilversmit, D. B., Micromethod for the direct determination of serum triglycerides, *J. Lab. and Clin. Med.*, 1957, **50**, 152.
18. Overbeek, G. A., and Vies, J. van der, Clearing factor and lipase, *Biochem. J.*, 1955, **60,** 665.
19. Horiuchi, Y., Studies on blood fat. II. Lipaemia in acute anemia, *J. Biol. Chem.*, 1920, **44,** 363.
20. Korn, E. D., Clearing factor, a heparin-activated lipoprotein lipase. I. Isolation and characterisation of the enzyme from normal heart, *J. Biol. Chem.*, 1955, **215,** 1.
21. Korn, E. D., Inactivation of lipoprotein lipase by heparinase, *J. Biol. Chem.*, 1957, **226,** 827.
22. Constantinides, P., Cairns, A., and Werner, A., Antilipemic activity of sulfated polysaccharides, *Arch. internat. pharmacod.*, 1954, **99,** 334.
23. Havel, R. J., and Bragdon, J. H., Heparin-like activity of polymetaphosphate, *Circulation*, 1954, **10,** 591.
24. Brown, R. K., Boyle, E., and Anfinsen, C. B., The enzymatic transformation of lipoproteins, *J. Biol. Chem.*, 1953, **204,** 423.
25. Rothstein, A., Interactions of arsenate with the phosphate transporting system in yeast, *J. Gen. Physiol.*, 1963, **46,** 1075.