sine, phenylalanine, and ornithine. In this connection it is interesting to note that the average weight of the liver between 150 and 175 days only increased 1.3%. An increase in free amino acid concentration during this period could be explained by a general increase in metabolic activity and enzyme maturation. A decrease in concentrations of the TCA cycle related amino acids, glutamic acid and aspartic acid, may be related to the sharp increase in demand for energy, but no proof is available. Correlation between free amino acid concentrations in the brain and the liver of fetal monkeys is virtually absent. The four amino acids whose concentrations increase in the brain during fetal development do not show this trend in the liver. On the contrary, while the concentrations of aspartic acid and glutamine increase in the brain, they decrease in the liver. Glutamic acid levels in the brain and the liver showed the same sharp increase shortly after birth. The concentrations of threonine, proline, and alanine decrease both in brain and liver.

The free amino acid content of umbilical cord serum from the same fetal monkeys (1) failed to correlate with our data on the brain and liver amino acid concentrations. The free amino acid pool available during development of the fetus is undoubtedly a reflection of the free amino acid concentration in the plasma of the mother (6), but the influence of fasting of the mother monkeys for 8 hr prior to surgery on the amino acid levels in the organs of the fetus is unknown.

It is however, difficult to conceive that the wide range of values for some amino acids are due to dietary influences. Although all animals in this study were growing at the same rate despite the differences in free amino acid concentrations, it is doubtful that these levels in the organs of the fetus are a proper reflection of the rate of protein synthesis at the cellular level.

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Production of Antibodies against Insecticide–Protein Conjugates* (33366)

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The increasing awareness to environmental pollution has resulted in widespread concern about means of detecting and minimizing contamination by pesticide residues. The work reported here constitutes an effort to apply immunologic methods for the assay of pesticide residues. Immune reactions are highly sensitive and specific and can be used as analytical tools without complex and expensive instrumentation. Hence, such a method might be particularly adaptable to field tests.

DDT [1,1,1,-trichloro-2,-2-bis(*p*-chlorophenyl)ethane] and Malathion[1] [*0,0,*-dimethyl-*S*-bis(Carboethoxy)ethylphosphorodithioate] are very widely used and represent two of the most important classes of insecticides: chlorinated hydrocarbons and organophosphates, respectively. These two compounds were chosen for the production of antibodies.

Small organic molecules are not antigenic

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[1] American Cyanamid.

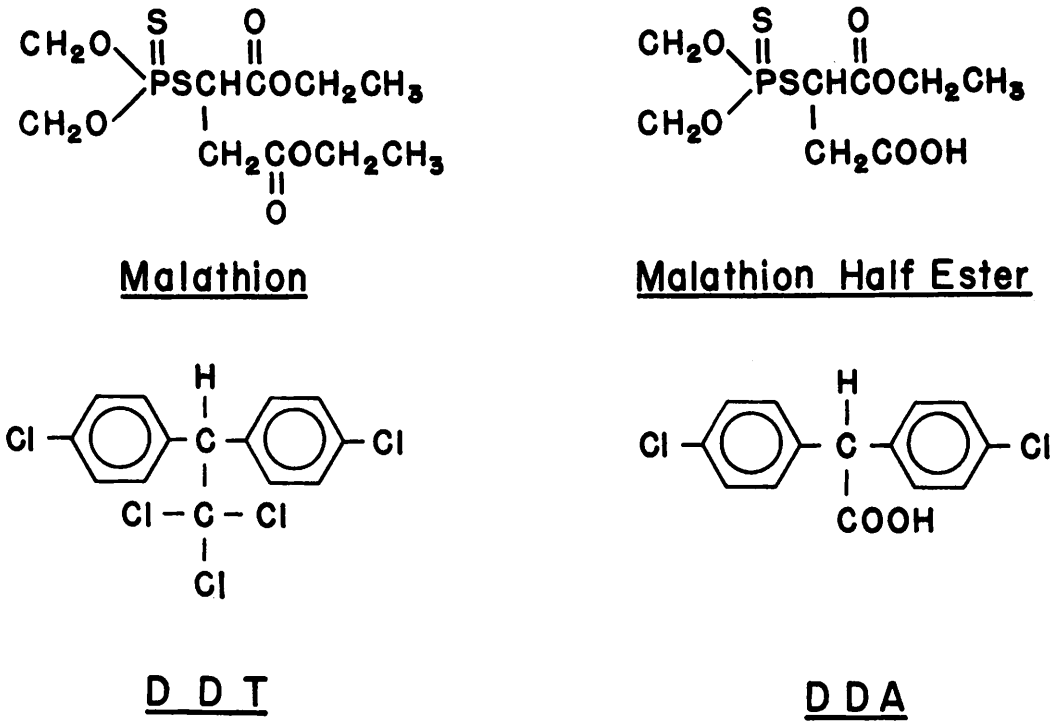

FIG. 1. Structural formulas of the compounds used.

per se. They can, however, be conjugated as haptens to protein carriers yielding an antigenic hapten–protein complex. Hapten specific antibodies may be obtained using such conjugates.

Initially our efforts were concentrated on trying to obtain insecticide–protein conjugates by reacting DDT and Malathion with enzymes which they inhibit. DDT has been reported to inhibit carbonic anhydrase (1). This noncompetitive inhibition was theorized by Keller to be due to binding of DDT to the enzyme molecule (2).

The insecticidal action of organophosphates is believed to be due to their inhibitory effect on enzymes via complex formation (3). Even though complex formation could not be proved chemically, rabbits were injected with DDT–carbonic anhydrase and Malathion–chymotrypsin mixtures in the hope of obtaining antibodies against the insecticide. Such antibodies were not obtained. It was then decided to use synthetic insecticide–protein conjugates for inoculation of the animals.

Experimental Methods. Synthesis of conjugate. Four proteins were used as carriers for the haptens: Rabbit serum albumin was chosen in the hope that with a homologous carrier the immune response would be solely against the hapten. Bovine serum albumin, fibrinogen fraction I, and β-globulin fraction III were also used since they were readily available and are known to be antigenic. All proteins were purchased from Nutritional Biochem. Corp.

DDT is highly inert. Malathion can phosphorylate proteins, but the reaction results in the split of the molecule and the liberation of the succinic acid moiety, which does not link to the protein. For these reasons structurally related derivatives of the insecticides were used in the hope that antibodies against the derivatives would cross react with the insecticides. DDA [2,2-bis(*p*-chlorophenyl) acetic acid] (Aldrich Chemical Co.) and Malathion half ester [*o,o,* dimethyl-*S*-Carboethoxy-Carboxyethylphosphorodithioate] (kindly donated by American Cyanamid Co.) were used as haptens (Fig. 1). Both these compounds contain a free carboxyl

group, which was reacted with thionylchloride to form the respective acyl chloride. Each acyl chloride was reacted with the protein to form the hapten–protein conjugate (approximately 60:1 hapten:fibrinogen molar ratio).

Inoculation. New Zealand white rabbits, 4-5 lb each and less than 1 year old, were inoculated intracutaneously in the neck and foot pads using an emulsion of the appropriate hapten–protein conjugate in saline with complete Freund's adjuvant (Difco). Each rabbit received approximately 2 mg of the conjugate in 1 ml of an emulsion containing equal parts of saline and adjuvant. Trial bleedings and booster shots were carried out at 3-week intervals, except in the last experiment, where this was done at 10-day intervals.

Precipitation. Precipitation tests were performed by layering 0.1 ml of the immune serum on 0.1 ml of the appropriate antigen dilution in a test tube. The mixture was then incubated for 0.5 hr at 37° and a further 0.5 hr at room temperature (25°) and examined for ring formation; it was then shaken again and, after 2 hr at room temperature, examined for turbidity.

To test for reactions directly against DDA, DDT, or Malathion a precipitin test was done on suspensions of these insecticides in water. To prepare these suspensions, stock solutions of DDT, DDA, and Malathion were made in ethyl alcohol so that the final concentration was 20 mg/ml in each case. A 0.1-ml aliquot of the stock solution was added to 0.9 ml of buffered (pH 7.3) saline and the resulting suspensions were serially diluted with buffered saline in 1:3 steps. Malathion was also tested by suspending it directly in warm water. The precipitation tests were then carried out as described above.

Absorption. Absorption of the immune sera with the carrier protein was done as follows, 1–5 mg of the protein were added to 1 ml of the serum which was then incubated for 0.5 hr at 37° and 0.5 hr at room temperature. Any precipitate formed was removed by centrifugation. This procedure was repeated until no further precipitate was found. Suitable controls were included in the precipitin or hemagglutination tests that followed to check for completeness of absorption.

Tanned cell hemagglutination. This test was done as described by Ollodart and Rose (4).

Tanned cell hemagglutination inhibition. Inhibition tests were carried out as follows: Stock solutions of DDA and DDT were made up in several organic solvents. Unfortunately, introduction of these solvents into the aqueous system in which the hemagglutination reactions were carried out often caused lysis of the cells, or made the interpretation of the results difficult. Best results were obtained with dimethyl sulfoxide (DMSO) and dioxane, although, occasionally, lysis would still occur within the first 4 hr, and nearly always after standing overnight. Readings were always made within the first 2 hr. Results were usually identical, whether DMSO or dioxane was used, but the latter caused faster cell lysis.

Twenty mg of DDA or DDT were dissolved in 1 ml of DMSO, or dioxane, and 2 ml of buffered saline were added. The resulting suspension was then serially diluted (1:3) in buffered saline. One tenth ml of each dilution was incubated for 1 hr at room temperature with 0.1 ml of diluted sera. A 0.1-ml aliquot of the coated cell suspension was added, the tubes were shaken and the test was read within 2 hr. A serial dilution of DMSO in buffered saline, containing no DDA or DDT, was used as the control.

Inhibition tests were similarly done with Malathion, except suspensions of this insecticide were made in water (0.2 ml of 95% Malathion in 0.8 ml of warm distilled water and shaken).

Results. The rabbits inoculated with the conjugates of rabbit and bovine serum albumin did not form any antibodies against the protein carrier, the hapten, or the hapten–protein conjugate. The reason for this lack of antigenicity is not known. The conjugates of bovine fibrinogen consistently produced a response in the rabbit against fibrinogen and the hapten–fibrinogen conjugate. As the results with β-globulin were less consistent, experiments were only continued with DDA–fibrinogen (DDA-FBN) and Malathion–fibrinogen (MLT-FBN).

After inoculation of DDA-FBN and MLT-FBN into separate groups of animals, the sera from the first two bleedings were found to agglutinate erythrocytes coated with fibrinogen and the hapten–fibrinogen conjugate. Following absorption with fibrinogen, the sera no longer reacted with the hapten–protein coated cells.

The sera from the third bleeding also agglutinated cells coated with fibrinogen and hapten–fibrinogen, but the latter agglutination could still be found after absorbing the sera with fibrinogen (Table I). These results indicated the presence of antibodies against the hapten–protein conjugate.

Tanned cell hemagglutination inhibition tests using sera absorbed with fibrinogen showed that DDA could inhibit the agglutination of DDA-FBN coated cells and Malathion inhibited the reaction against MLT-FBN (Table II). The sensitivity of the inhibition was between 0.1 and 1.0 μg for both DDA and Malathion. DDT did not inhibit the hemagglutination of DDA-FBN coated cells (Table II).

No direct reaction against DDA, DDT, or Malathion was found by either precipitin or tanned cell hemagglutination (Table III). This lack of direct reaction may be due to the fact that DDA and Malathion, like many other haptens, have only a single antigenic determinant. Consequently, they combine with a single antibody molecule, which is not sufficient to produce a visible reaction. Indirect tests, such as tanned cell hemagglutination inhibition, have to be resorted to in order to establish the presence of hapten specific antibodies.

It is also possible that the very low solubility of these compounds in water affects their ability to react directly. In the case of hemagglutination it is quite likely that these compounds are not able to coat the erythrocytes.

The immune serum from the rabbits inoculated with DDA-FBN did not agglutinate DDA-RSA (the DDA conjugate with rabbit serum albumin) (Table III). The antibodies produced against the conjugates are apparently specific for the hapten conjugate with

TABLE I. Tanned Cell Hemagglutination Pooled Sera from Four Rabbits, Third Bleeding.[a,b]

Reciprocal of dilution	Inoculated with DDA-FBN						Inoculated with MLT-FBN					
	Unabsorbed			Absorbed (FBN)			Unabsorbed			Absorbed (FBN)		
	FBN	DDA-FBN	T.O.[c]	FBN	DDA-FBN	T.O.	FBN	MLT-FBN	T.O.	FBN	MLT-FBN	T.O.
5	+3	+3	—	—	+1	—	+3	+3	—	—	+2	—
25	+2	+2	—	—	+1	—	+2	+3	—	—	+1	—
125	+2	+2	—	—	+1	—	+2	+2	—	—	+1	—
625	+1	+2	—	—	—	—	+1	+1	—	—	—	—
3125	+1	+1	—	—	—	—	+1	+1	—	—	—	—
15,625	+1	+1	—	—	—	—	+1	+1	—	—	—	—
78,125	—	+1	—	—	—	—	—	—	—	—	—	—
390,625	—	—	—	—	—	—	—	—	—	—	—	—

[a] Range of reaction +4 → 0.
[b] Preimmune sera were negative for all compounds.
[c] Tanned only.

TABLE II. Tanned Cell Hemagglutination Inhibition Pooled Sera[a] from Four Rabbits, Third Bleeding.[b]

Row no.:	1	2	3	4	5
Rabbits inoculated with:	DDA-FBN	DDA-FBN	DDA-FBN	MLT-FBN	MLT-FBN
Sera absorbed with:	FBN	FBN	FBN	FBN	FBN
Tanned cells coated with:	DDA-FBN	DDA-FBN	DDA-FBN	MLT-FBN	MLT-FBN
Sera inhibited with:	DDA	DDT	—	MLT	—
Reciprocal of dilution					
3	—	+1	+1	—	+1
9	—	+1	+1	—	+1
27	—	+1	+1	—	+1
81	—	+1	+1	—	+1
243	—	+1	+1	—	+1
729	—	+1	+1	—	+1
2187	—	+1	+1	—	+1
6561	+1	+1	+1	+1	+1
19,683	+1	+1	+1	+1	+1

[a] Pooled antisera diluted 1:50 in buffered saline.
[b] Range of reaction +4 → 0.

the particular protein carrier used (in this case, fibrinogen).

The antifibrinogen antibodies were found to be quite stable to freezing and thawing. In contrast, the antihapten antibodies are quite labile, activity being lost after a few days storage at 4 or —10°. Neither lyophilization nor sterilization by membrane filtration followed by refrigerated storage effectively prevents loss of activity. Preliminary results have shown that freezing with Dry Ice and storage at —30° for up to 1 month preserved the antihapten activity of the serum. More work on the stability of these antibodies is necessary before generalizations about optimal handling conditions can be made.

TABLE III. Precipitin Test.[a]

	DDA-FBN antisera			
Reciprocal of dilution	DDA	DDT	DDA-RSA	DDA-FBN
5	—	—	—	+2
25	—	—	—	—
125	—	—	—	—
	MLT-FBN antisera			
	MLT	FBN	MLT-FBN	
5	—	+2	+5	
25	—	+2	+5	
125	—	+2	+4	

[a] Range of reaction +4 → 0.

An additional difficulty encountered was the fact that these antihapten antibodies appeared to be transitory. In one experiment, two sets of 4 animals were inoculated, one with DDA-FBN and the other with MLT-FBN, bled and reinoculated at 10-day intervals as described before. The pooled sera showed that the maximum titer was obtained approximately 6 weeks after the initial inoculation, and no reaction was detectable 3 weeks after that.

Conclusions. Immune reactions against DDA and Malathion can be achieved by injecting rabbits with synthetic conjugates of these compounds with bovine fibrinogen. No direct reaction can be found against DDA or Malathion, but these haptens inhibit the agglutination of erythrocytes coated with their respective hapten–fibrinogen conjugate.

The lack of cross reaction between DDA and DDT could possibly be due to the greater insolubility of the latter in water. If there is in fact cross reaction with DDT, but it is too weak to be visible, perhaps concentrating the anti-DDA antibodies, or increasing the titer, could result in detectable cross reaction. If, however, the lack of cross reaction is due to the structural dissimilarity between the two

compounds, more closely related derivatives of DDT would have to be used as a hapten, i.e., Kelthane [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol].

The interpretation of immunological results with DDA are made difficult by its low solubility in water. Proper choice of solvents enabled us to get what appear to be meaningful results.

The lack of stability of the hapten specific antibodies cannot be explained at this time. As the antibodies formed against both types of insecticide derivative showed this lability, the problem deserves closer study, so that reasons for the lack of stability and the optimal conditions for the preservation of the sera can be determined.

The transient immune response against DDA-FBN and MLT-FBN is similar to that reported against insulin (5). We cannot explain at this time the reason for the transitory nature of the antibodies.

The immunological approach to the assay of pesticides could be extended to other pesticides and herbicides, with particular emphasis on those which lend themselves to covalent bonding with proteins, i.e., 2,-4-D[(2,4-dichlorophenoxy)acetic acid].

Summary. Antibodies against conjugates of DDA and Malathion half ester with bovine fibrinogen were produced by sensitizing rabbits with these conjugates. No direct reaction was found against DDA or Malathion, but these compounds inhibited the hemagglutination of their respective conjugate. The antihapten antibodies were transitory, and their activity was lost during storage, unless frozen and kept at very low temperatures. Due to the hydrophobic nature of DDA, organic solvents were used in the precipitin and hemagglutination tests involving this compound.

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Antithyroid Effects of 3-Amino-1, 2, 4-triazole* (33367)

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Astwood and associates (1) tested a large number of compounds for their antithyroid activity in 1945. Among these were 3-mercapto-1,2,4-triazole and related compounds. The triazole compounds exhibited some antithyroid activity but were less active than thiouracil, the standard for comparison at that time. In recent years, another triazole compound, 3-amino-1,2,4-triazole (ATZ), has become available and has gained importance as a herbicide, plant growth inhibitor, and defoliant. These effects are probably the result of reduction in chlorophyll (2) and riboflavin (3) content and in catalase activity in the plant (2). In rats, ATZ reduces hepatic and renal catalase and, to a lesser extent, hepatic peroxidase but not erythrocytic peroxidase (2, 4).

Alexander (5) called attention to the selective action of ATZ toward tissue hydroperoxidase and noted that the compound inhibits ^{131}I uptake and the organic binding of ^{131}I without affecting the iodide "trap" in rats. Pitt-Rivers (6) confirmed these effects.

The present study extends the observations

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