Biochemical Pharmacology, Vol. 26, pp. 1841–1846. Pergamon Press, 1977. Printed in Great Britain.

DETECTION BY RADIOIMMUNOASSAY OF NICOTINAMIDE NUCLEOTIDE ANALOGUES IN TISSUES OF RABBITS INJECTED WITH NICOTINE AND COTININE*

WEI-CHIANG SHEN, KATHLEEN M. GREENE and HELEN VAN VUNAKIS†
Department of Biochemistry, Brandeis University, Waltham, MA 02154, U.S.A.

(*Received* 16 *September* 1976; *accepted* 21 *January* 1977)

Abstract—The formation of the NAD and NADP analogues of nicotine and cotinine *in vitro* by the NAD glycohydrolase-catalyzed exchange reaction suggested that such compounds (and the related mononucleotides) might also be produced in animals receiving the alkaloid or its metabolite. Since only sensitive and specific analytical methods could detect small amounts of the nicotine and cotinine analogues in the presence of the naturally occurring and abundant NMN, NAD and NADP compounds, radioimmunoassays were developed and used for this purpose. In rabbits injected with cotinine, cotinine nucleotide analogues were found at pmole levels/g of wet tissue in extracts from liver, kidney and lung. In both liver and lung extracts, cotinine mononucleotide was identified as the major product. Cotinine nucleotides were also found in the tissues of rabbits injected with nicotine. The rapid metabolism of nicotine to continue *in vivo* probably contributes to the sparsity of the nicotine analogues in tissue extracts from these animals.

We have described the formation of nicotinamide adenine dinucleotide analogues of nicotine and cotinine (its major metabolite) by the NAD glycohydrolase (NADase, EC 3.2.2.5)-catalyzed exchange reaction [1–3]. When incubated with a rabbit liver microsomal fraction, these NAD analogues [(nicotine)AD and (cotinine)AD‡] and NADP analogues [(nicotine)ADP and (cotinine)ADP] were hydrolyzed to the corresponding mononucleotides [(nicotine)RP and (continine)RP] [3]. The mononucleotides were relatively stable to further hydrolysis, e.g. 80 per cent of the original (cotinine)RP was recovered when incubated with a rabbit liver extract for 18 hr.

In order to determine whether any correlation exists between the formation of these nucleotide analogues *in vivo* and clinical problems that may be related to cigarette smoking, it is essential that analytical methods for their detection and estimation be available. Radioimmunoassay is a highly specific, sensitive and simple analytical method which is being used to quantitate a variety of compounds in physiological fluids and tissues [4–6]. This method has proven especially useful for the analysis of compounds which occur in small amounts (pg to ng range) in the physiological fluids and tissues of human subjects.

In this paper we describe the development of radioimmunoassays for the detection and estimation of nicotine and cotinine nucleotide analogues. We have used these procedures to investigate nucleotide analogue formation in the tissues of rabbits injected with nicotine and cotinine. A preliminary report of this work has been presented [7].

MATERIALS AND METHODS

Materials. The nucleotide and riboside analogues of nicotine and cotinine were prepared as described [1–3]. Bovine serum albumin (BSA) was purchased from Reheis Chemical Co., Chicago, IL, U.S.A., and 1-ethyl-3-(dimethylaminopropyl)carbodi-imide (CDI) was obtained from the Ott Chemical Co., Muskegon, MI, U.S.A.

Preparation of conjugates for immunization. CDI (10 mg) was added to (nicotine)RP or (cotinine)RP (10 mg) and BSA (5 mg) dissolved in 0.1 ml H_2O. The reaction mixture (pH 7.0–7.2) was kept in the dark at 25° overnight. It was then diluted to 0.3 ml with buffer (0.14 M NaCl–0.01 M Tris–HCl, pH 7.2), passed through a Sephadex G-50 column (1.2 × 30 cm) and eluted with the same buffer. Fractions of the void volume with a high absorbance at 280 nm were pooled (total 3 ml). From spectral measurements, the concentration of protein was 1.8 mg/ml. Since small amounts of [^{3}H]mononucleotides were used in the synthesis of the immunizing conjugate, the degree of coupling could be estimated by radioactivity measurements and was found to be approximately 4 moles hapten/mole of BSA.

Immunization. The mononucleotide–BSA conjugates obtained after Sephadex G-50 chromatography were mixed with an equal volume of complete Freund's adjuvant. The emulsion mixture (1.25 ml) was injected into the toe pads and leg muscle of four New Zealand albino rabbits. After 6 weeks, the ani-

* Publication No. 1126 from the Dept. of Biochemistry, Brandeis Univ., Waltham, MA 02154. Supported by a contract from the National Cancer Institute (NIH-NCI-E-72-3243) and by grant DA0007 from the National Institute of Mental Health.

† PHS Research Career Awardee (Award 5K6-AI-2372 from the National Institute of Allergy and Infectious Diseases).

‡ The Abbreviations used in the text: (nicotine)AD, nicotine adenine dinucleotide; (nicotine)ADP, nicotine adenine dinucleotide phosphate; (nicotine)RP, nicotine mononucleotide; (cotinine)AD, cotinine adenine dinucleotide; (cotinine)ADP, cotinine adenine dinucleotide phosphate; and (cotinine)RP, cotinine mononucleotide.

mals were given booster injections and bled 1 week later. Antibodies with the properties required to develop specific and sensitive radioimmunoassays were obtained after the second course of injections.

Labeled hapten. ([^{3}H]nicotine)AD and ([^{3}H]cotinine)AD were prepared from [^{3}H]nicotine and [^{3}H]cotinine (sp. act. 2.4 Ci/m-mole) by incubating these compounds with 3 mg NAD and 8 mg of pig brain NADase in 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.4. After 1 hr at 37°, the reaction was stopped by placing the tube in a boiling water bath for 3 min. The denatured protein was removed by centrifugation, and the clear supernatant solution diluted to 15 ml with H_2O and chromatographed on an anion exchange resin. After washing the column with an excess of H_2O, a linear buffer gradient (100 ml H_2O to 100 ml of 0.2 M formate buffer, pH 3.6) was applied. Fractions that contained (nicotine)AD or (cotinine)AD were combined and lyphilized. The residue was dissolved in a small amount of H_2O and stored at −20°. These preparations undergo large dilutions prior to use in the radioimmunoassay procedure and therefore need not be desalted. Since the antibodies produced from rabbits immunized with the nicotine or cotinine mononucleotide–BSA conjugate bind the homologous NAD analogue and mononucleotide equally well, the tritiated NAD analogues were used as the labeled antigens in the radioimmunoassays.

Radioimmunoassay procedures. The double antibody technique was used to separate free labeled antigen from antibody-bound labeled antigen. The conditions of the assay are similar to those described previously [8]. Labeled hapten (0.1 ml, approximately 15,000 cpm) and 0.1 ml of serially diluted inhibitor standard (or 0.1 ml of diluted tissue extract) were incubated for 1 hr at 37° with 0.1 ml of rabbit antisera (diluted 1/1000 for anti-cotinine nucleotide and 1/200 for anti-nicotine nucleotide). Normal rabbit serum (0.1 ml of 1/50 dilution) was then added to each tube as carrier to ensure the completeness of the immune precipitation. The hapten–antibody complex was precipitated by the addition of 0.1 ml of goat anti-rabbit γ-globulin previously titered to give complete precipitation of the γ-globulin. After standing overnight at 4°, the tubes were centrifuged, decanted and carefully wiped. The precipitates were dissolved in 0.2 ml of 0.1 N NaOH and transferred to scintillation fluid, and the radioactivity was determined in a liquid scintillation spectrometer. When no inhibitor was present, approximately 2200 cpm was precipitated with the anti-nicotine nucleotide and 2500 cpm by the anti-cotinine nucleotide sera. Approximately 200 cpm was precipitated non-specifically in the presence of normal rabbit serum.

The results obtained in the analysis of crude extracts are expressed as nucleotide equivalents/g of wet tissue since the antisera cannot differentiate between the homologous mononucleotide, NAD, or NADP analogue (see Table 1). After separation by chromatographic procedures, the individual analogues can be quantitated.

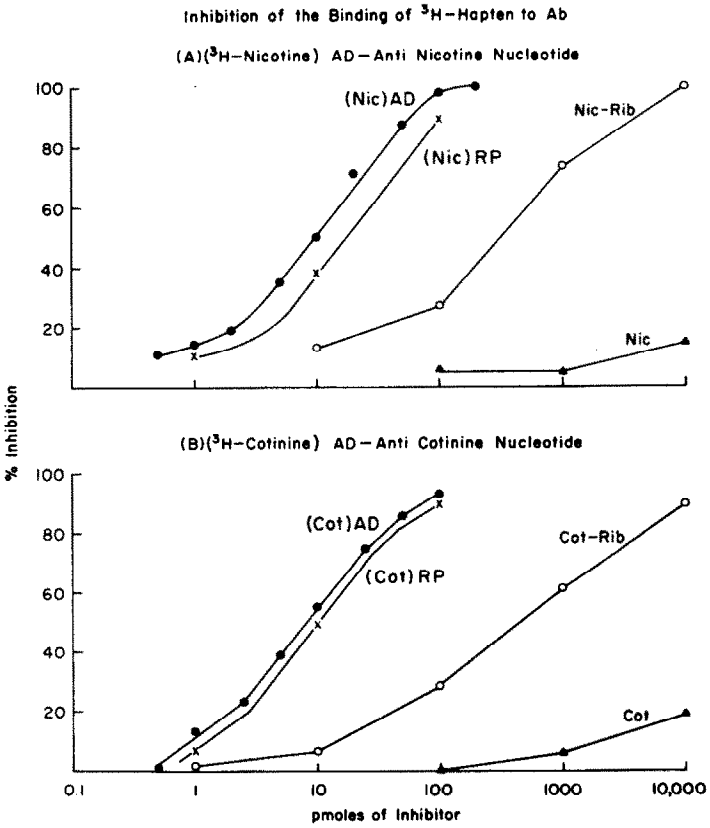

Fig. 1. (A) Inhibition of the ([^{3}H]nicotine)AD-(antinicotine nucleotide) binding by various nicotine derivatives. (B) Inhibition of the ([^{3}H]cotinine)AD-(anti-cotinine nucleotide) binding by various cotinine derivatives. The radioimmunoassay procedure is described in Materials and Methods.

Table 1. Inhibition of the nicotine nucleotide and cotinine nucleotide antigen–antibody reactions

Compound	Amount required for 50 per cent inhibition (nmoles)	
	([^{3}H] nicotine)AD-(anti-nicotine nucleotide)	([^{3}H] cotinine)AD-(anti-cotinine nucleotide)
(Nicotine)AD	0.010	10.0 (45.8%)*
(Nicotine)ADP	0.013	
(Nicotine)RP	0.017	10.0 (38.4%)*
Nicotine–ribose	0.320	
Nicotine	10.0 (14.7%)*	
(Cotinine)AD	10.0 (45.2%)*	0.008
(Cotinine)ADP		0.008
(Cotinine)RP	10.0 (42.8%)*	0.010
Cotinine–ribose		0.450
Cotinine		10.0 (19%)*
NAD	10.0 (3.6%)*	10.0 (0%)*
NMN	10.0 (4.6%)*	10.0 (0%)*
Nicotinamide	10.0 (0%)	10.0 (0%)*

* Per cent inhibition at 10.0 nmoles.

RESULTS

Antibodies specific for the NMN, NAD and NADP analogues of nicotine or cotinine were produced in rabbits immunized with nicotine or cotinine mononucleotide–BSA conjugates. Antibodies that could bind ([^{3}H]nicotine)AD or ([^{3}H]cotinine)AD were produced in all the animals. Those that possessed good specificity and sensitivity were used in the radioimmunoassays for the nicotine nucleotides and cotinine nucleotides.

The inhibition of binding of ([^{3}H]nicotine)AD to anti-nicotine nucleotide sera by varying amounts of nicotine derivatives is shown in Fig. 1A and Table 1. The inhibition of the anti-cotinine nucleotide reaction by cotinine derivatives is shown in Fig. 1B and Table 1. In each case, the homologous mononucleotide, NAD and NADP analogues were the most effective inhibitors and had almost equal activities (10–17 pmoles of the nicotine analogue and 8–10 pmoles of the cotinine analogues were required for 50 per cent inhibition of the homologous antigen–antibody reactions). Barring the occurrence of secondary reactions during the assay procedure, these results would indicate that the conformation of the nicotine (or cotinine) nucleotide moiety is similar in the NMN, NAD and NADP analogues. Despite the size of the adenosine moiety, it is not preventing the nicotine

Table 2. Nicotine and cotinine nucleotide analogues in the tissues of rabbits injected with nicotine or cotinine*

Compound Administered	Rabbit No.	Liver		Lung		Kidney		Heart	
		Nic	Cot	Nic	Cot	Nic	Cot	Nic	Cot
Nicotine	1	1.6	16.0	1.0	3.3	1.4	6.6	0.6	1.6
	2	2.1	5.8	0.5	1.6	0.6	4.6	0.5	1.6
	3	1.6	4.8	3.6	2.9			1.6	3.5
Cotinine	4	1.0	27.0	0.8	10.0	0.6	8.6	1.2	3.6
	5	1.4	48.0	2.2	35.0	2.5	12.5	1.2	3.0
	6	1.4	40.0	2.5	10.5	2.3	15.5	1.1	3.6
Control	NR1	2.6	3.2	0.5	1.5	1.0	3.6	1.5	2.5
	NR2	2.3	3.8	0.5	0.5	1.0	3.6	2.0	3.5
	NR3	2.0	3.6	0.5	0.5				

* New Zealand albino rabbits were injected daily (i.v.) with 0.5 ml saline which contained either 5 μmoles nicotine or 50 μmoles cotinine (higher doses of nicotine could not be used due to the toxicity of the alkaloid). The control rabbits were not injected. After 23 days, the rabbits were killed and tissues were removed, rapidly frozen and stored at −70°. To prepare the extracts, chopped tissue (10 g) and nicotinamide (5 mM added to inhibit NADase activity) were homogenized in 20 ml of cold water in a Sorvall Omni-Mixer. The homogenate was then poured with stirring into a tube which contained 20 ml of boiling water. After heating in a boiling water bath for 2 min, the protein precipitate was removed by centrifugation and washed once with 20 ml H_2O. The supernatant fractions were pooled and applied to a column containing Dowex 1 formate resin (1.5 × 10 cm). The column was washed with 50 ml H_2O and then with 75 ml of 0.2 M formate buffer, pH 3.6. The buffer wash was collected and lyophilized. The residue was dissolved in 2 ml H_2O and desalted on a Sephadex G-10 column eluted with 0.05 M NH_4HCO_3. The effluent was lyophilized and redissolved in an appropriate amount of H_2O for radioimmunoassay. Values are expressed as pmoles of nucleotide equivalents/g wet tissue.

or cotinine mononucleotide moiety from reacting fully with the antibody. The phosphate group contributes to the binding energy; compared to the mononucleotides, the ribosides must be present at 30- to 40-fold higher concentrations to give 50 per cent inhibition of their respective antigen–antibody reactions. The alkaloids are essentially inactive; less than 20 per cent inhibition is observed with 10 nmoles nicotine or cotinine.

The poor cross-reaction between these antibodies and the naturally abundant NAD and NMN (Table 1) enabled us to use the antisera to detect the cotinine nucleotide analogues in tissue extracts prepared from rabbits which had been injected with nicotine or cotinine (Table 2). Cotinine nucleotides were found in the extracts of liver (23–44 pmoles of nucleotide analogues/g of wet tissue corrected for background), kidney (5–12 pmoles/g) and lung (9–34 pmoles/g) prepared from the animals injected with cotinine (50 μmoles/day for 23 days). We had shown previously [1] that rabbit liver microsomes can be used as a source of NADase for the coenzyme analogue formation.

The formation of nucleotide analogues varies among the individual rabbits. For example, the concentration of cotinine nucleotides in lung extracts from cotinine-treated rabbits ranged from 9 to 34 pmoles (Table 2). Nucleotide analogues were not detected in heart extracts. Analysis of tissue extracts from normal rabbits shows that the background contribution from the various organs was low and constant.

The pyridine–ribose bond in NAD and NADP analogues can be split by mild alkaline hydrolysis to yield free pyridine derivatives [9]. In order to confirm the presence of cotinine nucleotide analogues in tissues, the liver extract from a cotinine-treated rabbit was hydrolyzed and assayed for the nucleotide and the metabolite (Table 3). The amount of free cotinine produced as a result of the hydrolysis was equal to the cotinine nucleotide present before hydrolysis. There were no significant differences before and after hydrolysis of an extract from a normal rabbit.

It should be stressed that the cotinine antiserum used to quantitate the free metabolite is highly specific and permits analysis of cotinine in the presence of the nucleotide analogues as well as the parent alkaloid and several other metabolites [10]. The results obtained by the use of two antibodies with vastly different specificities provide strong evidence that the nucleotide analogues of cotinine rather than those of other metabolites are present in the tissue extracts.

Table 4. Cotinine nucleotides in tissues of rabbits injected with cotinine*

Tissue	(Cotinine)RP (pmoles/g tissue)	(Cotinine)AD (pmoles/g tissue)
Liver	24	1
Lung	12	2.6

* Tissues from a rabbit injected with cotinine were homogenized and the nucleotides purified as described in Table 2. The Dowex 1 resin was washed with 50 ml H_2O, followed by 50 ml of 0.05 M formate buffer (Fraction 1) and then by 75 ml of 0.2 M formate buffer (Fraction 2). Each fraction was lyophilized, desalted and assayed for cotinine nucleotide by radioimmunoassay. Fraction 1 contained (cotinine)RP, while Fraction 2 contained (cotinine)AD.

We had previously shown that a mixture of authentic (cotinine)ADP, (cotinine)AD and (cotinine)RP could be separated by ion-exchange chromatography [3]. When the liver and lung extracts from cotinine-treated rabbits were chromatographed, the major portion of the serological activity was recovered in the (cotinine)RP fraction (i.e. with the 0.05 M of formate buffer eluant). The remainder of the activity (5–15 per cent) chromatographed as (cotinine)AD (Table 4). The high concentration of salt in the buffer required to eluate (cotinine)ADP interfered with the assay of small amounts of (cotinine)ADP since dilution to achieve the ionic strength required for the antigen–antibody reaction was not possible. Analysis for (cotinine)ADP was therefore not carried out. However, since the recovery of the serological activity in the (cotinine)AD fractions was complete, it is unlikely that appreciable quantities of this analogue were also present. With the naturally occurring compounds, NAD is far more abundant in tissues than NADP [11]. If a similar ratio exists among the analogues so that the quantities of (cotinine)AD known to be present are indicative of the quantities of (cotinine)ADP that might be expected, then the detection of the latter compound would be difficult.

DISCUSSION

Rabbits were immunized with conjugates prepared by covalently linking the mononucleotide analogues of nicotine or cotinine to BSA with carbodi-imide.

Table 3. Cotinine and cotinine nucleotides in rabbit liver extracts before and after alkaline hydrolysis*

	Cotinine-treated rabbits			Control rabbits		
	Before hydrolysis	After hydrolysis	Difference	Before hydrolysis	After hydrolysis	Difference
Cotinine†	11.3	42.6	+31.3	1.4	0.5	−0.9
Cotinine nucleotide‡	35.8	3.5	−32.3	5.0	5.5	+0.5

* Before hydrolysis, aliquots of the concentrated liver extract from a rabbit injected with cotinine and from a control rabbit (Table 2) were diluted and assayed for both cotinine and cotinine nucleotides by the radioimmunoassay procedures. After hydrolysis, aliquots (0.2 ml) of each concentrated liver extract were heated in a boiling water bath for 5 min with 0.12 ml of 1 N NaOH. The pH was adjusted to 7 with 1 N HCl at 0°, and the solution diluted to an appropriate volume and assayed as described for the unhydrolyzed samples.

† Measured in pmoles/g tissue.

‡ Measured in pmoles of cotinine nucleotide equivalents/g wet tissue.

This procedure was similar to that used by Halloran and Parker [12, 13] to couple mono-, oligo- and polynucleotides to proteins. Evidence that phosphoamide bonds were formed between the terminal phosphate groups of the nucleotides and amino groups of the protein was presented by these workers.

The specificities of our antisera are consistent with this mode of binding. The alkaloids appear to be completely exposed in the immunizing conjugates and contribute significantly to the binding energy required for the hapten–antibody interaction. The structural differences that exist between (nicotine)RP and (cotinine)RP are clearly recognized by the antibodies. For example, while 50 per cent inhibition of the binding of ([^{3}H]nicotine)AD to anti-nicotine nucleotide is obtained with 10 pmoles (nicotine)AD, 100 pmoles (cotinine)AD is inactive. The combining sites of the antibodies encompass both the ribose and phosphate groups. Compared to the mononucleotides, the ribosides are only about 3 per cent as effective in inhibiting the homologous reactions. Free nicotine and cotinine do not inhibit significantly even when very large concentrations are used (see Table 1).

(Cotinine)RP is the major nucleotide analogue present in tissues of rabbits injected with cotinine. Since the anti-cotinine nucleotide sera cannot differentiate between (cotinine)RP, (cotinine)AD and (cotinine)ADP, the combination of ion-exchange chromatography followed by immunochemical analysis was used to identify and quantitate the nucleotides. Analysis with an anterisum specific for cotinine served to confirm the identity of the product released after alkaline hydrolysis of the pyridine–ribose bond.

Cotinine nucleotide analogues were also found in rabbits injected with nicotine. The fact that nicotine is rapidly converted to cotinine in animal tissue may account for these findings [14, 15]. Similar results have been reported in the study of 3-acetylpyridine-treated rabbits. The NAD analogue could not be detected in the liver extracts presumably because 3-acetylpyridine is rapidly metabolized to nicotinic acid in liver [16].

Small amounts of material reactive with the anti-nicotine–nucleotide sera were found in lung extracts from one of the nicotine-treated rabbits and in the lung and kidney extracts prepared from the cotinine-treated rabbits. It is possible, although it has never been reported, that cotinine can be reduced to nicotine in lung and kidney. However, before we can conclude that nicotine nucleotides are present, the serologically reactive material must be analyzed further. This was possible with the cotinine nucleotides since they formed in larger amounts. In these studies, the amount of nicotine given to the rabbits as a single injection was limited by the toxicity of the alkaloid. The cotinine rabbits received ten times the dose of nicotine. However, since nicotine is so rapidly metabolized to products that are less toxic [17, 18], it might be possible to administer much larger quantities of drug over the course of many hours and thus favor the production of nicotine analogues.

The procedure used to extract the tissues (liver, lung, heart and kidney) prior to radioimmunoassay is not applicable to the preparation of brain samples, because the high lipid content interferes with the ion-exchange chromatography. Alternative purification procedures are being developed to study analogue formation in this tissue. Since NADase transglycosidic activity in brain is high [19] and since nicotine has been found to accumulate in rat brain shortly after the intravenous injection of radioactive nicotine [20], the formation of nucleotide analogues of nicotine or its metabolites in brain could be significant. Especially noteworthy is the suggestion that the toxicity of 3-acetylpyridine is due to the formation of the NAD analogues in the nervous tissue [16].

The formation of NAD and NADP analogues *in vivo* has been reported by many laboratories [16, 21–22]. However, to our knowledge, the formation of mononucleotides has not been extensively studied. One major difference between our studies and others dealing with nicotinamide nucleotide analogue formation is that our animals received the pyridine derivatives (i.e., nicotine and cotinine) on a daily basis for approximately 3 weeks to simulate in some degree the exposure a habituated smoker would have to these compounds.

Although the molecular events leading to nicotine toxicity have not yet been described, nucleotide analogues of this alkaloid and its metabolites may be involved. For example, the formation of nucleotide analogues may be implicated in the teratogenic effect of nicotine [23]. Pyridine derivatives, such as 3-acetyl pyridine and 6-aminonicotinamide, which can form NAD analogues by the NADase exchange reaction, have been shown to produce specific developmental defects in the chick embryo [24, 25].

In smokers, cotinine has a much longer half-life in the bloodstream than nicotine (i.e. 1–2 days, compared to 30–60 min) and is present in much larger concentrations (up to 650 ng/ml of sera) [10, 26]. After an individual has smoked 1–2 cigarettes, blood levels of nicotine can approach 60–70 ng/ml of sera for short periods of time [26–28]. With relatively high levels of cotinine continuously present in the bloodstream of habituated smokers and with the levels of nicotine peaking many times during the day depending on individual smoking habits, the formation of nucleotide analogues in smokers is a distinct possibility. Once the presence of these analogues in human tissues is clearly established, their implication in diseases associated with smoking will be sought.

Acknowledgements—The authors are indebted to Dr. Lawrence Levine for advice and constructive criticism. We thank Mr. Ronald L. Carroll for his assistance in carrying out the animal experiments.

REFERENCES

1. W.-C. Shen and H. Van Vunakis, *Biochemistry* **13**, 5362 (1974).
2. W.-C. Shen and H. Van Vunakis, *Res. Commun. Chem. Path. Pharmac.* **9**, 405 (1974).
3. W.-C. Shen, J. Franke and H. Van Vunakis, *Biochem. Pharmac.* **26**, 1835 (1977).
4. R. S. Yalow and S. A. Berson, *J. clin. Invest.* **39**, 1157 (1960).
5. V. P. Butler, Jr. and S. M. Beiser, *Adv. Immunol.* **17**, 255 (1973).
6. J. J. Langone, H. Van Vunkalis and L. Levine, *Accs chem. Res.* **8**, 335 (1975).
7. W.-C. Shen and H. Van Vunakis, *Fedn Proc.* **35**, 667 (1976).

8. H. Van Vunakis and L. Levine, in *Immunoassays for Drugs Subject to Abuse* (Eds. S. J. Mule, I. Sunshine, M. Braude and R. E. Willette), p. 23. CRC Press, Cleveland, Ohio (1974).
9. N. O. Kaplan, S. P. Colowick and C. C. Barnes, *J. biol. Chem.* **191,** 461 (1951).
10. J. J. Langone, H. B. Gjika and H. Van Vunakis, *Biochemistry* **12,** 5025 (1973).
11. G. E. Glock and P. McLean, *Biochem. J.* **61,** 388 (1955).
12. M. J. Halloran and C. W. Parker, *J. Immunol.* **96,** 373 (1966).
13. M. J. Halloran and C. W. Parker, *J. Immunol.* **96,** 379 (1966).
14. H. McKennis, Jr., in *Tobacco Alkaloids and Related Compounds* (Ed. U. S. von Euler), p. 53. Pergamon Press, Oxford, England (1965).
15. J. W. Gorrod and P. Jenner, *Essays Toxic.* **6,** 35 (1975).
16. N. O. Kaplan, A. Goldin, S. R. Humphrey, M. M. Ciotti and J. M. Venditti, *Science, N.Y.* **120,** 437 (1954).
17. H. Schievelbein, *Beitr. Tabakforsh* **6,** 199 (1962).
18. M. S. G. Clark, M. J. Rand and S. Vanoy, *Archs int. Pharmacodyn. Thér.* **156,** 363 (1965).
19. N. O. Kaplan, *Meth. Enzym.* **2,** 660 (1955).
20. C. G. Schmiterlöw anl E. Hansson, in *Tobacco Alkaloids and Related Compounds* (Ed. U. S. von Euler), p. 75. Pergamon Press, Oxford, England (1965).
21. L. S. Dietrich, I. M. Friedland and L. A. Kaplan, *J. biol. Chem.* **233,** 964 (1958).
22. R. P. DiAugustine, *Molec. Pharmac.* **12,** 291 (1976).
23. R. W. Menges, L. A. Selby, C. J. Marienfeld, W. A. Aue and D. L. Greer, *Envir. Res.* **3,** 285 (1970).
24. M. L. Murphy, C. P. Dagg and D. A. Karnofsky, *Pediatrics, N.Y.* **19,** 701 (1957).
25. W. Landauer, *J. exp. Zool.* **136,** 509 (1957).
26. J. J. Langone, H. Van Vunakis and P. Hill, *Res, Commun. Chem. Path. Pharmac.* **10,** 21 (1975).
27. P. F. Isaac and M. J. Rand, *Nature, Lond.* **236,** 308 (1972).
28. A. K. Armitage, C. T. Dollery, C. F. George, T. H. Houseman, P. J. Lewis and D. M. Turner, *Br. med. J.* **4,** 313 (1975).