**Acknowledgment.**—The authors wish to acknowledge partial support of this research through Grant C-2457 from the National Cancer Institute of the National Institutes of Health.[3]

(3) Analyses by Micro Tech Laboratories, Skokie, Illinois. Melting points are not corrected.

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## Penetration of Brain and Brain Tumors by Intravascular Injection of Alkylating Agents. IV[1]

ALBERT H. SOLOWAY, EMERY NYILAS, RAYMOND N. KJELLBERG, AND VERNON H. MARK

*Neurosurgical Service of the Massachusetts General Hospital and Department of Surgery, Harvard Medical School, Boston, Massachusetts*

*Received June 8, 1962*

Maximum effectiveness of cytotoxic agents in the treatment of cancer may be achieved if they preferentially concentrate in the neoplasm. Following this hypothesis, cerebral perfusion of anticancer agents for the therapy of brain tumors has certain distinctive advantages. Whereas the normal and neoplastic tissues of the same organ generally show no differential permeability, primary and secondary neoplasms in the central nervous system (CNS) have a markedly increased permeability to many substances in contrast with adjacent normal areas.[2,3] Therefore, it may be possible to devise cancerocidal agents which, by cerebral perfusion, will achieve a maximum differential concentration in the tumor.

Lipid solubility has been noted to be an important factor in determining the penetration of a compound into brain tissue.[4-6] The

(1) (a) Our thanks are due to Mrs. Janette Messer and the Misses Betsy Ferris and Ellen Dukat for their willing cooperation in this entire study. This work was supported by National Cancer Institute Grant CY-4707 (Cl), CY 2. (b) Paper III: A. H. Soloway, B. Whitman, and J. R. Messer, *J. Med. Pharm. Chem.*, **5**, 191 (1962).

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(5) A. Krogh, *Proc. Royal Soc. London*, **B113**, 140 (1946).

(6) L. C. Mark, J. J. Burns, L. Brand, C. I. Campomanes, N. Trousof, E. M. Papper, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.*, **123**, 70 (1958).

technique of partitioning compounds between a buffered aqueous phase and a lipid solvent such as chloroform or benzene has been used in estimating this lipid solubility.[7,8] A compound with high lipid solubility concentrates at least as rapidly in the brain as in many other tissues including brain tumor. The converse is not invariably true. However, among the compounds with low lipid solubility there are substances which are restricted in entering the brain but not its tumor.[9]

Compounds in this latter category are the ones which may hold promise for the treatment of brain tumors by isolated cerebral perfusion. In order to screen agents for such therapy, it was essential to evaluate whether brain penetration can be related to the compound's lipid solubility, especially with regard to the alkylating agents. It was also important to determine whether the partitioning procedure used for measuring lipid solubility could be applied to alkylating agents with very low half-lives in aqueous media. This investigation has used radioactively labeled compounds to undertake such a correlation between lipid solubility and tumor:brain ratio of these substances.

## Experimental

To determine lipid solubility, approximately 100 mg. of each substance was partitioned between 50 ml. of a buffer system containing a mixture of mono- and dihydrogen phosphate ions (pH 7.2) and 50 ml. of benzene. After vigorous mixing in a separatory funnel, the two layers were separated immediately to prevent appreciable hydrolysis of the chloroethyl group. The single exception to this sequence was 5-[di-(2-chloroethyl)-amino]-uracil which proved to be quite insoluble in either solvent. This mixture was shaken for 2 hr., the insoluble material was separated and the amounts in each phase were determined. The temperature of each incubation was room temperature and did not exceed the range of 21–24°. The molar concentration depended upon the compound partitioned and varied from $3 \times 10^{-3}$ to $7 \times 10^{-3}$. Only the phosphate buffer has been used throughout this study; admittedly, however certain alkylating agents might react with it.[10] Therefore it is conceivable that the buffer in this way could influence the partition values.

After separation of these two phases, 40 ml. of each was evaporated in a tared flask. The residues were dried over phosphorus pentoxide unless, in the case of certain aqueous mixtures, the product was hygroscopic. In these cases the residue was heated at 105° under reduced pressure until constant weight was achieved. Identical aliquots of the buffer solution itself were treated in a comparable manner

(7) A. H. Soloway, *Science*, **128**, 1572 (1958).

(8) A. H. Soloway, B. Whitman and J. R. Messer, *J. Pharmacol. Exptl. Therap.*, **129**, 310 (1960).

(9) W. H. Sweet, A. H. Soloway and G. L. Brownell, *Acta Union Intern. Contre le Cancer*, **16**, 1216 (1960).

(10) C. Golumbic, J. S. Fruton and M. Bergmann, *J. Org. Chem.*, **11**, 518 (1946).

TABLE I

PARTITION COEFFICIENTS OF ALKYLATING AGENTS

| Compound | Aqueous[a] | Benzene[a] | Aqueous/benzene partition coefficients[b] |
|---|---|---|---|
| N-Methyl-di-(2-chloroethyl)-amine | 28 | 58 | 0.5 |
| N-Methyl-di-(2-chloroethyl)-amine N-oxide | 80–96 | 10–11 | 9.0 |
| *p*-[Di-(2-chloroethyl)-amino]-1-phenylalanine | 90–94 | 9–15 | 8.0 |
| *p*-[Di-(2-chloroethyl)-amino]-L-phenylalanine ethyl ester | 6–7 | 81–92 | ≦0.1 |
| 1,6-Di-(methanesulfonoxy)-D-mannitol | 80–106 | 4–5 | >15.0 |
| 1,6-Di-(chloroethylamino)-1,6-dideoxy-D-mannitol | 88–104 | 1–2 | >15.0 |
| γ-[*p*-(Di-[2-chloroethyl]-amino)-phenyl]-butyric acid | 20–30 | 67–83 | 0.3 |
| 2-[Di-(β-chloroethyl)-amino]-1,3,2-tetrahydrooxazaphosphorine-2-oxide | 48–66 | 30–42 | 2.0 |
| 2,5-Di-(1-aziridinyl)-3,6-di-*n*-propoxy-1,4-benzoquinone | 10–20 | 78–80 | 0.2 |
| 5-[Di-(2-chloroethyl)-amino]-uracil[c] | 68–71 | 9–10 | 7.0 |
| Triethylenethiophosphoramide[d] | | | ≦0.1 |

[a] Weight in mg. and the range obtained from several experiments. [b] Average partition coefficient of the ratios from these experiments. [c] 6–20 mg. undissolved in the partition of this compound. [d] See reference 11.

to obtain a base value for the aqueous phase. The ratio of the weight of the aqueous phase residue in excess of this value, to the benzene residue is the partition co-efficient. The aqueous and benzene ranges in mg. obtained from usually two or three separate determinations of each of these clinically-active carcinostatic agents are recorded in Table I. The coefficients are determined from these values.

To determine whether there is a correlation between lipid solubility of the alkylating agents and tumor:brain ratios, two compounds which had been prepared previously in this laboratory with radioactive labels were used in this investigation, $P^{32}$-triethylenethiophosphoramide (I)[11] and *p*-(di-[2-$C^{14}$-β-chloroethyl]-amino)-L-phenylalanine (II).[12] C3H mice with subcutaneously-transplanted ependymomas were utilized to obtain tumor:brain ratios of these agents. The animals were injected intraperitoneally with I of specific activity of 4.0 μc./mg. at a dose of 6-9 μc. (6.1 mg. of compound/ml.) and with II of specific activity of 0.63 μc./mg. at a dose of 1-2 μc. (3.2 mg. of compound/ml.). After intervals of 30 min., 2 hr. and 24 hr. the mice were sacrificed. In the case of the $P^{32}$-labeled compound, fresh samples of brain and tumor were dissected, weighed and counted

(11) V. H. Mark, R. G. Ojemann, R. N. Kjellberg, and A. H. Soloway, *Neurol.*, **10**, 772 (1960).

(12) A. H. Soloway and E. Nyilas, *J. Org. Chem.*, **26**, 1091 (1961).

TABLE II

MOUSE DISTRIBUTION STUDY OF ALKYLATING AGENTS

| Sacrifice time, min. | *p*-(Di-[2-chloroethyl]-amino)-l-phenylalanine Tumor:Brain $C^{14}$ Ratio[a] | Triethylenethiophosphoramide Tumor:Brain $P^{32}$ Ratio[a] |
|---|---|---|
| 30 | 16.7 ± 4.0 | 1.3 ± 0.6 |
| 30 | 25.7 ± 1.2 | 1.3 ± 0.1 |
| 120 | 12.0 ± 0.7 | 1.3 ± 0.4 |
| 120 | 11.5 ± 1.0 | 1.4 ± 0.4 |
| 1440 | 14.6 ± 1.4 | 2.2 ± 0.4 |
| 1440 | 17.6 ± 1.3 | 2.6 ± 0.2 |

[a] The value for the ratio is the average of four ratios obtained for each animal and the standard deviation of these determinations.

directly to determine the tumor:brain ratio. For each mouse, the entire brain and 3 or more samples of tumor were used to obtain the average concentration in this tissue of the isotopically-labeled compound. The values in Table II are recorded with the standard deviation of these ratios. With the $C^{14}$-labeled compound, the low energy of the $C^{14}$ *beta* particle precluded the determination of the radioactivity in this manner. Tissue samples to be counted for $C^{14}$ were placed in a small tissue homogenizer containing water. The sample was ground, transferred quantitatively to a volumetric flask, and made up to volume. Aliquots containing approximately 10-20 mg. of tissue were withdrawn, plated on a copper planchet, weighed and counted (Tracerlab Autoscaler, Model No. SC 50 B1). In this manner, tissue counts were based on the dry weight of the sample. Ratios were determined in the same manner as was calculated for the $P^{32}$-compound and are recorded in Table II with the standard deviations.

**Discussion.**—The utility of the partitioning procedure can be seen by examining Table I. The occurrence of the hydrolysis of the $\beta$-chloroethyl group would result in a more hydrophilic compound and consequently a large aqueous/benzene partition coefficient. If hydrolysis does occur, it is not of sufficient extent to obscure the fact that N-methyl-di-(2-chloroethyl)-amine (III) has a much higher lipid solubility that its N-oxide (IV). The results are corroborated by the pharmacology of these agents. Compound III is known to produce central nervous system (CNS) toxicity[13] and the high lipid solubility of this compound may contribute to its facile penetration of the brain and its deleterious effect on this organ. Compound IV, however, shows much lower toxicity and appreciably larger doses may be administered without many of the undesirable side effects of III. It is certainly apparent that changes can be produced in the chemical structure of a compound increasing or decreasing its hydrophilic properties without basically altering the cytotoxic action of the mole-

(13) R. L. Clark, in "Cancer Chemotherapy," C. C Thomas, Inc., Springfield, Illinois, 1961, p. 16.

cule. In addition to a comparison of III and IV, examination of Table I shows that a similar relationship exists between *p*-[di-(2-chloroethyl)amino]-L-phenylalanine and its ethyl ester. The free amino acid has a much larger partition coefficient than the ester.

These coefficients obviously do not have any quantitative significance especially for the alkylating agents with their highly reactive groups. It is of use, however, strictly on a qualitative basis and may, in this manner, assist the synthetic chemist and clinician in the design and use of active agents which will be restricted in their penetration of normal brain. Groups such as carboxylic and sulfonic acids, ureido functions, carbohydrates, and amino acids increase the hydrophilic properties of a compound. Introduction of such moieties into a biologically potent carcinostatic agent may permit a change in the physical properties without altering their biological attributes.

From an examination of Table II it is apparent that the more lipid-soluble compound I penetrates brain nearly as rapidly as the tumor, whereas II is restricted in its entrance to the CNS. This is based not only upon the ratio but upon the percentage in tissue of the total dose administered. In the case of I, within 30 minues of injection, the activity/mg. of tumor and normal brain is comparable to the activity/mg. of animal in the administered dose. By two hours, the activity in the tissues was reduced by 20% and by 24 hours only one-tenth of the injected dose/mg. was present in tumor and slightly less in normal brain. With II, the activity/mg. of tumor within 30 minutes of injection is approximately one-half of the activity/mg. of the administered dose, whereas the level in normal brain is only 2 or 3% of this value. Interestingly enough as well, this compound does not disappear as rapidly from tissue as I. By 24 hours only half of the initial tissue levels had gone.

Though the analyses for $P^{32}$ and for $C^{14}$ cannot be used completely as a measure of the number of unchanged molecules remaining in the tissues, yet they do indicate the fate in tissue of the alkylating agent whether still active or following its metabolism. Compounds I[14] and II[15] are present to a large extent in a biologically active form certainly within the first hour of injection in animals. Consequently, the ratios at 30 minutes which do not vary appreciably from those at 2 and 24 hours, must in part be a measure of the active alkylating agent in tissue. The metabolism of I has been shown to involve loss of the sulfur atom to produce triethylenephosphoramide. This would not

(14) L. B. Mellett and L. A. Woods, *Cancer Res.*, **20**, 524 (1960).

(15) V. H. Mark, Y. Miyazaki, R. N. Kjellberg, A. H. Soloway, and W. H. Baker, *Surg., Gyn. and Obs.*, in press.

affect the $P^{32}$ label. In the case of II, the $C^{14}$ label is in the active alkylating portion of the molecule and only its position is of interest.

**Conclusion.** From the work relating to the penetration of the CNS by organic boron compounds, the human biopsy studies with $P^{32}$-triethylenethiophosphoramide and these data it would appear that lipid solubility is a good criterion in determining whether a compound will enter the brain readily. However, in using cancer chemotherapeutic agents, the primary factor is whether the compounds are able to interfere with the neoplastic process in brain tumors. If such active materials are highly lipid soluble, it may be possible to modify this property by the introduction of hydrophilic groups. An investigation is currently underway to screen agents against this malignant ependymoma in C3H mice. Preliminary results have been reported.[16]

(16) R. N. Kjellberg, V. H. Mark, G. A. Austin, G. A. Bendixen, R. B. Ojemann, A. H. Soloway, R. S. Shaw, J. W. Raker, and W. S. Sweet, Harvey Cushing Society Meeting, Mexico City, April 17, 1961.

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## Constituents of Cocculus Leaeba DC—Correction for Mistaken Botanical Identity

A. V. SUBBARATNAM* and WILLIAM B. COOK

*Department of Chemistry, Montana State College, Bozeman, Montana and Organic Chemistry Division, National Chemical Laboratory of India, Poone-8, India*

*Received September 5, 1962*

In a previous communcation[1] we reported the isolation and characterization of allantoin, m.p. 236–237°, a bitter yellow crystalline substance, m.p. 279–286° (dec.) identical with aristolochic acid present in the Austrian variety of *Aristolochia clematitis*[2] and *Aristolochia indica*[3] of Indian origin, a small quantity of another bitter yellow crystalline substance, m.p. 245–250° (dec.) and a green viscous essential oil consisting mainly of a hydrocarbon, $C_{15}H_{24}$, b.p. 85–87°

* The Wilson Laboratories, 4221 S. Western Blvd., Chicago 9, Illinois.

(1) Presented before the Chemistry Section, at the 44th Indian Science Congress, Calcutta, January, 1957 (Abstract No. 3, Part IV) and I.U.P.A.C. Symposium on the Chemistry of Natural Products, Australia, 1960 (Abstract 50).

(2) M. Pailer, L. Belohlav, and E. Simonitsch, *Monatsh.*, **87**, 249 (1956).

(3) P. R. Krishnaswami, B. L. Manjunath, and S. J. Venkata Rao, *J. Ind. Chem. Soc.*, **12**, 476 (1935). See also (a) *ibid.*, **12**, 494 (1935), (b) **15**, 646 (1938), and (c) A. Somasekar Rao and M. S. Muthana, *J. Ind. Inst. Sci.*, **37**(*A*), 266 (1955).