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THE PASTEUR PROBE: AN ASSAY FOR MOLECULAR ASYMMETRY *

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Abstract. The crucial role of a sterically specified informational macromolecule argues for net optical activity as an assay for the presence of biogeny on a planet. The logical sufficiency of this assay can also be argued [1-3]. Recent laboratory results [4-7] demonstrate the feasibility of scanning for enantiamorphs of possible metabolites with optically active reagents to give diastereoisomers resolvable by gas liquid chromatography. In combination with a mass spectrometer, this technique can be used to detect and identify ratios of stereoisomers, becoming a powerful method for the detection and understanding of signs of life. The Pasteur Probe, an instrument for the biological exploration of Mars, utilizing these principles, is described and laboratory results are presented. Several levels of instrument design and experiment performance are discussed. These range from an essentially single purpose instrument of about 20 pounds to the use of a computer-managed laboratory for the operation of the Pasteur Probe experiment. The relative merits of both approaches are considered.

РЕЗЮМЕ: Важную роль может сыграть использование оптической активности макромолекул при попытке определения наличия жизни на планете. Вопрос о том, достаточен ли этот метод с логической точки зрения также может дискутироваться (1,2,3). Недавние лабораторные результаты (4,5,6,7) указывают на возможность сканирования для анантиаморфов возможных метаболитов с оптически активными реагентами, дающими диастереоизомеры, которые можно определить газовой и жидкостной хроматографией.

В сочетании с масс-спектрометром эта методика может применяться для того, чтобы обнаружить и идентифицировать соотношения стереоизомеров, что в свою очередь весьма важно для обнаружения и интерпретации признаков жизни.

Дается описание пастеровского зонда-прибора для биологического исследования Марса на основе указанных принципов и приводятся результаты лабораторных исследований. Обсуждается

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несколько конструкций прибора и характеристик эксперимента, в частности, одноцелевой прибор весом около 20 фунтов (9кг) и управляемая электронно-вычислительной машиной лаборатория для проведения эксперимента с пастеровским зондом. Рассмотрены относительные достоинства обеих методов.

The crucial role of a sterically specified informational macromolecule argues for net optical activity as an assay for the presence of biogeny on a planet [1-3]. Fortunately, this argument does not depend on optical rotation per se since paradoxically the direct measurement of optical activity by polarimetry is an inherently insensitive approach. Most biologically interesting compounds such as the animo acids and sugars, have small specific rotation and thus at least 10 μg of optically pure material is needed. However, the basic criterion is not optical rotation but asymmetry based on molecular statistics. Whenever carbon, or more generally a tetravalent atom, is incorporated in a biogenic macromolecule, it has a fair chance of being an asymmetric center, that is, of having a distinctive substituent on each of its four valences. In fact, the larger the molecule, the more efficient the molecular information storage that has evolved, the more probable this event becomes. Such macromolecules then must have a uniquely specified three-dimensional shape if this information is to be preserved and replicated, or, in the case of enzymes, the molecule is to function. The orientation at each asymmetric center must be specified to define the conformation of the molecule. That is to say, a particular stereoisomer must be specified for a biologically functioning molecule. This same property causes these molecules to preferentially discriminate among the isomers of monomeric substrates. On earth, where biogeny has dominated the statistics of organic molecules, we find the ratio of D-to-L glucose residues is at least $10^{15}:1$ (i.e., L-glucose is not known to occur in nature. Net optical activity is almost a sufficient condition for deducing the presence of life on a planet. Nonbiological chemical processes would ordinarily generate racemic mixtures, chemical enantiomorphs in equal proportion.

We have to consider the possibilities of spontaneous resolution of some racemate on a planet, this in turn biasing further chemical synthesis. However, this alternative hypothesis will have to provide a model to defeat the expected re-racemization of local pools of a particular isomer in thermodynamic equilibrium. Biological processes can, of course, change these statistics. This does indicate, however, that in investigating residues of isomers of monomeric substrates, several places should be sampled and several members of the molecular class analyzed. For example, the analytical system should be capable of analyzing the ratio of at least a few amino acids if that were the target residue. Recent laboratory results [4-7] demonstrate the feasibility of scanning for enantiomorphs of possible metabolites with optically active reagents to give diastereo-isomers resolvable by gas liquid chromatography.

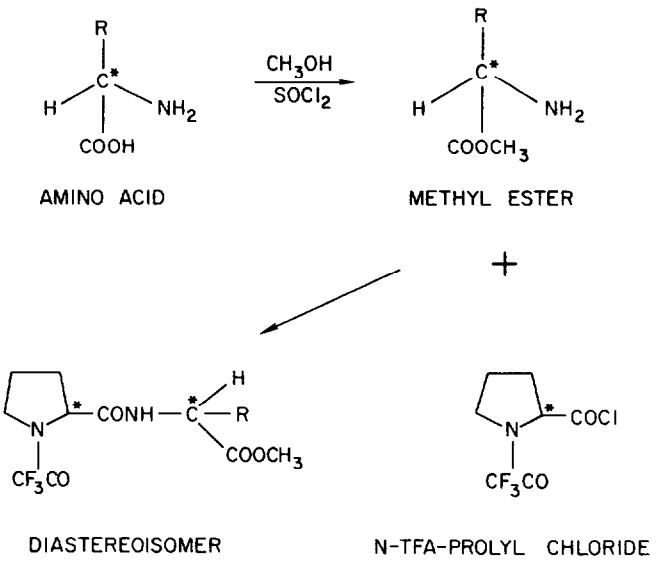


Fig. 1. Preparation of diastereoisomers.

We have now found a sensitive technique for demonstrating the asymmetry of D, L amino acids. Using N-trifluoracetyl-L-prolyl chloride as the resolving agent, the diastereoisomers of amino acids can be separated by gas liquid chromatography (GLC) and as little as 0.1 μg of each antipode can be detected. The method of preparing diastereoisomers is summarized in fig. 1 and a typical gas chromatographic separation of a synthetic mixture of neutral D, L amino acids as their N-trifluoracetyl-L-prolyl peptide methyl esters is illustrated in fig. 2.

Amino acids isolated from soil could be identified and their steric purity determined by comparison of retention times on the gas chromatograph with a known sample. However, a peak derived from material other than amino acids could cause confusion and, therefore, an additional method of identification is necessary. We have now used mass spectrometry to identify the GLC peaks as well as providing a method of accurately determining the ratio of the optical antipodes. For this purpose we prepared an artificial mixture of D and L enantiomeric resolving agents, in which the L reagent was labelled with 2 deuterium atoms (L*). For each symmetrical molecule (e.g., glycine), the D and L* reagents are unresolved and the label ratio will remain uniform through the peak. However, if an asymmetric molecule is encountered, which gives rise to resolvable diastereoisomers, the deuterated reagent will be concentrated in one peak, distorting the ratio. If the target molecule is racemic (d, l), two peaks will also be formed (one containing L*d plus Dl, the other L*l plus Dd), but the label ratio in each peak will remain constant (fig. 3). We chose trifluoracetyl-thiazolidine-4-carboxylic acid chloride as the reagent, because both enantiomers are available and deuterium can be incorporated into position 2 with deuteroformaldehyde.

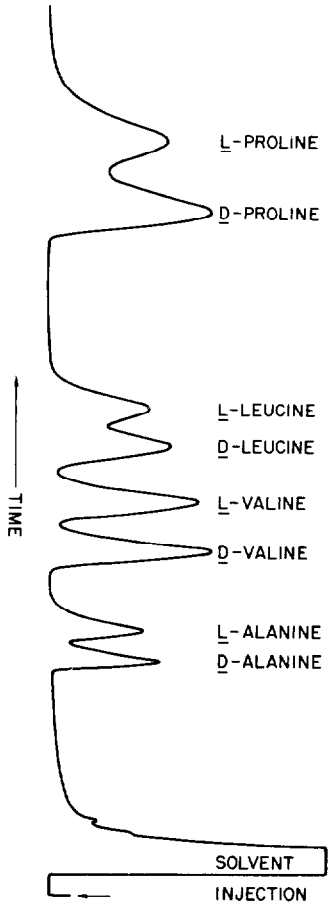


Fig. 2. Resolution of diastereoisomers.

Also, mass spectrometric fragmentation patterns of its condensates with amino acid esters yielded characteristic peaks which could be used to identify both the reagent and the amino acid (fig. 4). Monitoring fragment a for the ratio [184 : 186] as well as the ratio $[b : b+1]$ for the base peak (M - 156), a recording of steric purity was obtained. In addition, the position of the base peak was also used to confirm the identity of the optically active amino acids present (table 1, fig. 5).

The experiment described so far, which we have called the Pasteur Probe, consists of an observation of the existing statistics of asymmetric molecules at or near the Martian surface. It would be directly applicable to hydrolysates of soil samples or to condensates from the atmosphere. The experiment can be extended to indicate growth by inoculating the Martian soil with substantial quantities of a variety of racemic compounds. After varying periods of time, small samples of the surrounding soil would be retrieved and

$$DL^* + dl \rightarrow (Dd, L^*l) + (Dl, L^*d)$$

$$DL^* + d \rightarrow Dd + L^*d$$

$$DL^* + l \rightarrow L^*l + Dl$$

Fig. 3. Use of labelled enantiomeric resolving agent.

extracted, then analyzed for the relative abundance of the inoculated species. So far, this is a typical metabolism experiment; however, we now examine the optical purity of the recovered material.

Almost without exception, biological metabolic systems show substantial selectivity in the degradation of the metabolites (and appearance of new products). Knowing which materials have been degraded will also tell us a good deal about the orientation of the metabolic system. A large number of DL-compounds could be used together, the main limitation being background noise and possible mutual toxicity. The sensitivity of this approach is augmented by its direct interface with the Martian crust. Furthermore, in consequence of diffusion, the material introduced will not be in large excess everywhere, answering a plausible argument against swamping a small sample used in a metabolic experiment with any prespecified environmental factor, even, for example, water. Inoculation in terrestrial soil, using the GLC to monitor the stereo-specific metabolism of several racemic amino acid substrates, has verified this approach. In most cases, gross changes in the ratio of enantiomorphs could be demonstrated after 18-24 hr.

In a typical assay, soil (10 g), the racemic amino acid substrate (10 mg) and distilled water (10 ml) were shaken at room temperature. From time to time aliquots (approximately 1 ml) were removed and diluted with water

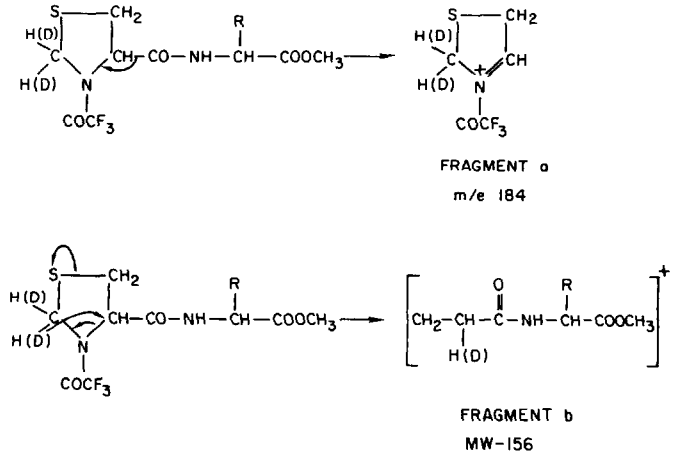


Fig. 4. Mass spectral fragmentation of TFA-thiazolidine-4-carboxylic acid condensation products.

Table 1
Mass spectral monitoring of GLC fractions corrected for isotopic abundance *.

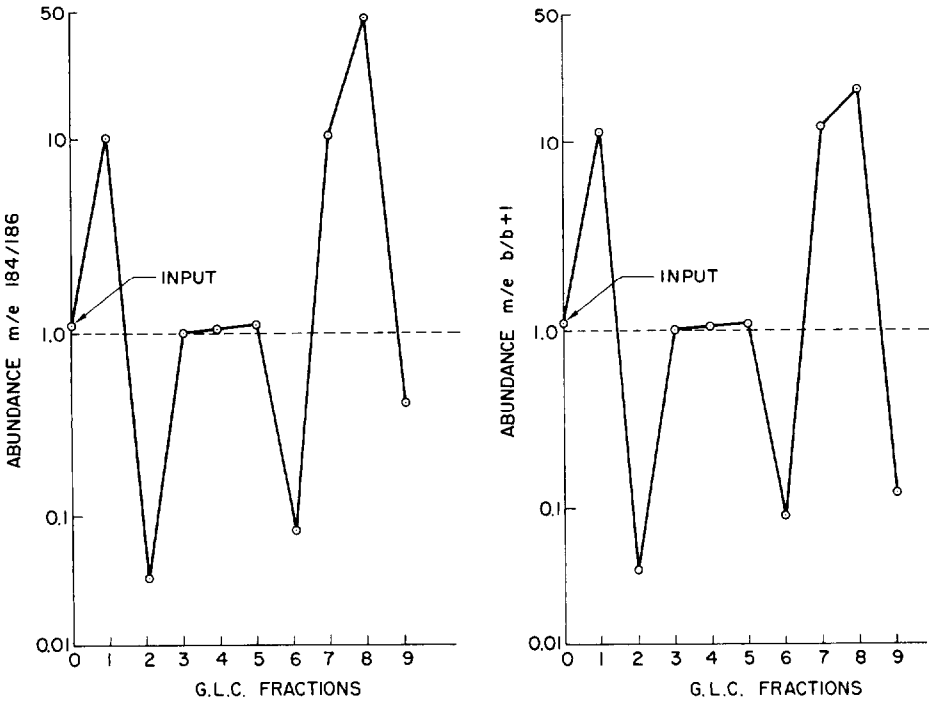
GLC fraction	Ratio (a : $a+2$) m/e 184 : 186	Fragment (b) m/e	Ratio (b : $b+1$)	Molecular weight ($b+156$) and identity of amino acid	Optical identity of fraction
1	28 : 2.5	158	100 : 8.5	314-alanine	L
2	1 : 24	158	4.5 : 100	314-alanine	L
3	38 : 41	144	97 : 100	300-glycine	DL
4	55 : 56	172	100 : 96	328-aminobutyric acid	DL
5	33 : 32	172	100 : 98	328-aminobutyric acid	DL
6	2.5 : 33	200	8.5 : 100	356-leucine	D
7	23 : 2	200	100 : 8	356-leucine	D
8	100 : 2	184	100 : 5	340-proline	L
9	12 : 31	184	12 : 100	340-proline	L

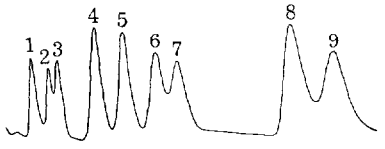
* GLC analyses were carried out on a Wilkens 600C aerograph, fitted with a micro collector and using a 5' × $\frac{1}{8}$" S.S. Column containing 5% SE 30 on chromosorb W. The separation temperature was 180°C and the N_2 flow was 28 ml/min. Mass spectra were determined on a Bendix-time-of-flight spectrometer and the collected sample fractions introduced directly into the ion source.

(10 ml). The soil was centrifuged down and the supernatent solution lyophilized. The residual amino acid was analyzed by conversion into diastereoisomers as previously described and injected into a gas chromatograph. By computing the peak areas of the two diastereoisomers, a measure of residual D/L amino acid concentration could be obtained. Fig. 6 demonstrates the susceptibility of D, L-glutamic acid to micro-organisms in Bowers Clay. Table 2 and fig. 7 demonstrate the susceptibility of a mixed D, L amino acid substrate. Fig. 7b, c and d show typical GLC separations on which these curves were based. Our results show that the L-antipodes of the substrates are preferentially attacked, but that different amino acids are used at different rates. Stereospecific action is lost after heat sterilization of the soils, confirming that a biological process is involved. While the exact nature of the microbiological system responsible for the stereospecific attack is as yet not known, the kinetics of the experiment suggest an exponential increase of activity, which would be consistent with growth of micro-organisms.

Several levels of instrument design and experiment performance are possible for the Pasteur Probe. The experiment is not restricted to amino acids. The logical basis of the experiment is the role of molecular asymmetry in defining the conformation of *any* polymer. We need make no fixed assumptions about amino acids (as such) as the methods will generally be applicable to any carbon molecule with free -OH- or -NH_2 groups. The same approach should be easily generalized to other optically active species, such as alcohols [8], amines [9] and carbohydrates.

The simplest and lightest weight hardware would utilize only a gas chromatograph and compare the chromatograms obtained first with one and then with the other of a pair of enantiomeric couplers. While this uses conventional reagents and detectors, it places a stringent demand on the homoge-





Gas chromatograph *

* GLC fraction:
1. D reagent-L-alanine
2. L* reagent-L-alanine
3. D reagent-glycine and L* reagent-glycine
4. D reagent-L aminobutyriacid and L* reagent-D-aminobutyriacid
5. D reagent-D aminobutyriacid and L* reagent-L-aminobutyriacid
6. L* reagent-D leucine
7. D reagent-D leucine
8. D reagent-L proline
9. L* reagent-L proline

Fig. 5. Analytical resolution of labelled input reagent.

neity of the sample and quantitative reproducibility of the entire process. There is the possible difficulty of confusion if a wide variety of organic molecules is present in the sample. Somewhat heavier more complex instrumentation could utilize differential labels of the enantiomeric reagents, for example, "levo-tritium" and "dextro-C_{14}". Even though a wide variety of substances may be present in the sample, the ratio recording of the chromatograph output would be influenced only by an optically active species. Overlapping peaks would interfere only insofar as they attenuate the shift in ratio by diluting the difference in label. The resolution between diastereoisomers, or among the various chemical species, need not be perfect to give decisive results. We

Table 2
Susceptibility of DL-amino acids to micro-organisms in soils.

Soil sample	DL amino acid	Unused D/L amino acid concentration after period of incubation (hr) *												
		0	2	4	6	8	10	12	14	16	18	20	22	24	48
Bowers clay †	Proline	1.0		1.0		1.1				1.2	1.9	3.0	6.8	9.2	
Bowers clay sterilized ‡	Proline	1.0								1.0					
Bowers clay	Glutamic acid	1.0		1.0		1.1		1.3		1.3	1.5	2.5	2.7	5.0	
Bowers clay sterilized ‡	Glutamic acid	1.0						1.0						1.0	
Stanford soil ‡†	Glutamic acid	1.0						1.8						16.0	
Stanford soil sterilized ‡	Glutamic acid	1.0						1.0						1.0	
Stanford soil	Amino butyric acid	1.0				1.1								2.9	4.7

* GLC analyses were carried out on a Wilkens 600C aerograph using a 5' × $\frac{1}{8}$" column (0.5% EGA on chromosorb W). During the analyses the nitrogen flow was 46 ml/min and the oven temperature was programmed from 140°C to 200°C at a rate of 4°/min. Under these conditions the retention times (min) for the N-TFA-L-prolyl derivatives were D-valine (7.6), L-valine (8.9), D-proline (13.0), L-proline (14.2), D-glutamic acid (20.2), L-glutamic acid (21.4), D-amino butyric acid (9.0), and L-amino butyric acid (10.5).

† Soil collected at Moffett Field, California and characterized by NASA Ames. The organic nitrogen analysis was 1435 ppm and the organic carbon was 6380 ppm. The soil had a pH 6.08.

‡ Soil samples were sterilized by heating at 135°C for 24 hr.

‡† Garden soil collected at Stanford in December 1965.

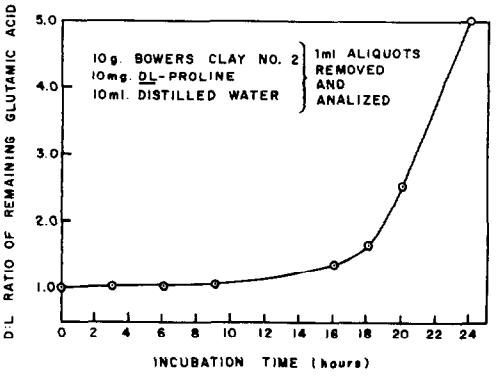


Fig. 6. Susceptibility of D : L-glutamic acid to micro-organisms in Bowers Clay.

would, however, not be able to identify what the species is merely from the relative mobility on the GLC.

The most powerful instrumentation would combine a gas chromatograph with a mass spectrometer. The fragments identified in fig. 4 could be cracked before admission to the mass spectrometer requiring only a modest mass spectrometer with unit mass resolution in the lower mass range, overlapping the requirements of atmospheric composition analysis. Without cracking one would require discrimination of fragments of mass = 186 from mass = 184. The greatest utility of the mass spectrometer would permit the identification of the molecular species characteristic of the chromatograph peaks. This requires at least unit resolution over a mass range of several hundred. Fig. 8 is a flow diagram of the Pasteur Probe based on such a configuration. We have shown the utilization of the Pasteur Probe as an experiment to test for the presence of life on a planet. Such a test requires, however, a multitude of experimental investigations covering the widest variety of methodologies to be sure of confronting nature's local evolutionary strategy. No single criteria will ever be solely satisfactory. If one adds, as one must, the characterization of life, if present, as an additional scientific goal of the biological exploration of Mars, one is led to the concept of a computer-managed, reprogrammable, automated biological laboratory. Such a laboratory could best perform an experiment such as the Pasteur Probe if it provided reprogrammable computer-control of a set of laboratory functions and operations which would permit an indefinitely large set of possible variations of the experiment and the experimental conditions. The choice of the optimum variation will no doubt originate from earth after the initial data are received. This is to be distinguished from the automation of a prewired,

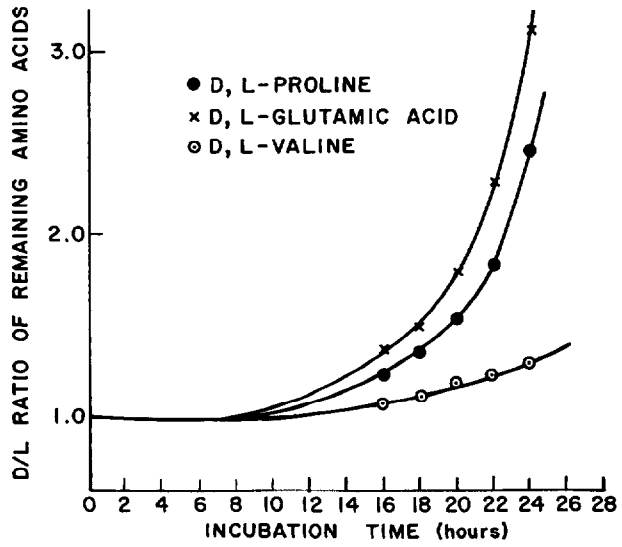


Fig. 7a. Susceptibility of mixed D : L-amino acid.

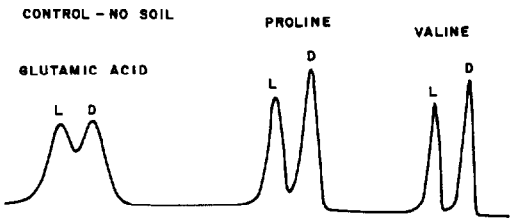


Fig. 7b. GLC Separative-control no soil.

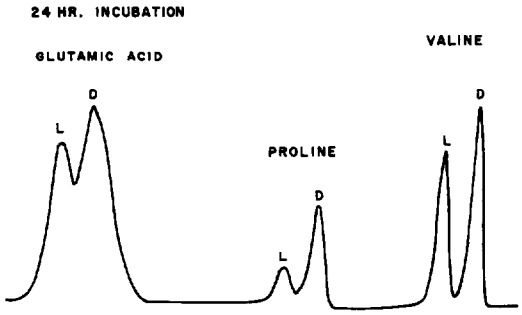


Fig. 7c. GLC Separative 24 hr incubation.

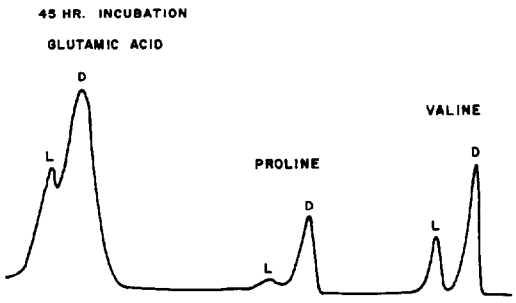


Fig. 7d. GLC Separative 48 hr incubation.

discrete set of experiments, the sequencing of which is perhaps controlled by local computation of data or from earth. Besides its impact on the particular experiment under discussion, such a laboratory would have other interesting unique characteristics. Participating scientists would be competing not for payload weight or engineering support, but for pro-rated time on the system. Computer logic would direct the execution of the main subroutines of, say, sampling and biochemical analysis, but would be supervised and reprogrammable by command from earth to allow full advantage to be taken of stepwise or scientifically competitive programs. Such a concept allows for the broadest possible national and international scientific participation. The scientist in his own laboratory could direct and evaluate the results of an experiment carried out on Mars. He could initiate an experiment any time

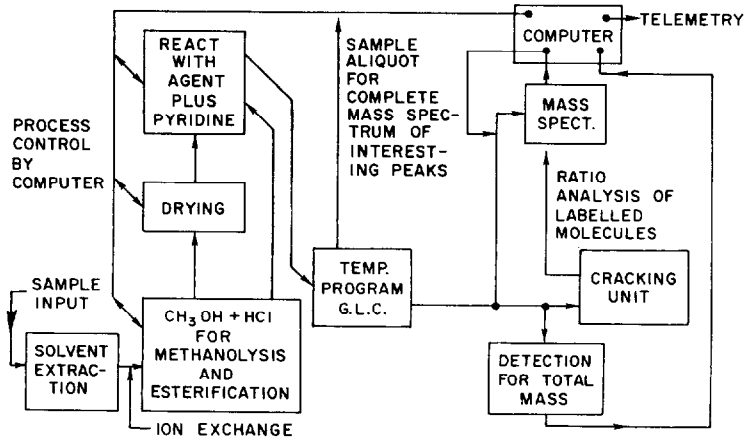


Fig. 8. Flow diagram - Pasteur Probe.

during the lifetime of the laboratory without the need of having had to design space-qualified hardware years earlier.

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